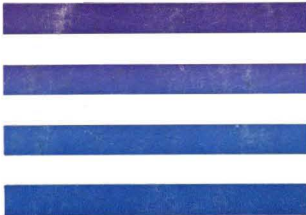

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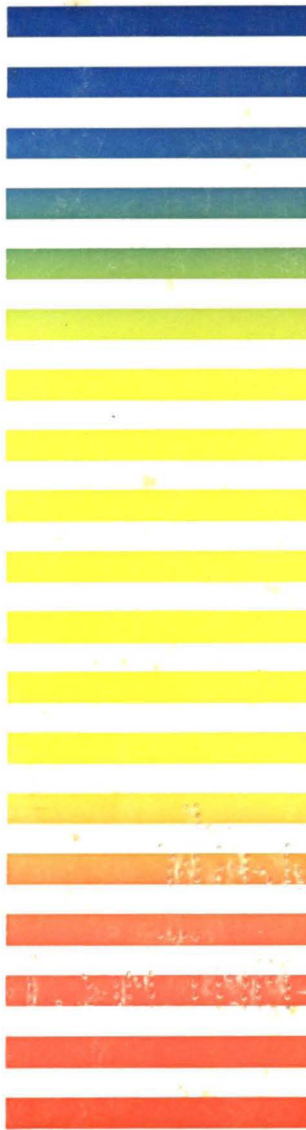

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SPECIAL VOLUME

EIGHTH INTERNATIONAL SYMPOSIUM ON AFFINITY CHROMATOGRAPHY AND BIOLOGICAL RECOGNITION

Jerusalem (Israel), October 29–November 3, 1989

Guest Editor

M. WILCHEK

(Rehovot, Israel)

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PREFACE

The *Eighth International Symposium on Affinity Chromatography and Biological Recognition* was held at Kibbutz Ramat Rachel in Jerusalem, Israel, from October 29th to November 3rd, 1989. This particular site was chosen because it combines both the ancient and modern history of Israel; for many people around the world, Israel is symbolized by the city of Jerusalem and by the communal way of life on a kibbutz.

The meeting attracted some 200 scientists from 20 different countries. The programme included 45 lectures and 40 posters. The lectures were given by leading scientists in the field, as well as by many newcomers who have published interesting work in the fields of biorecognition and affinity chromatography. Many of these are presented in this volume. According to the letters which we received after the meeting, the programme was thoroughly enjoyed and appreciated by the participants.

Most of the scientists were located in the guest house of the kibbutz, in the same building as the lecture hall, bar and restaurant. Consequently, many contacts were formed among the participants, often over a glass of wine or beer, sometimes continuing into the early hours of the morning. During the daytime hours, some of the scientists took time off and joined the accompanying persons to visit the beautiful city of Jerusalem. All the participants went on a half-day archeological tour of Jerusalem, which was preceded by an exciting lecture on the close association of the geography, history and archeology of Jerusalem. The breathtaking tour of Jerusalem led us to an underground museum in the Jewish Quarter of the Old City which comprised excavations of many wealthy mansions which were razed during the time of the destruction of the Second Temple, nearly two millennia ago. An afternoon tour of Kibbutz Ramat Rachel was also organized.

The meeting was held under the Honorary Chairmanship of Professor Ephraim Katzir. I would like to take this opportunity to thank Professor Shmuel Shaltiel, the co-Chairman of the meeting, for his cooperation, advice and involvement in the organization of the programme. It is also my pleasure to thank the organizing committees, both local and international, for their suggestions and assistance, and especially for their trust in me, in giving me "academic" freedom in the many decisions regarding the meeting. The members of the international advisory committee were as follows: Irwin M. Chaiken, Pedro Cuatrecasas, Tom C. J. Gribnau, Shin-ichi Ishii, Jan-Christer Janson, Herbert P. Jennissen, Chris R. Lowe, Klaus Mosbach, Werner Müller, Indu Parikh, Jerker Porath, William H. Scouten and Jaroslava Turková. The members of the local advisory committee were Haim Aviv, Shmaryahu Blumberg, Zelig Eshhar, Leon Goldstein, Marian Gorecki, Itzhak Kahane, Noah Lotan, Menachem Rubinstein, Nathan Sharon and Abraham Warshawsky.

I would also like to thank the Israel Academy of Sciences and Humanities, the Maurice and Gabriela Goldschleger Conference Foundation at The Weizmann Institute of Science, the Israel Ministry of Science and Development, the International Society for Biorecognition Technology and the National Science Foundation (U.S.A.), and also the many companies who gave support to the meeting. The latter include Pharmacia, Glaxo, Isolab, Merck, Becton Dickinson, InterPharm, Belovo,

BioProbe International, Miles, Sandoz, Smith Kline & French, Monsanto, BioMakor, BioTechnology General, Boehringer Mannheim, Ciba Corning Diagnostics, Cetus, Elsevier Science Publishers, I.B.F. Biotechnics, Rohm Pharma, Organon, Pierce Europe, Zymed, Amgen, Enzo Biochem and Roreh Central Research. Pierce Chemical donated the Pierce Award for Affinity Chromatography, which was presented to Dr. Chris Lowe of the University of Cambridge.

Of course, many thanks are due to Dr. Edward A. Bayer and Dr. Fortune Kohen, the scientific secretaries of the meeting, and to Mr. Yitzchak Berman, the coordinator. Special thanks are due to Mrs. Dvorah Ochert, the organizing secretary, for her efforts and hard work which made this symposium so successful and pleasant for the participants. I would also like to thank the speakers and chairmen without whose good-natured flexibility there would never have been a meeting at all.

Finally, I have to apologise personally to all of the participants of the meeting with whom I could not spend as much time as I would have liked, owing to the variety of organizational matters which occupied much of my time during the symposium. I hope to speak to all of them at the next meeting in Yokohama.

Rehovot (Israel) MEIR WILCHEK

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Review

Application of avidin–biotin technology to affinity-based separations

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1. INTRODUCTION AND PERSPECTIVES

In the past decade, the unprecedented interaction between the egg-white glycoprotein avidin and the vitamin biotin has provided the basis for establishing a new technology which has broad application in virtually all fields of biology and biotechnology[1–5]. The basis of this new technology resides in the exceptionally high affinity constant between avidin (or its bacterial counterpart streptavidin) and biotin, which is several orders of magnitude greater than other types of affinity interactions (Table 1). Originally designed to facilitate and improve purification (affinity chromatography) and localization (affinity cytochemistry) procedures for biologically active macromolecules, the application of avidin–biotin technology has also led to major advances in medical diagnostics (immunoassay, histopathology and gene probes). In addition, a variety of new applications have accumulated, including affinity targeting, cross-linking and immobilization studies, cell cytometry, blotting technology, drug delivery, bioaffinity sensors, fusogenic studies and hybridoma technology.

The general idea of the approach is that biotin, coupled to low- or high-molecular-weight molecules, can still be recognized by avidin, either as the native protein or in derivatized form containing any one of a number of reporter groups (*e.g.*, fluorescent groups, electron-dense markers, enzymes, immobilizing matrices). In this manner, mediation through the avidin–biotin complex often leads to a dramatic enhancement of signal and/or sensitivity levels.

The use of the avidin–biotin system as a "universal" tool in the biological

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TABLE 1

SOME AFFINITY PAIRS AND THEIR DISSOCIATION CONSTANTS

Binding protein	*Target molecule*	K_D *(M)*
Avidin	Biotin	*ca.* 10^{-15}
Streptavidin	Biotin	*ca.* 10^{-15}
Receptors	Hormones, toxins, etc.	10^{-9}–10^{-12}
Antibodies	Antigens	10^{-7}–10^{-11}
Transport proteins	Vitamins, sugars, etc.	10^{-6}–10^{-8}
Lectins	Carbohydrates	10^{-3}–10^{-6}
Enzymes	Substrates	10^{-3}–10^{-5}

sciences is the product of concepts which were defined in the mid-1970s. In rapid succession, a number of laboratories developed methodologies for the biotinylation of membranes[6], nucleic acids[7,8], antibodies and other proteins[9]. The biotinylated binders were then analysed in some way with an appropriate avidin-conjugated probe. Today, the same basic approach is commonly employed for a wealth of different applications (Fig. 1). Conceptually, little has changed from the original applications; currently reported contributions usually describe changes in the choice of target material, binders and probes, and new strategies for introducing biotin and avidin into the desired experimental system. There is no doubt, however, that the application of avidin–biotin technology is still on the increase[10].

2. EARLY ISOLATION STUDIES

In historical terms, the first example of the use of avidin–biotin technology was the isolation of avidin on a biocytin–Sepharose column[11]. Already in this instance was it clear that there would be problems in applying avidin–biotin technology for isolation purposes; although avidin bound readily enough to the biotin-containing column, the conditions required for its removal were extremely drastic (6 *M* guanidinium

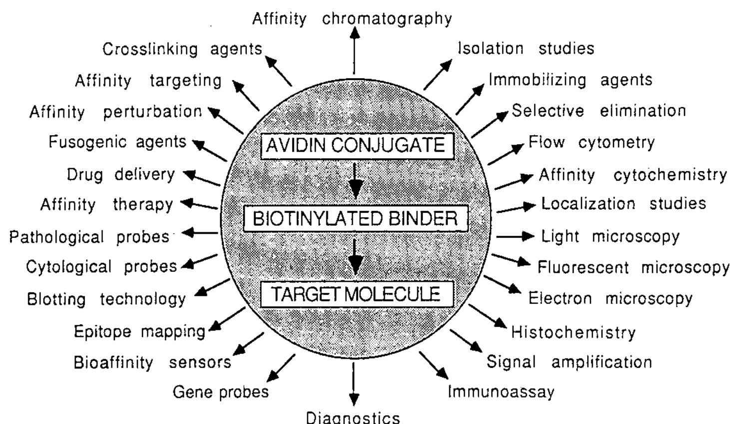

Fig. 1. General approach and major applications of avidin–biotin technology.

hydrochloride, pH 1.5). Clearly, if one wants to use an avidin column for the purification of target material via biotin-containing binder, in most instances it will be impossible both to regenerate the original column (avidin–Sepharose) and to recover active biotinyl binder. It was also unclear what if any short- or long-term advantages would be gained by preparing such binder–biotin:avidin–resin complexes. Thus, in the area of isolation studies, the unprecedented affinity constant which characterizes the avidin–biotin complex has remained its most important virtue as well as its most confounded vice. Nevertheless, such a system was used to isolate biotin-containing peptides from proteins which were modified with different biotinyl derivatives for sequencing purposes[12]. In addition, a similar approach was used to assist in the purification of peptides synthesized by the solid-phase method[12,13]. The approach which we used in these studies is shown schematically in Fig. 2.

Despite the difficulties encountered in applying avidin–biotin technology for separation purposes, many successful isolation procedures have been reported and the list continues to grow (Table 2). One approach has been to use avidin columns for the isolation or purification of target material by releasing the latter from the column. In many instances, researchers have depended on the stability of the complex which fastens the binder to the column, and even relatively harsh procedures (*e.g.*, high concentrations of detergents) can be employed to release the target molecule. In other strategies, avidin-containing columns have been used selectively to purge biotinylated molecules or cells from a complex mixture in solution or suspension. The first example of this approach was the removal of biotinylated lectins and antibodies from solution for analytical purposes[9]. An effective way of releasing the biotinylated molecule from an avidin column has also been developed[14]; columns consisting of immobilized avidin monomers take advantage of the lower affinity constant[15] which permits the subsequent release of the biotin-containing material under mild conditions. Another field in which avidin–biotin mediation is rapidly undergoing extensive development is the isolation or subtractive elimination of genes.

3. LARGE-SCALE ISOLATION OF ANTIGENS

In recent work, we have performed a pilot study in which avidin-derivatized matrices were proposed as universal columns for isolation and immobilization purposes[16]. In one of the examples, we chose to demonstrate the use of an avidin–Sepharose column for the large-scale isolation of an antigen using the appropriate biotinylated antibody. This relatively simple strategy is shown schematically in Fig. 3. In this study, we used transferrin as a model antigen and commercially obtained antibodies (BioMakor, Jerusalem, Israel) as the biotinylated binder. Using this approach, we were able to achieve remarkably high yields and purity using a single-step isolation procedure. A yield of about 0.75 mg of transferrin was obtained for every 1 ml of avidin–Sepharose column (which contained 2 mg/ml of biotinylated antibody and 1.5 mg/ml of avidin). Interestingly, the procedure proved more efficient than "conventional" affinity chromatography in which the antibody was directly attached to the resin. This was reflected in both the amount of antigen bound to the column and the percentage of antigen released. Essentially all of the target material could be released from the column using 0.1 *M* acetic acid.

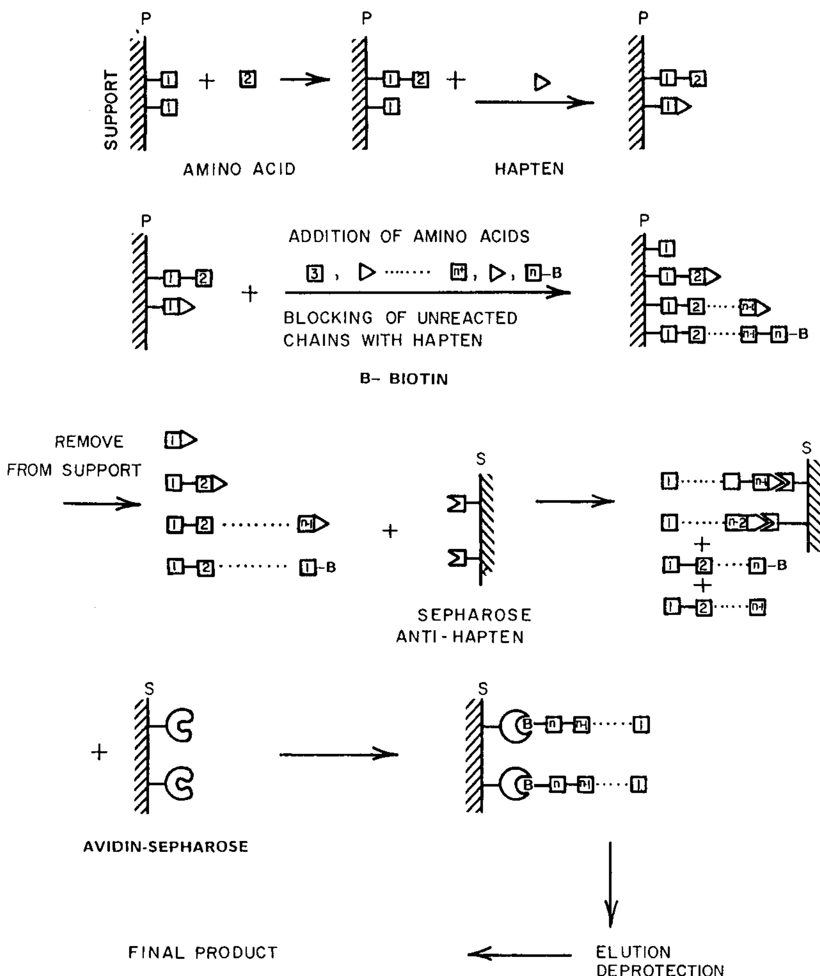

Fig. 2. Use of antibody and avidin columns for the isolation of solid-phase synthesized peptides. After each step in which tBoc-amino acid is added, the residual unreacted amino groups are blocked with a reactive hapten-containing reagent (*e.g.*, dinitrofluorobenzene). In the last step of the synthesis, either biotinyl methionine is added to the N-terminal residue or a terminal methionine is biotinylated using biotinyl N-hydroxysuccinimide ester. After removal of the synthetic peptide from the solid phase, the mixture is passed through an anti-hapten antibody column to remove all of the truncated peptides containing the hapten. The effluent which contains the complete peptide (containing the biotinyl methionine residue) is then passed through an avidin column. After removal from the column, the biotinylmethionine is split off the column with cyanogen bromide to yield the required peptide. This procedure, of course, is applicable to peptides that do not contain methionine. In such cases, an alternative procedure can be used by adding biotinyl homoserine or N^{α}-biotinyldiaminobutyric acid and the resultant derivative can be cleaved with acid. In yet another alternative, N^{α}-tBoc-biocytin can be included in the last step and removed by Edman degradation. In many instances it may even be advantageous to leave the biotin on the synthetic peptide for identification purposes.

TABLE 2

EXAMPLES OF BIOLOGICALLY ACTIVE MATERIAL PURIFIED OR SEQUESTERED USING AVIDIN COLUMNS

Biotin-containing systems	*Systems mediated via biotinylated binder*
Native biotin-containing enzymes, subunits and peptides	Surface glycoproteins
	Soluble antigens
Sodium transport enzyme (biotin-containing)	Membrane antigens
	Antibodies
Biotinylated lectins	Enzymes
Biotinylated antibodies	IgE receptor
Biotinylated enzymes	Hormone receptors
Biotinylated mitogens	Opioid receptor
Biotinylated DNA	Cell subpopulations
Biotinylated RNA	Antibody-producing cells
Biotinylated tRNA	DNA
Biotinylated rRNA fragments	Plasmid DNA
Biotinylated nucleosomes	Recombinant plasmids
Biotinylated membranes	Transcription factors
Iminobiotinylated membrane proteins and glycoproteins	Spliceosomes
	DNA-binding proteins
Biotinylated synthetic peptides	

4. AVIDIN *VERSUS* STREPTAVIDIN

In many systems, the use of the egg-white glycoprotein avidin has led to high levels of non-specific binding. Consequently, we have witnessed a trend to replace avidin with another biotin-binding protein, streptavidin, the bacterial analogue from *Streptomyces avidinii*[17]. Despite the fact that streptavidin is currently about 100 times more expensive than avidin, the replacement is sometimes justified as non-specific binding can be averted. Some of the major characteristics of the two proteins are shown in Table 3.

The major differences between avidin and streptavidin lie in the fact that streptavidin is a neutral non-glycosylated protein whereas avidin is highly alkaline and usually consists of a single oligosaccharide chain per subunit. In many systems,

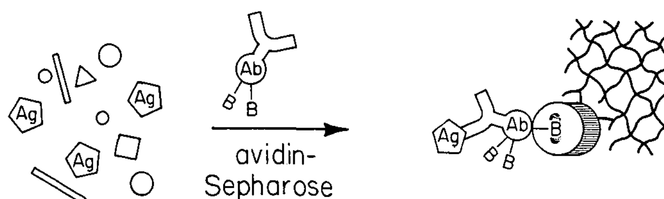

Fig. 3. Schematic diagram illustrating the isolation of an antigen on an avidin-containing resin via a biotinylated antibody. In this particular instance, avidin–Sepharose is loaded with biotinylated anti-transferrin antibody (B-Ab) and serum which contains the antigen (Ag) is applied to the column. The transferrin binds to its immobilized antibody and can be purified using acetic acid. Under these contitions, only the antigen is released from the column; the antibody remains owing to the stability of the avidin–biotin complex.

TABLE 3

SOME IMPORTANT CHARACTERISTICS OF AVIDIN AND STREPTAVIDIN

Values given for oligosaccharide, sugar and amino acid residues refer to the number of the respective groups per subunit.

Property	*Avidin*[a]	*Streptavidin*[b]	*Property*	*Avidin*[a]	*Streptavidin*[b]
Molecular weight:					
Tetramer	67 000	67 000	A_{282} (1 mg/ml)	1.54	2.7
	(60 000)	(60 000)		(1.70)	(3.4)
Subunit	16 500	16 500	Trp	4	4
	(15 000)	(15 000)	Tyr	1	6
Biotin bound[c]	1	1	p*I*	>10	<7
K_D (*M*)	*ca.* 10^{-15}	*ca.* 10^{-15}	Lys	9	8 (4)
			Arg	8	4
Oligosaccharide	1	0			
Man	4–5	0			
GlcNAc	3	0			

[a] Native avidin (non-glycosylated avidin in parentheses).
[b] Native streptavidin (truncated core streptavidin in parentheses).
[c] Per subunit.

egg-white undergoes extensive interaction with negatively charged macromolecules, *e.g.*, nucleic acids and acidic proteins. In other systems, lectins or other sugar-binding materials might interact "non-specifically" with avidin.

In view of the high cost of the bacterial protein, attempts have been made to rectify the two major undesirable characteristics of avidin. In terms of correcting the positive charge, the lysines of avidin can be easily derivatized by acetylation, succinylation, etc. Thus, a variety of avidin derivatives are commercially available with average p*I* values of 7 or lower. On the other hand, it is much more difficult to remove the carbohydrate residue from avidin. The native avidin tetramer is not generally susceptible to the action of commercially available glycosidases. However, we have recently demonstrated the isolation of a non-glycosylated form of the avidin tetramer from a commercial preparation of avidin (from Belovo, Bastogne, Belgium) which contained a heterogeneous combination of glycosylated and non-glycosylated subunit types[18]. Unfortunately, this procedure was applicable to only one batch of avidin dating from 1983 and more recent batches of avidin are heavily glycosylated. We are currently designing a procedure which may lead to the preparation of gram amounts of non-glycosylated avidin, and we are hoping that this product will eventually become commercially available.

5. STRUCTURAL STUDIES ON AVIDIN AND STREPTAVIDIN

The last few years have seen dramatic progress in the understanding of the molecular basis for the avidin–biotin interaction[10]. The primary sequences of both avidin and streptavidin are now known[19,20]. The genes for both proteins have been cloned and expressed in *E. coli*[20,21].

There is a remarkable conservation in the primary structure of the two proteins.

Avidin and the truncated form of streptavidin show an overall homology of about 40%. The conserved residues are usually confined to short homologous stretches which form relatively defined domains (Fig. 4). These homologous domains are more or less coincident with amino acid residues thought to be important to the construction of the binding site. Chemical modification studies have shown that the single tyrosine in avidin (Tyr-33) and its homologue in streptavidin (Tyr-43) play a role in biotin binding[22]. The majority of the tryptophans of both proteins, specifically those homologous to the two proteins, also appear to be involved in the binding site[23,24]. In addition, the homologies seem to be extended to the respective predicted secondary structures, which indicate a high preponderance of β-strands in both proteins connected by successive turn structures[20].

Although crystallizations of avidin have been reported since the early 1970s[25], the three-dimensional structure of streptavidin was the first to be elucidated, and only recently by two independent groups[26,27]. These studies confirmed the predicted model[20] in that the streptavidin subunit consists essentially of an extremely stable β-barrel consisting of a series of eight juxtaposed β-structures connected by turns. The biotin site is inside the barrel and, in binding biotin, some of the turns fold over to stabilize the complex.

X-ray crystallographic studies confirmed that one of the important residues for biotin binding is indeed the above-described tyrosine (Tyr-43), which is hydrogen-bonded with the carbonyl oxygen of the ureido ring. On the other hand, one of the fascinating discoveries from the X-ray studies on streptavidin is that none of the binding-site tryptophans seems to form direct bonds with biotin. Instead, four tryptophans appear to stabilize via hydrogen bonding other binding site residues which in turn interact directly with the biotin. Another surprising feature is that one of the four tryptophans (Trp-120) is donated by a neighbouring subunit. This also appears to add another factor of stability to a molecule which is already extremely resistant to dissociation and denaturation[28].

Just how close is the conservation of the three-dimensional structures and formation of the respective binding sites of avidin and streptavidin? Does the theme of secondary tryptophan interaction hold true for egg-white avidin as well as for the bacterial protein? Crystals are now available which are suitable for X-ray studies[29], and it is hoped that within a short time the three-dimensional structure of avidin will also be solved, which would allow comparative analyses of the binding site residues. The data obtained so far indicate subtle differences in the fine structures of the two proteins.

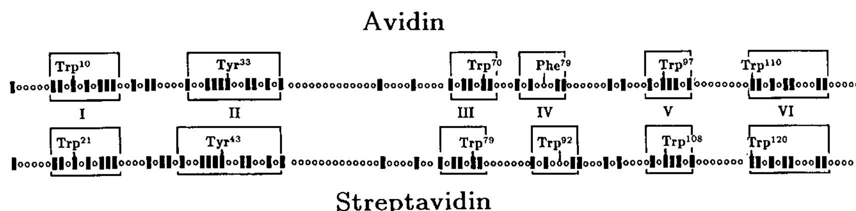

Fig. 4. Schematic description summarizing the sequence homology in egg-white avidin and a truncated form of bacterial streptavidin. Homologous amino acid resides in the two proteins are designated by ■ and unconserved residues by ○. Conserved regions are boxed in; each of the six regions contains a single aromatic amino acid residue of interest, and their respective positions are shown.

6. OTHER NATIVE BIOTIN-BINDING PROTEINS

There are other proteins in nature which bind biotin at different affinity levels (albeit much lower than those of avidin and streptavidin). These include the egg-yolk biotin-binding protein[30], the enzymes biotinidase[31] and biotin holocarboxylase synthetase[32], anti-biotin antibodies[33,34] and the biotin receptor in yeast[35] and other cells. Eventually, the primary and perhaps three-dimensional structures for many of the biotin-binding proteins will be known. In this context, the first 30 residues of the egg-yolk protein have been determined and the N-terminus bears striking resemblance to the N-terminal sequences of avidin and streptavidin[36]. The era of genetic engineering will undoubtedly contribute exciting information to this field. Indeed, a recent study of an EGF homologue from sea urchin has already revealed an unexpected and astonishing similarity in the sequence of the C-terminal domain with those of avidin and streptavidin[37]. On this basis, the authors suggested a biotin-binding function, despite the fact that the gene product has yet to be isolated.

It is hoped that such studies will eventually lead to a general definition of the contribution of given residues to the binding of biotin. This information should open the door to a flurry of site-directed mutagenesis studies, which should add a new dimension to this area. We hope that by deciphering the nature of the high–affinity avidin-biotin complex, we may be able both to improve its application further and to understand better other lower order affinity interactions.

7. ABSTRACT

During the last decade, avidin–biotin technology has become a commercially viable tool for research, medical and industrial applications. From the beginning, mediation via the avidin–biotin complex was proposed for affinity-based separations. This particular application, however, has been slow in gaining acceptance. One of the reasons is that the strength of binding between avidin and biotin is sometimes inappropriate for the desired affinity system. Another problem involves certain "undesirable" structural properties in the avidin molecule which may lead to high levels of "non-specific" binding. Recent progress in understanding the molecular requirements for binding biotin may eventually lead to the design of avidin-like proteins which will exhibit preferred recognition properties according to the desired application.

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Review

Hydrazido-derivatized supports in affinity chromatography

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1. INTRODUCTION

Biospecific affinity chromatography is now a common technique in all areas of biomedical research. Over the years, much effort has been focused on the development and understanding of affinity chromatographic systems and the practical uses thereof. In particular, the chemistry and "structure" of the support and the chemistry of immobilization of ligands have received considerable attention.

The two major requirements for the immobilization of ligands onto insoluble matrices for subsequent use in affinity chromatography are (a) a stable linkage between the matrix and the ligand and (b) retention of specific binding characteristics of the immobilized ligand. Numerous immobilization chemistries have been developed in an attempt to fulfill these requirements. However, because of the extreme diversity of the ligands being immobilized, not one methodology can be considered as universal. On the other hand, certain generalizations may be made and it is one such generalization that serves as the basis for the methodology described in this article, which concerns the immobilization of glycoconjugates specifically via their glycosylation.

Glycoconjugates are ubiquitous in nature and are involved in many cellular and extracellular events including enzymatic activities, the immune system, cell–cell recognition, hormone–receptor interactions etc. From this point of view, glycoconjugates are finding increasing importance in affinity chromatographic systems. The common feature of all glycoconjugates is the presence of one or more sugar moieties covalently

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linked to a non-sugar moiety. In addition, the oligosaccharide moiety(ies) is, in many instances, not involved in the biological activity one wishes to preserve or investigate in a chromatographic system[1–3]. This non-involvement of the oligosaccharide in ligand binding forms the basis of the methodology described below for the specific and site-directed immobilization of glycoconjugates. Particular reference is given to glycoproteins since the immobilization of proteins in general is more problematical with respect to retention of activity than the immobilization of small ligands.

2. PRINCIPLES OF THE METHOD

Specific and site-directed labelling of the oligosaccharide moieties of glycoproteins with fluorescent dyes, biotin etc. is not new and has been reviewed recently[3,4]. The methodologies employed for the site-directed immobilization of glycoproteins via their glycosylation are of course the same as those used for "tagging" glycoproteins. Surprisingly, however, this approach to the immobilization of glycoproteins, or glycoconjugates in general, has not been widely reported.

The method relies on the oxidation of glycoproteins (or other glycoconjugates), specifically on the oligosaccharide moieties. Basically two procedures are available. Galactose oxidase can be used to form a C_6 aldehyde on terminal galactose or N-acetylgalactosamine residues[5]. Since in many instances the terminal sugar of a glycoprotein is a sialic acid, particularly for glycoproteins of mammlian origin, neuraminidase treatment is required, prior to oxidation with galactose oxidase, to expose the penultimal galactosyl residue. The second procedure used to generate aldehydes on the oligosaccharide moieties is chemical oxidation of vicinal diols using sodium metaperiodate[6]. Under suitably mild conditions, periodate oxidation is reported to be specific for the generation of an exocyclic C_7 aldehyde on sialic acids[7]. The aldehydes thus generated, by either enzymatic or chemical means, may then be condensed with nucleophiles such as primary amines or hydrazine derivatives. The chemistry involved is shown schematically in Fig. 1.

The choice between a primary amino-derivatized support or a hydrazido-derivatized support is again one of specificity. Primary amines exist on lysine residues of the protein moiety of glycoproteins and are available for condensation with the aldehydes generated on the oligosaccharide moieties. This results in a competition between the support and the ligand-associated primary amines for the ligand-associated aldehydes and may cause inter- or intra-molecular cross-linking of the ligand. In addition, the Schiff base that is formed from the condensation of an aldehyde and a primary amine is unstable and needs to be reduced, preferably with sodium cyanoborohydride, to a secondary amine. The use of this reagent in itself may have a deleterious effect on the bioactivity one wishes to preserve[8].

Greater specificity can be achieved using hydrazido-derivatized matrices. This is due to the low pK of a hydrazido function (*ca.* 3, ref. 9) compared to the pK of a primary amine which is *ca.* 9–10. Both primary amines and hydrazines will only condense with aldehydes when in the unprotonated form. Therefore the marked difference in the pK values of these two groups allows one to significantly reduce the formation of Schiff bases between the oligosaccharide moieties and the protein moiety of the ligand by performing the coupling reaction under mildly acidic conditions, *ca.* pH 4.5–5.5, where the ligand-associated primary amines are protonated and unreac-

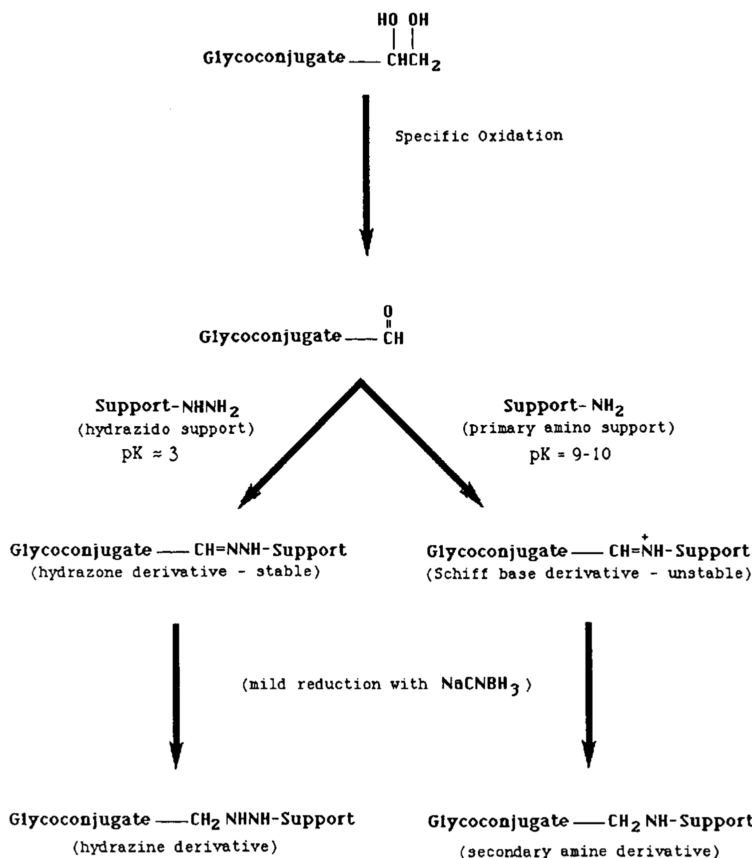

Fig. 1. Flow diagram summarizing the chemistry involved in the site-specific immobilization of glycoconjugates via their glycosylation onto hydrazido-derivatized and amino-derivatized matrices.

tive. In addition, the product of condensation between a hydrazine and an aldehyde is a stable hydrazone, obviating the need for reduction (although this can be performed if desired). The increased nucleophilicity of a hydrazine also results in an increased rate of reaction with an aldehyde, which may be advantageous in some situations. Another important advantage of hydrazido supports is that the linkage to the immobilized ligand, whether in the non-reduced or reduced forms, is non-ionizable and therefore does not add ion-exchange properties to the matrix as happens with the primary amino-derivatized supports[10].

With these points in mind, the use of hydrazido-derivatized supports is recommended over the use of primary amino-derivatized supports. Further discussion will therefore be restricted to hydrazido supports and specific examples from the literature on the uses of these supports for the immobilization of glycoconjugates will be described.

3. IMMOBILIZATION OF GLYCOPROTEINS

The first description of the immobilization of glycoproteins to hydrazido supports appears to be the immobilization of glucoamylase onto carboxymethylcellulose hydrazide[11]. This approach to immobilization was reported to be superior to immobilization via amino acid side-chains with respect to retention of enzymatic activity. However, some loss of activity as well as some precipitation of the oxidized glucoamylase was reported, as had previously been described for horseradish peroxidase[12] and α-amylase[13]. Similarly, some loss in affinity has also been described for avidin immobilized via its oligosaccharide moieties[14]. These results contrast to a report by Zaborsky and Ogletree[2] in which oxidation of glucose oxidase resulted in complete retention of both enzyme protein and enzymatic activity. Junowicz and Charm[15] also reported complete retention of activity of DNAase B after oxidation and coupling to hydrazido supports. Some question therefore remains as to the efficacy of this approach to the immobilization of glycoenzymes. However, no systematic study on the effect of oxidation on the activity of glycoenzymes has yet been presented and it is most likely this reaction which results in a loss of enzymatic activity, rather than the immobilization *per se*. All of the studies presented so far have used chemical oxidation of the oligosaccharides which may result in oxidation of some amino acid residues, thereby leading to a decrease in enzymatic activity. It is conceivable that the more specific enzymatic oxidation of the oligosaccharides would prove effective in studies on glycoenzymes.

4. IMMOBILIZATION OF ANTIBODIES

Unlike the immobilization of glycoenzymes, the results obtained for the immobilization of antibodies clearly demonstrate that site-directed immobilization via the oligosaccharide moieties is superior to amino acid-directed immobilization chemistries. Quash *et al.*[16] were the first to describe the immobilization of polyclonal immunoglobulin G (IgG) via the oligosaccharide moieties. In this report, IgG was immobilized onto hydrazido-derivatized latex particles for use in agglutination experiments. Unfortunately, no quantitation of residual activity or comparison to other methods of immobilization were described.

Recently, however, several authors have presented comparative studies on the immobilization of polyclonal IgGs for use as immunoaffinity matrices. Prisyazhnoy *et al.*[17] reported a 300% increase in activity of rabbit anti-mouse IgG immobilized onto Sepharose-hydrazide when compared to the same antibodies immobilized through SH groups onto maleimide-Sepharose. A similar increase in activity was reported by Hoffman and O'Shannessy[18] for rabbit anti-human IgG immobilized onto the hydrazide derivative of Affi-Gel 10. These authors also showed that the moles antigen bound per mole of immobilized antibody varied depending on the molecular weight of the antigen. Little *et al.*[19] also showed increases in specific antigen-binding activities from 35 to 400%, depending on the antigen/antibody pair, and demonstrated the stability of the immobilized antibodies to various eluting agents such as potassium thiocyanate, urea and high and low pH.

In each of these studies, therefore, a significant increase in antigen binding capacity of the immobilized antibodies was demonstrated for "oriented" *versus*

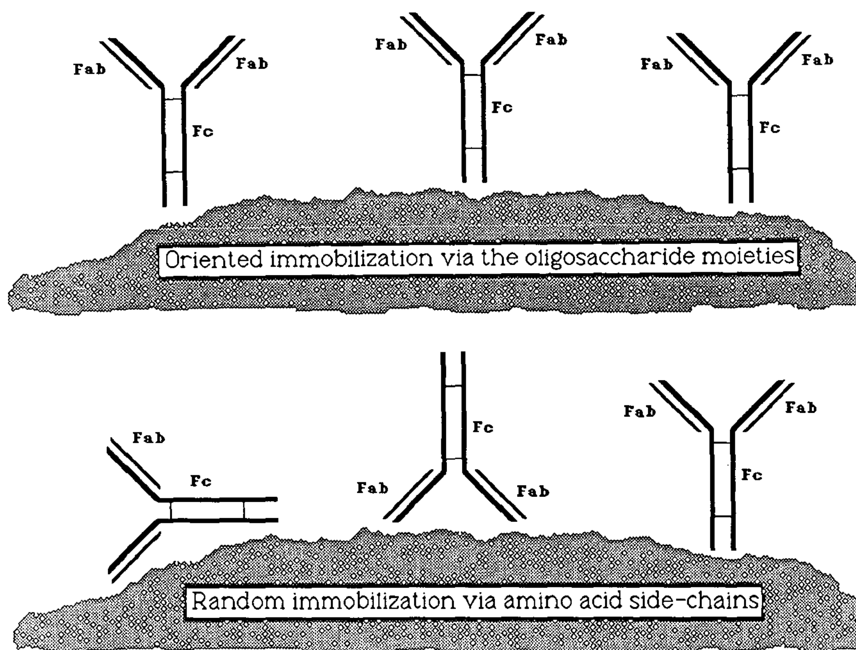

Fig. 2. Schematic depicting the inferred differences in the orientation of antibodies immobilized via "random" amino acid-directed chemistries and via "oriented" oligosaccharide specific chemistries.

"random" coupling procedures (see Fig. 2). The oligosaccharide moieties of polyclonal IgGs are primarily located on the Fc portion of the molecule such that site-directed immobilization via the oligosaccharide moieties should result in the antigen binding Fab regions being oriented away from the matrix, resulting in greater accessibility of antigen. This is similar to the immobilization of IgG onto protein A supports, followed by cross-linking of the IgG to the protein A (ref. 20). Although the oriented *versus* random immobilization model is somewhat oversimplified, the increased retention of antigen binding activity, coupled with the excellent stability of such conjugates, demonstrates the efficacy of this technique for the immobilization of antibodies.

Several other reports on the use of this procedure have been published but no quantitative data presented. Interestingly, two reports on the immobilization of monoclonal antibodies by this procedure did not demonstrate an increase in antigen-binding activity as would be expected from the results reported for the immobilization of polyclonal antibodies, although the activity was comparable to other immobilization techniques[21,22]. The reasons for the lack of increase in activity with monoclonal antibodies are not known. Table 1 lists the known examples from the literature of the site-directed immobilization of glycoproteins via their glycosylation.

5. GENERAL COMMENTS ON THE IMMOBILIZATION OF GLYCOPROTEINS

The immobilization of glycoproteins onto hydrazido supports requires prior oxidation of the oligosaccharide moiety(ies), an indication of the specificity of the

TABLE I

IMMOBILIZATION OF GLYCOPROTEINS ONTO HYDRAZIDO-DERIVATIZED SOLID SUPPORTS

Glycoprotein	*Support*	*Ref.*
Glucoamylase	Cellulose	11
DNAase B	Cellulose	15
Polyclonal IgG, viral agglutinins	Latex	16
Avidin	Sepharose	14
Glucose oxidase, glucoamylase	Polyethyleneimine	23
Polyclonal IgG	Agarose	24
Polyclonal IgG, avidin, HRP[a]	Agarose	25
Polyclonal IgG	Agarose	18
Invertase	Cellulose	26
Polyclonal IgG	Sepharose, Separon	17
Monoclonal IgG	Matrex-Pel-102	21
Polyclonal IgG	Agarose	19
Polyclonal IgG	Methacrylate	22
Monoclonal IgG_1	Agarose	27
Polyclonal IgG	AvidGel AX	28

[a] HRP = Horse radish peroxidase.

reactions. In addition, a number of authors have shown that the product formed on reaction of an oxidized glycoprotein with a hydrazido support is stable, without reduction, to a number of common eluents such as urea and thiocyanate, as well as to extremes of pH (2–10). The immobilization of glycoenzymes via their glycosylation has also been shown to increase their stability, particularly with respect to temperature.

The reaction of an oxidized glycoprotein with a hydrazido support is a specific acid-catalysed reaction and shows a pH maximum at around 3 (ref. 25). Acetate buffer appears to be the most suitable for the immobilization and buffers containing primary amines, such as Tris, should be avoided. In addition, immobilization is independent of the p*I* of the glycoprotein since fetuin (p*I* = 3.3), human IgG (p*I* = 5.8-7.3) and avidin (p*I* = 10.5) have all been shown to bind to the hydrazide derivatives of Affi-Gels (Bio-Rad)[25]. However, the rate of immobilization of a glycoprotein does appear to be dependent on the degree of glycosylation and possibly the "type" of glycosylation. In this respect, the increased binding of glycoproteins at lower pH values may in part be due to a partial unfolding of the protein, thus exposing the oxidized oligosaccharide and facilitating binding to the hydrazido support.

6. IMMOBILIZATION OF NUCLEOTIDES, NUCLEOSIDES AND RNA

Periodate oxidation of RNA has been shown to be specific for the 3′-terminal *cis*-diol resulting in the formation of a reactive dialdehyde, which may then be condensed with primary amines, hydrazines or other suitable nucleophiles. This technique has long been used for the isolation, purification and analysis of tRNAs[29]. Similarly, RNA species immobilized onto hydrazido supports have been used for the affinity purification of, for example, hybridizable DNA[30], ribosomal proteins[31–34] and the C_5 protein sub-unit of RNAase P[35]. Since only one reactive site (dialdehyde) is

generated per RNA molecule, this technique leads to an extremely site-specific and oriented immobilization of RNA, with the result that the RNA is literally projecting into the liquid phase, anchored only by the 3′-terminal sugar.

Nucleosides, nucleotides and coenzymes possessing vicinal hydroxyls have also been immobilized onto hydrazido supports following oxidation with sodium periodate[36]. Examples of the use of such supports include the purification of glucose-6-phosphate dehydrogenase on agarose-NADP and the adsorption of heavy meromyosin onto agarose-ATP[36]. Pyridoxal 5′-phosphate has also been immobilized onto various hydrazido supports and used for the purification of apo-aspartate aminotransferase[15]. In the case of pyridoxal 5′-phosphate, no oxidation step is required as this compound contains an aldehydic function.

7. MISCELLANEOUS COMPOUNDS IMMOBILIZED ONTO HYDRAZIDO SUPPORTS

The immobilization of sugars onto hydrazido supports for the affinity purification of lectins has been described[37]. In this case, use is made of the linear–cyclic equilibrium of the reducing end sugar, which in the linear form exists as an aldehyde. The aldehyde will condense with the hydrazido support and the hydrazone produced is stabilized by performing the reaction in the presence of sodium cyanoborohydride (see Fig. 1). In a similar manner, heparin has been immobilized onto hydrazido supports and used in studies on heparin-binding proteins[38]. It is worth noting at this stage that multi-site attachment of ligands to the hydrazido support is thought to stabilize the ligand–matrix complex. Single-site attachment of ligands may result in a less stable bond, depending on proximal function groups, and reduction of the hydrazone in such situations is recommended.

Parikh and Cuatrecasas[39] described the preparation and use of ganglioside-agarose derivatives. The gangliosides were oxidized with periodate, allowed to couple to a polyhydrazido-agarose [poly(L-lysyl-DL-alanyl-hydrazido)-agarose] and subsequently reduced with sodium borohydride. Such preparations were useful for the affinity purification of cholera toxin. The use of polyhydrazido-agarose derivatives has been reported to decrease the "leakage" of ligands from such supports. Neoglycoproteins, such as glycosyl albumin, have also been immobilized in a site-specific manner onto hydrazido derivatives of cellulose[40].

Ligands containing functional groups other than aldehydes may also be immobilized onto hydrazido-derivatized supports. Examples of this include the immobilization of the tresyl ester of T-2 fungal toxin for the affinity purification of anti-T-2 antibodies[41] and the immobilization of proteins and other ligands through carboxylic acid functions using carbodiimide activation of the ligand[42,43]. It is worth noting that hydrazido derivatives of a number of support matrices have been used as intermediates in the synthesis of other "activated" supports, such as acyl azides[43].

8. CONCLUDING REMARKS

In the foregoing brief discussion, the methodology for the site-directed immobilization of glycoconjugates onto insoluble matrices, and the uses thereof, were presented. As previously stated, no single immobilization chemistry can be considered as universal. However, it is clear from the literature examples cited that site-directed

immobilization of glycoproteins, particularly antibodies, has inherent advantages not the least of which is retention of biological activity. In the case of nucleosides, nucleotides and RNA, immobilization onto hydrazido-derivatized supports would appear to be the method of choice. Hydrazido-derivatized matrices, along with the associated chemistries, should therefore be added to the repetoir of chemistries available for immobilization of ligands onto insoluble matrices in the preparation of affinity supports.

9. ABSTRACT

Many chemistries have been developed for the immobilization of ligands onto insoluble matrices for subsequent use in affinity systems. One such chemistry which has received little attention involves the use of hydrazido-derivatized solid supports. Hydrazine derivatives are strong nucleophiles which will react with a number of functional groups including aldehydes which may be generated on the oligosaccharide moieties of glycoconjugates by specific oxidation reactions. This paper presents a brief overview of the chemistries involved and the uses of hydrazido-derivatized solid supports for the site-directed immobilization of glycoconjugates. Specific examples from the literature on the uses of affinity matrices prepared by this method are cited.

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Studies on the interaction of a surface-bound ligand with a multi-valent high-molecular-weight ligate

The biotinylcellulose–avidin system

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ABSTRACT

Two types of biotinylated cellulose disks were examined: filter-paper disks to which biotin had been covalently attached directly to the paper surface (biotinylcellulose) and disks on which biotin was attached to polyacrylamide side-chains grafted onto the filter-paper surface (biotinylpolyacrylamide–cellulose). The amount of avidin taken up from solution by these disks was linearly related to the avidin input concentration and could be estimated by exposure to [^{14}C]biotin. The avidin-binding capacity of the disks depended on the surface density of covalently attached ligand and exhibited hyperbolic, Langmuir-type behaviour for both types of disks. The [^{14}C]biotin binding capacity of avidinylated disks, on the other hand, showed anomalous, biphasic behaviour: at higher ligand densities, a decrease in [^{14}C]biotin binding was observed. The largest anomalies were obtained with biotinylpolyacrylamide–cellulose disks. Calculated ratios of bound [^{14}C]biotin *vs.* amount of avidin tetramer (B/A_4) showed a similar biphasic behaviour. A constant value of $B/A_4 = 3$ was obtained at low ligand densities, whereas B/A_4 decreased monotonously with increasing ligand density and asymptotically approached $B/A_4 = 1$. The data could be explained by assuming that at high ligand densities tetrameric avidin interacts with more than one surface-bound biotin residue.

INTRODUCTION

The specificity and ease of operation of solid-phase affinity systems has fostered their widespread use in the analysis and separation of both large and small biomolecules[1–8]. However, there are numerous instances where such systems are reported to exhibit severe functional anomalies. Mainly these entail failures in recognition, where

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the soluble ligand does not bind to a support to which it ought to bind. Alternatively, specifically bound ligands cannot be eluted under reasonably mild conditions[9–11]. This paper deals with mechanisms underlying phenomena of this nature.

In previous papers we described a simple, rapid assay for biotin based on filter-paper disks modified to contain covalently attached biotin[12,13]. The assay involves sequential exposure of these biotinylated disks to avidin and biotin as indicated in Fig. 1. Thus, biotinylated disks can be saturated with avidin by specific adsorption of the protein through one or more of its four binding sites and then allowed to take up biotin from an unknown test sample before being exposed to excess of radioactive biotin in order to determine the fraction of biotin sites on filter-bound avidin remaining unoccupied. The rationale for this sequential competition assay was the extremely low dissociation constant (*ca.* 10^{-15} *M*) of the avidin–biotin complex[14]. Thus, for all practical purposes, avidin-bound biotin will not exchange with free biotin in solution. Simple modification of the method allowed it to be used for the determination of avidin in unknown test solutions[13].

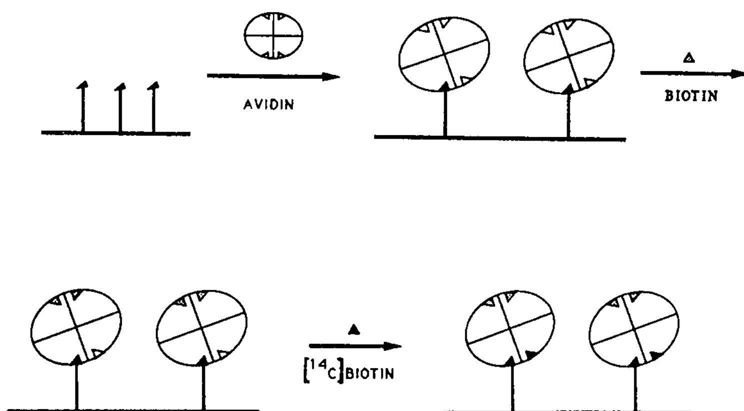


Fig. 1. Sequential competition assay for biotin.

The major limitation of he method as originally described was its narrow linear assay range. However, attempts to extend the useful range of the assay by increasing the density of covalently bound biotin in order to raise the avidin capacity of the disks did not produce the hoped for result. Although the amount of avidin adsorbed per disk did indeed increase as the density of matrix biotin was raised, this was not paralleled by a corresponding rise in the ability of the disks to take up free biotin from solution. On the contrary, in some instances a drastic decrease in biotin-binding capacity was observed at very high surface-bound biotin densities. This paper reports a systematic investigation of the deterioration phenomenon and proposes an explanation that probably has more general validity. The proposed model also suggested a practical approach to the problem of designing disks with wider linear ranges for biotin and avidin.

EXPERIMENTAL

Materials

Chromatographically purified avidin (10–15 U/mg protein), bovine serum albumin (BSA, fraction V), egg-white lysozyme and *d*-biotin were obtained from Sigma (St. Louis, MO, U.S.A.). *d*-Carbonyl[^{14}C]biotin (50 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, U.K.). Potassium *tert.*-butoxide, obtained from Fluka (Buchs, Switzerland), was stored in small batches in hermetically sealed vials in a desiccator and used only once. 1-Tosyloxy-3-isocyanopropane was purchased form Fluka. All common reagents were of the purest grade available.

Disks of 12 mm diameter were cut from sheets of Whatman Grade 542 filter-paper (ashless, hardened) (Whatman, Maidstone, U.K.). Anhydrous dimethyl sulphoxide (DMSO) (Merck, Darmstadt, F.R.G.) was routinely treated with molecular sieve before use. Acetaldehyde was redistilled and stored in full tightly stoppered bottles at −20°C for up to 2 months.

Polyacrylation of cellulose disks

Polyacrylamide side-chains were grafted onto cellulose filter-paper disks by a modification of the procedures of Mino and Kaizerman[15] and Müller[16]. Untreated disks (7 g; about 1000 12-mm disks) were placed in a three-necked, round-bottomed flask connected to a mechanical shaker. An aqueous solution of acrylamide (3–6 g in 240 ml of water) was then added and the suspension was exhaustively deaerated under a water pump and then flushed with nitrogen. Graft polymerization was initiated by the addition of stock cerium(IV) ammonium nitrate solution (0.1 *M*) to a final initiator concentration of $4 \cdot 10^{-3}$ *M* (Type I disks) or $4 \cdot 10^{-5}$ *M* (Type II) disks (Table I). The polymerization reaction was allowed to proceed for 2 h at room temperature under nitrogen with gentle mechanical shaking. The grafted disks were then exhaustively washed with water and taken for hydroxymethylation. The nitrogen content of such disks was determined by the Kjeldahl method[17].

Hydroxymethylation of polyacrylamide side-chains on grafted disks

Hydroxymethyl groups were introduced on the polyacrylamide side-chains of the grafted disks essentially as described by Amarant and Bohak[18]. Washed, grafted disks (7 g) were suspended in an aqueous solution (200 ml) 5.2 *M* in formaldehyde and 0.05 *M* in sodium carbonate and allowed to react for 2 h at 50°C in a 250-ml

TABLE I

BASIC TYPES OF POLYACRYLAMIDE–CELLULOSE DISKS

Parameter	*Type I disks*			*Type II disks*		
Batch no.	1	2	3	4	5	6
Ce^{IV} concentration (*M*)		$4 \cdot 10^{-3}$			$4 \cdot 10^{-5}$	
Acrylamide monomer concentration (%)	1.25	2.0	2.5	1.25	2.5	5.0
Acrylamide content (μmol per disk)	5.5	7.5	10.8	5.6	7.7	8.9

stoppered flask with mechanical shaking. The disks were then exhaustively washed with water, methanol and diethyl ether and air dried. The dry disks were stored in a desiccator over phosphorus pentoxide.

Introduction of isonitrile functional groups

Isonitrile (–NC) functional groups were introduced on cellulose and hydroxymethylated polyacrylamide–cellulose disks as described previously[12,13,19–21]. Appropriate disks (7 g; about 1000 disks) were suspended in 85 ml of DMSO, allowed to swell with gentle mechanical shaking for 30 min in a stoppered flask and then brought to a final potassium *tert.*-butoxide concentration of 0.005–0.05 *M* by slow addition of 50–500 mg of the compound. Ionization of hydroxyl groups was allowed to proceed to equilibrium at 40°C for 15 min before addition of 1-tosyloxy-3-isocyanopropane (1.3 g, 0.006 mol) dissolved in DMSO (2.5 ml). The malodorous reaction was allowed to proceed for 4 h at 40°C with gentle mechanical shaking in a hood and the disks were removed, placed in a funnel, washed once with DMSO, several times with methanol, then diethyl ether and air dried. The disks could be stored indefinitely in closed vials at 4°C.

Biotinylation

Isonitrile disks (7 g) were suspended in 80 ml of ice-cold 0.1 *M* Tris buffer (pH 7) containing 50 mg of *d*-biotin in a tightly stoppered vessel. Cold, redistilled acetaldehyde (0.35 ml) was then added and the reaction was allowed to continue at 4°C for 18–24 h with gentle mechanical shaking. The disks were removed to a new vessel and then continuously flushed with tap water for 24 h in order to remove all traces of unbound biotin. The disks were sequentially washed with deionized water, methanol and diethyl ether on a suction funnel and air dried. Disks stored in the dry state are stable indefinitely, even at room temperature.

Avidinylation of biotinylated disks

Saturative avidinylation for biotin assay purposes was carried out with either commercial, chromatographically purified egg-white avidin or diluted egg-white as the avidin source[13], as follows.

About 20 disks were immersed for 3–4 h at 37°C in 10 ml of a solution containing 40–80 $\mu g\ ml^{-1}$ purified tetrameric avidin in 0.5 *M* potassium phosphate buffer (pH 7) made 0.005 *M* in cetyltrimethylammonium bromide (CTAB) and 0.01 *M* sodium azide. The mixture of polyvalent anion plus cationic detergent minimizes non-specific adsorption of avidin (a glycoprotein) to the cellulosic matrix of the disk.

Egg-white from one fresh egg (*ca.* 30 ml) was slowly mixed with one volume of 2% (v/v) Triton X-100 while magnetically stirring at room temperature. The resulting homogenate was then diluted 1:1 with 0.5 *M* potassium phosphate buffer (pH 7) made 5 m*M* in CTAB and 0.01 *M* in sodium azide and the gelatinous precipitate formed was removed by centrifugation at 8000 *g* and room temperature for 20 min. The clear supernatant (*ca.* 120 ml) was used without further treatment as a source of avidin tetramer. Depending on the disk capacity, overnight exposure to 1–2 ml of this solution at room temperature sufficed for saturative avidinylation. Avidin exposures of longer than 24 h are to be avoided for reasons that will become evident under Results and Discussion. Avidinylated disks were exhaustively washed with water prior to use.

Sequential competition assay for biotin[12,13]

The maximum capacity of a given batch of avidinylated disks for biotin was determined by immersion (for 30 min) in excess of [^{14}C]biotin (100 ng/ml per disk) followed by extensive washing with water, drying and counting in a β-scintillation spectrometer. Additional disks of the same batch were immersed in unknown biotin solutions (or dilutions thereof) for 3 h (five disks per 5-ml sample) and then washed extensively with water. The residual biotin-binding capacity of disks previously exposed to unknown biotin solution was determined by saturative exposure to [^{14}C]biotin as above. Subtraction of this residual value from the originally determined maximum disk capacity directly measured the amount of biotin in a sample (see Results and Discussion). Given the unusually high affinity of avidin for biotin (K_D = 10^{-15} M)[14], there is essentially no exchange of unlabelled biotin with ^{14}C-labelled ligand.

Avidin assay[13]

The ability of biotinylated disks to remove avidin from crude mixtures forms the basis of a simple, quantitative assay for avidin.

Pretreatment of disks and glassware. Biotinylated disks were immersed in a solution containing 4 mg/ml each of lysozyme and BSA in PTA buffer [0.5 M potassium phosphate–1% (v/v) Triton X-100–0.01 M sodium azide, pH 7] for 10–15 min in order to minimize subsequent non-specific adsorption of avidin and other glycoproteins. Similarly, presoaking of glassware, micropipette tips and plastic microplates in a five-fold diluted solution of detergent-treated egg-white (see above) prevented loss of sample avidin to the walls of these containers. Quantitative measurements of standard avidin solutions are not obtained when these pretreatments are omitted.

Uptake of avidin from solution by biotinylated disks. Biotinylated disks pretreated as described above were placed, one disk per well, in the wells of a tissue-culture microplate of appropriate size. Avidin-containing test solutions were diluted 1:1 with double-strength PTA buffer and 200 μl of diluate were then placed on each disk. The plates were covered, incubated at 37°C for 3 h, washed successively with PTA buffer and water and then immersed in a standard solution of radioactive biotin to saturate the biotin-binding sites of avidin molecules taken up from the test solution. Based on results with purified avidin solutions, the relationship between the amount of radioactivity bound to disks and the avidin concentration in the test samples was linear.

Determination of –NC functional group content of isonitrile–cellulose and isonitrile–polyacrylamide–cellulose disks.

The –NC content of disks was estimated from the amount of [^{35}S]methionine bound by a four-component condensation (4CC) reaction[19,21]. Sets of five disks were suspended in 10 ml of 2 mM [^{35}S]methionine (specific radioactivity *ca.* 10^6 dpm μmol^{-1}) in 0.1 M potassium phosphate–0.5 M potassium acetate (pH 8) in a stoppered vial. Ice-cold acetaldehyde was added to a final concentration of 0.45 M (25 μl ml^{-1}) and the reaction mixture was shaken gently overnight at 4°C. Disks were removed, washed exhaustively with water and then successively washed with methanol and diethyl ether. The disks were air-dried and counted in a β-scintillation spectrometer at ^{35}S settings.

Direct determination of disk-bound avidin tetramer (A_4).

The avidin content of diluted egg-white solution was determined as described and then radioactively labelled with an equimolar amount of [^{14}C]biotin. Exposure of biotinylated disks to the labelled avidin solution made it possible to determine directly the precise amount of avidin tetramer bound to a disk.

RESULTS AND DISCUSSION

Preparation of biotinylated disks

The two basic types of functionalized disks used were isonitrile–cellulose, in which –NC functional groups were attached to the cellulose matrix itself, and isonitrile–polyacrylamide–cellulose, in which the –NC functional groups were attached to polyacrylamide side-chains grafted on the cellulosic matrix.

Polyacrylation of cellulose disks. Cellulose disks bearing polyacrylamide side-chains were prepared by graft polymerization of acrylamide according to a procedure[16] based on that of Mino and Kaizerman[15] for materials carrying surface primary or secondary alcoholic groups. According to the proposed mechanism, graft polymerization is started by a radical formed on the α-carbon of the hydroxylic function by the action of Ce^{IV} ion (reduced to Ce^{III} ion). The surface density of initiated polyacrylamide chains would hence depend on the concentration of Ce^{IV} ion.

Fig. 2 shows that the nitrogen content of grafted disks increases monotonously with increasing acrylamide monomer concentration (at $[Ce^{IV}] = 10^{-3}$ M) and approaches asymptotically a limiting value of about 22 μmol per disk. In addition, the nitrogen content of the disks was found to be relatively insensitive to the concentration of Ce^{IV} in the range 10^{-3}–10^{-5} M (Table I). The concentration of the polymerization initiator therefore appears to control the surface density of polyacrylamide chains, but not the amount of acrylamide incorporated. This finding suggests that the Type II disks defined in Table I (*i.e.*, those prepared in the presence of 10^{-5} M Ce^{IV}) will have fewer, but longer, polyacrylamide side-chains than Type I disks (prepared in the presence of 10^{-3} *M* Ce^{IV}).

Introduction of isonitrile functional groups on cellulose and polyacrylamide–cellulose disks. The procedure used to introduce isonitrile functional groups on hydroxylic polymeric supports (*e.g.*, cellulose and other polysaccharides) has been described

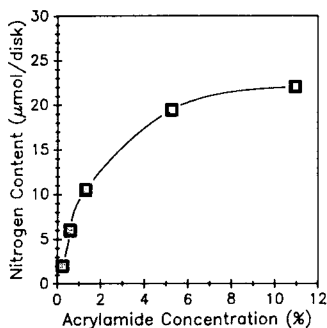
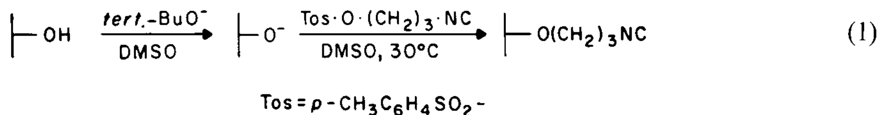


Fig. 2. Nitrogen content of polyacrylamide–cellulose disks as a function of acrylamide monomer concentration (Ce^{IV} ion concentration 4×10^{-3} *M*).

elsewhere[12,13,19,21]. In essence, it involves partial ionization of hydroxylic groups on the support with a strong base in a polar aprotic solvent (potassium *tert.*-butoxide in DMSO) followed by nucleophilic attack of the polymeric alkoxide ions on an isonitrile containing a good leaving group in the ω-position, *viz.*, 1-tosyloxy-3-isocyanopropane (p-$CH_3C_6H_4SO_2O(CH_2)_3NC$):

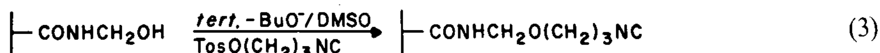


(1)

(where Bu = C_4H_9).

The procedure outlined in eqn. 1 could also be used to introduce –NC functional groups onto polyacrylamide side-chains of polyacrylamide–cellulose disks following partial hydroxymethylation of side-chain amide groups by treatment with concentrated formaldehyde at alkaline pH[18,20]:

(2)



(3)

Fig. 3 shows that the –NC content of both cellulose and hydroxymethylated polyacrylamide–cellulose disks is a linear function of *tert.*-butoxide concentration. The linear dependence on base concentration allowed considerable flexibility in preparing disks having a wide range of functional group densities.

Biotinylation of functionalized disks. Biotin was covalently attached to functionalized disks by a 4CC reaction carried out in an aqueous buffer at neutral pH[19–21]. As shown in eqns. 4 and 5, 4CC reactions involve the simultaneous participation of amine, carboxylate, aldehyde and isonitrile, and lead to the formation of a stable N-substituted peptide bond between the carboxylate (R^1COOH) and amine (R^2NH_2)

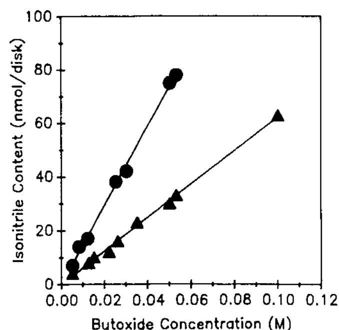
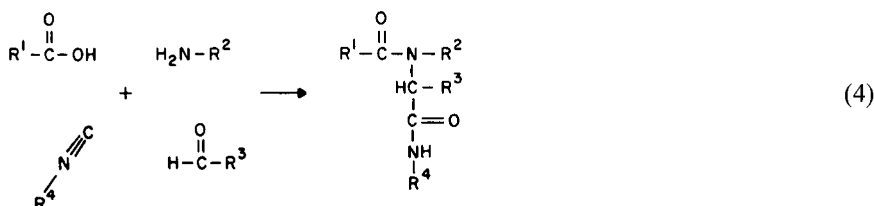
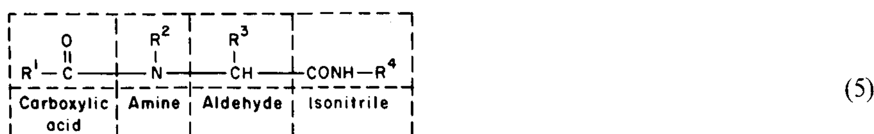


Fig. 3. Dependence of the isonitrile content of cellulose (▲) and polyacrylamide–cellulose disks (●) on the concentration of potassium *tert.*-butoxide, at a constant concentration of 1-tosyloxy-3-isocyanopropane (0.05 *M*).

moieties in which the aldehyde and the isonitrile components (R^3CHO and R^4NC) appear as the side-chain attached to the amide nitrogen. In our case, R^1COOH is biotin, NH_2 groups are supplied by Tris buffer, the carbonyl component is acetaldehyde and R^4NC is the functionalized cellulose or polyacrylamide–cellulose support.



(4)



(5)

Representative data relating some properties of biotinylcellulose and biotinyl-polyacrylamide–cellulose disks to functional group density are presented in Tables II and III (the data are discussed below).

Properties of biotinylcellulose disks

Fig. 4 summarizes the avidin- and biotin-binding properties of biotinylcellulose disks of varying functional group density (see also Table II). As far as their ability to bind avidin was concerned, such disks exhibited simple Langmuir-type saturation behaviour with increasing –NC group density. On the other hand, a biphasic response

TABLE II

BASIC CHARACTERISTICS OF BIOTINYLATED CELLULOSE DISKS

Batch No.	*–NC content*[a] *(nmol per disk)*	*Binding capacity*		*B/A*$_4$ *(mol/mol)*	*Disk designation*
		Avidin (μg per disk)	*[*^{14}*C]Biotin (ng per disk)*		
1	1.5	1.1	12	3	"High" efficiency
2	2.3	1.6	17	3	
3	4.0	2.6	29	3	
4	5.0	3.4	36	3	
5	6.1	4.0	44	3	
6	7.7	3.9	42	2.9	
7	9.1	4.0	43	3	
8	11.3	3.8	43	2.9	
9	13.8	3.7	38	2.7	"Low" efficiency
10	20.4	3.9	36	2.4	
11	40.0	4.0	26	1.8	
12	65.0	4.0	23	1.6	
13	100.0	3.9	22	1.6	

[a] Calculated from the amount of [^{35}S]methionine bound (see Experimental).

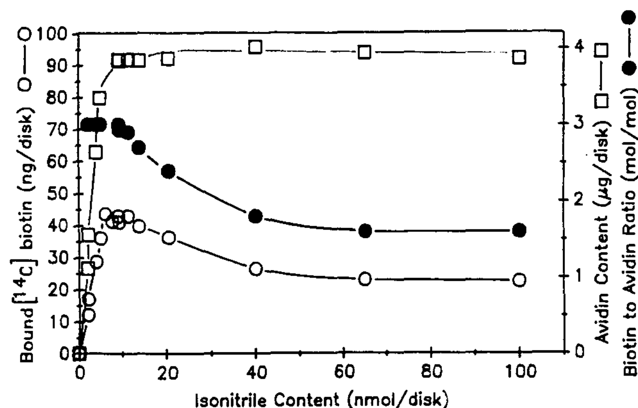


Fig. 4. Effect of functional group density on the subsequent ability of biotinylcellulose disks to bind avidin and radioactive biotin. ○ = [^{14}C]Biotin-binding capacity; □ = bound avidin content; ● = Biotin to tetrameric avidin ratio (B/A_4).

was observed when biotinylcellulose disks previously exposed to saturating amounts of avidin were challenged to radioactive biotin. The intitial rise in [^{14}C]biotin-binding capacity observed as the –NC group density increased to about 10 nmol per disk was followed by a monotonous decrease in biotin-binding capacity with a further increase in –NC density. This anomaly is emphasized when calculated molar ratios of biotin to avidin (B/A_4) are examined. Up to a critical –NC value (*ca.* 10 nmol per disk), B/A_4 assumes the theoretical value of 3 (reflecting the attachment of individual avidin tetramers to biotinylated disks via a single biotin-binding site). Beyond the critical inflection point, however, the apparent B/A_4 values decline asymptotically toward a value of *ca.* 1.5. Two types of biotinylcellulose disks could be defined operationally by the data in Fig. 4 and Table II: "high" efficiency disks ($B/A_4 \approx 3$) with functional group densities not exceeding 10 nmol per disk and "low" efficiency (*i.e.* high –NC group density) disks characterized by low biotin-binding capacities and sub-optimum (<3) B/A_4 ratios.

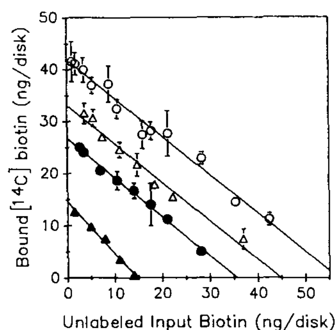


Fig. 5. Biotin assay ranges of avidinylated biotinylcellulose disks with diverse functional group densities. Isonitrile and avidin contents of tested batches of disks were (per disk): (▲) NC = 2.1 nmol, A_4 = 1.12 μg; (●) NC = 4.0 nmol, A_4 = 2.64 μg; (△) NC = 5.0 nmol, A_4 = 3.35 μg; (○) NC = 11.3 nmol, A_4 = 3.9 μg.

TABLE III

BASIC CHARACTERISTICS OF BIOTINYLATED POLYACRYLAMIDE–CELLULOSE DISKS

Disk Type[a]	*Batch No.*	*Acrylamide content (μmol per disk)*	*−NC content (nmol per disk)*	*−NC/AAm*[b]	*Binding capacity*		*B/A*$_4$ *(mol/mol)*
					Avidin (μg per disk)	*[^{14}C]Biotin (ng per disk)*	
Type I	1	5.5	3.9	0.0007	2.4	7.5	0.9
			13.3	0.0020	–	17	–
			42.5	0.0077	1.5	20	0.9
			76.7	0.0140	6.0	24	1.1
	2	7.5	10.2	0.0140	6.0	17	0.3
	3	10.8	7.1	0.0007	2.9	10	1.0
			15.8	0.0015	4.0	20	1.4
			40.0	0.0037	5.7	34	1.6
			48.0	0.0045	5.7	32	1.5
			300	0.0280	6.6	5.0	0.8
Type II	4	5.6	52.0	0.0090	8.2	90	3.0
	5	7.7	1.9	0.0003	0.7	7.2	3.0
			23.0	0.0030	4.5	46	3.0
			38.0	0.0050	7.3	63	2.8
			53.5	0.0070	7.7	77	2.7
	6	8.9	58.0	0.0065	8.7	95	3.0

[a] See Table I.

[b] –NC/AAm = side-chain isonitrile to acrylamide molar ratio.

Fig. 5 shows biotin assay ranges of "high"-efficiency biotinylcellulose disks of varying functional group content. As detailed under Experimental, sequential competition assay[12,13,22] for biotin consists of saturative avidinylation of the disks followed by their serial exposure to unknown or standard biotin solution and enough [^{14}C]biotin to ensure occupation of all remaining biotin sites on bound avidin molecules. The data show that every batch of disks examined gave a linear biotin-binding response whose range was a simple function of disk –NC density. As biotinylated disks remove avidin from solution, the batches of "high"-efficiency biotinylcellulose disks described above could also be used for the determination of avidin. As indicated in Fig. 6, it was always possible to obtain a range of avidin concentration over which the relationship between the amount of avidin added and final amount of radioactive biotin bound was linear (see Experimental for details). Moreover, the slopes of the linear regression lines so obtained were all identical. The avidin assay range of these disks was nonetheless limited as there was a tendency to depart from linear behaviour at avidin inputs below the saturation binding value imposed by their respective functional group densities (see also ref. 22). These limitations to the assay range prompted us to construct the grafted disks explored below.

Properties of Type I biotinylpolyacrylamide–cellulose disks

Fig. 7 indicates that Type I biotinylpolyacrylamide–cellulose disks (Table III) resemble biotinylcellulose disks in certain of their properties. Specifically, their avidin- and free biotin-binding capacities exhibit hyperbolic saturation behaviour and a biphasic response in relation to disk functional groups density, respectively. However, the avidin saturation value of these disks is roughly 1.5 times higher than that of the best biotinylcellulose disks obtained and, further, the inflection point of the [^{14}C]biotin-binding curve after saturative avidinylation occurs at a disk –NC content of *ca.* 40 nmol rather than the 10 nmol characteristic of biotinylcellulose disks.

Another striking property of Type I biotinylpolyacrylamide–cellulose disks evident from examination of Fig. 7 is their generally low apparent B/A_4 value. At even the optimum –NC functional group density this value never reaches 2, and falls well below 1 as the –NC content is raised even higher (see also Table III). In other words, disks with high enough functional group densities in effect become inactive. Thus, as indicated by the biotin and avidin assay curves in Figs. 8 and 9, the ligand-binding

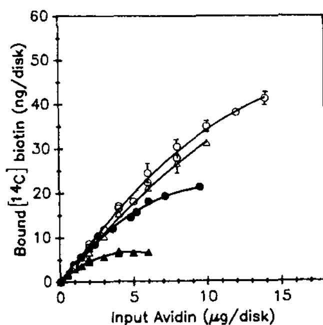


Fig. 6. Avidin assay ranges of biotinylcellulose disks. Batches of disks as in Fig. 5.

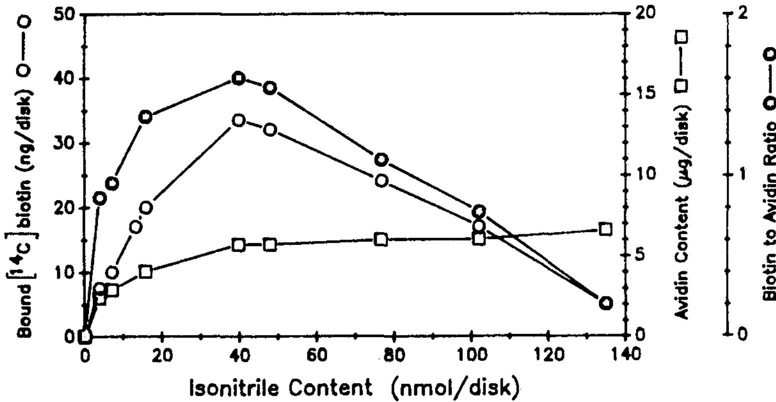


Fig. 7. Effect of functional group density on the subsequent ability of Type I biotinylpolyacrylamide–cellulose disks to bind avidin and radioactive biotin. (○) [^{14}C]Biotin binding capacity; (□) = bound avidin content; (**O**) = biotin to tetrameric avidin ratio. Details of disk preparation are presented under Experimental and in Tables I and III.

capacity progressively increases with increasing disk –NC content up to the inflection point (Fig. 7), and then declines nearly to zero. Also note the extended linear avidin assay range of the Type I polyacrylamide–cellulose disks in Fig. 9 compared with that of the cellulose disks in Fig. 6, in addition to the dependence of the slopes of the avidin response curves on disk –NC content. "High"-efficiency biotinylcellulose disks show no such dependence in the linear portions of their response curves.

Properties of Type II biotinylpolyacrylamide–cellulose disks

It will be recalled that the acrylamide contents of Type I and Type II polyacrylamide–cellulose disks were very similar (Table I and Fig. 2). As the concentration of polymerization initiator (Ce^{IV} ion) used to start the growth of polyacrylamide side-

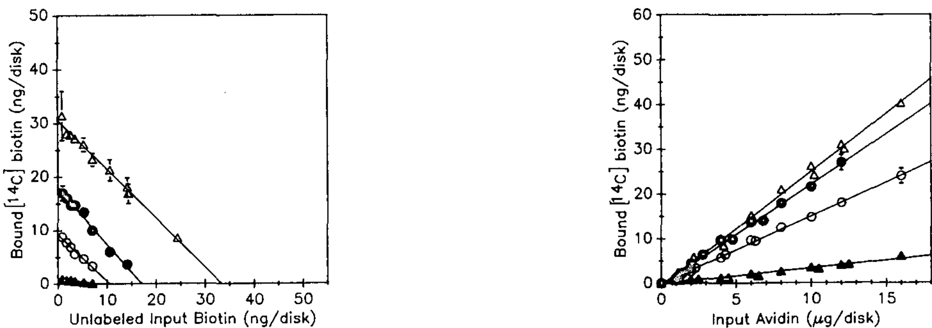


Fig. 8. Biotin assay ranges of avidinylated Type I biotinylpolyacrylamide–cellulose disks with diverse functional group densities. Isonitrile and avidin contents of tested batches of disks were (per disk): (○) NC = 7.1 nmol, A_4 = 2.9 μg; (**O**) NC = 15.8 nmol, A_4 = 4.0 μg; (△) NC = 48.0 nmol, A_4 = 5.7 μg; (4) (▲) NC = 301 nmol, A_4 = 6.6 μg (see also Table III).

Fig. 9. Avidin assay ranges of Type I biotinylpolyacrylamide–cellulose disks. Batches of disks as in Fig. 8.

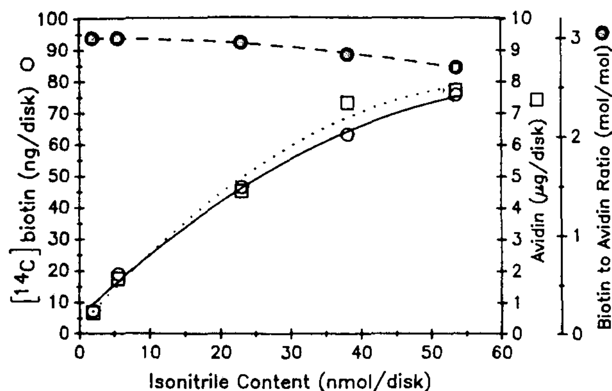


Fig. 10. Effect of functional group density on the subsequent ability of Type II biotinylpolyacrylamide–cellulose disks to bind avidin and radioactive biotin. (○) [^{14}C]Biotin binding capacity; (□) bound avidin content; (●) biotin to tetrameric avidin ratio. Details of disk preparation are presented in under Experimental and in Table III.

chains on Type II disks was 100-fold lower than in the case of Type I disks, it is reasonable to suppose that Type II disks possessed a smaller number of longer polyacrylamide side-chains compared with disks of Type I. The functional consequences of this difference are indicated by the avidin- and biotin-binding curves in Fig. 10. Thus, disks of about 50 nmol –NC content bound *ca.* 7 μg of avidin and were capable of ligating *ca.* 80 ng of free biotin after saturative avidinylation, compared with *ca.* 30 ng for similar Type I disks (see Table III). In other words, while similar to Type I disks with regard to avidin-binding capacity, Type II disks appeared to possess a much higher capacity for free biotin. In addition, their apparent B/A_4 values did not deviate much from the theoretical value of 3 over the entire –NC range tested. In comparison, the best B/A_4 value for Type I disks (50 nmol –NC content) was only 1.7 and was very dependent on –NC content (Fig. 7 and Table III). Moreover, as Fig. 11 shows, the linear range of standard avidin assay curves obtained with different batches of Type II disks was as extended as that observed with Type I disks (Fig. 9), while the slopes of these curves were independent of –NC content and similar in magnitude

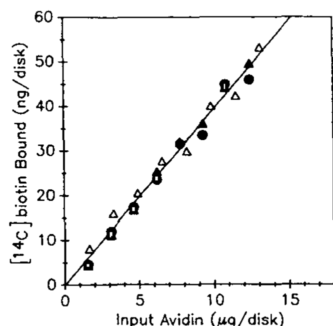


Fig. 11. Avidin assay ranges of Type II biotinylpolyacrylamide–cellulose disks. The three batches of disks used respectively contained (▲) 5.5, (●) 38 and (△) 55 nmol of isonitrile groups per disk.

to those for "high"-efficiency cellulose disks (Fig. 6). Accordingly, from a practical point of view, Type II polyacrylamide–cellulose disks become the vehicles of choice for avidin and biotin assay as they combine the extended linearity of Type I polyacrylamide–cellulose disks with the enhanced sensitivity of "high"-efficiency cellulose disks.

Time dependence of avidin and biotin assays with biotinylcellulose disks

As shown schematically in Fig. 12, differences in biotinylated disk behaviour can be explained by assuming both single- and multi-site attachment of avidin tetramers to cellulose (Fig. 12A) and polyacrylamide–cellulose (Fig. 12B and C) disks. Thus, the probability of multi-site avidin attachment to more than one polyacrylamide side-chain in a polyacrylamide–cellulose disk would be low when such chains are widely spaced (as in the case of Type II disks), and increase in frequency with increasing side-chain density (*i.e.*, Type I disks). In other words, the observed B/A_4 ratios of 3 in Type II polyacrylamide–cellulose disks and <3 in Type I polyacrylamide–cellulose disks are in keeping with the predictions of the above model. The same basic model also explains the behaviour of "high"- and "low"-efficiency cellulose disks, including the fact that the B/A_4 values of "high"-efficiency cellulose disks attain the limiting value of 3.

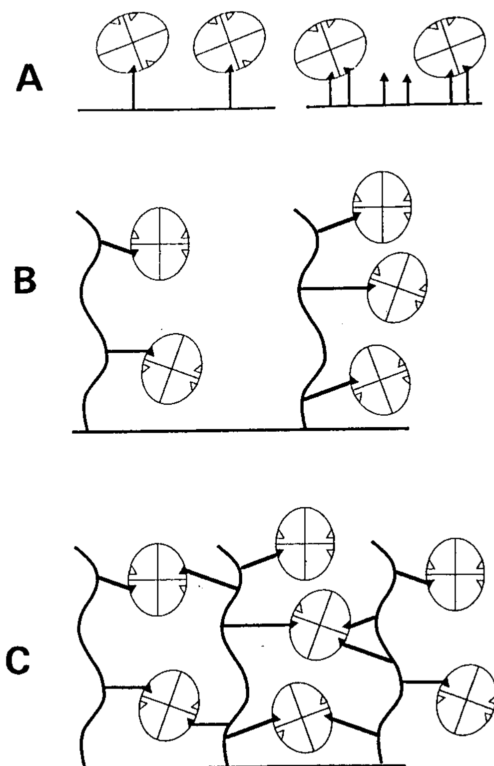


Fig. 12. Single- and multi-site attachment of avidin to (A) biotinylated cellulose and (B and C) polyacrylamide–cellulose disks.

One of the undesirable characteristics of "low"-efficiency avidinylated biotinylcellulose disks was their irreproducibility as biotin assay vehicles. Depending on the time and conditions of pre-exposure to avidin, the biotin-binding capacity of such disks varied from relatively good to almost zero. This observation can also be accommodated by the model described above by assuming that time-dependent changes from single- to multi-site avidin attachment can occur. Experimental support for this assumption comes from the kinetic data summarized in Fig. 13. The indicated curves represent parallel experiments with "high"-(triangles) and "low"-efficiency (circles) biotinylcellulose disks. They demonstrate time-dependent changes in the amount of avidin bound (A), in subsequent saturative [^{14}C]biotin binding (B) and in calculated B/A_4 ratios (C) as a function of time of exposure to excess of avidin. As can be seen,

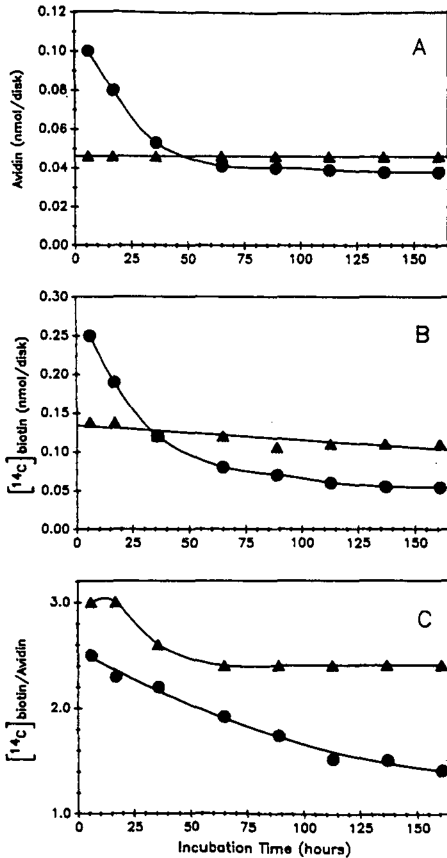


Fig. 13. Decay of disk-bound avidin function with time. Biotinylcellulose disks of low and high functional group density were derived from batches of disks averaging (▲) 7.6 and (●) 108 nmol of isonitrile groups per disk, respectively. Disks were immersed in an excess of ^{14}C-labelled avidin at 37°C for the indicated times. Some of the multiple number of disks removed at each time point were directly subjected to radioactivity determination in order to obtain their bound avidin content (A), whereas others were further exposed to excess of [^{14}C]biotin before counting in order to determine the total biotin binding capacity of each disk (B). The experimental values obtained were then used to calculate the number of biotin-binding sites per avidin (A_4) molecule (C).

the avidin-binding capacity of "high"-efficiency disks was constant with respect to time, the [^{14}C]biotin capacity of such disks was almost independent of time and the B/A_4 ratio varied from 3 at exposure times under 30 h to about 2.6 at longer exposure times. Presumably, disks with even lower functional group densities would have exhibited a time-independent B/A_4 value of 3. By contrast, "low"-efficiency disks initially showed a relatively high capacity for avidin, which declined steeply with time and reached a final plateau value lower than that of "high"-efficiency disks. "Low"-efficiency disks initially bound more free biotin than their "high"-efficiency counterparts, but lost biotin-binding capacity with time and approached a value only half that of the "high"-efficiency disks. Thus, the initial B/A_4 values of the "low"-efficiency disks were no higher than 2.5 and, in contrast to those of "high"-efficiency disks, steadily declined with time and approached a value of about 1.

Avidin molecules can be arranged in a two-dimensional array with either their long (5.5 nm) or short (4.1 nm) axis[14] lying perpendicular to a surface. In the former arrangement, each molecule of avidin occupies a smaller equivalent surface area (13.2 *versus* 23.8 nm^2) and hence permits a higher maximum molecular packing density at saturation. The only way to attain such maximum avidin packing is through single-site attachment (Fig. 12). Accordingly, the data for "low"- and "high"-efficiency cellulose disks in Fig. 13 can be interpreted in the light of the time-induced changes in avidin orientation depicted schematically in Fig. 14. Thus, the finding that the avidin content of "low"-efficiency disks decreased by a factor of about 2 with time can mean that half of the initially bound tetramers were forced off the disks by a shift from one to two-site attachement. Also, the fact that the B/A_4 ratio of "low"-efficiency disks continues to decrease with time even after disk avidin content has stabilized implies that the transition from one- to two-site attachment is followed by some degree of two- to three-site attachment (Fig. 14). The latter stage would not be expected to induce significant changes in the surface orientation of bound avidin molecules.

The "high"-efficiency cellulose disks used to obtain the kinetic data in Fig. 13 were similar in functional group density to batch number 8 disks in Table II and are thus borderline in behaviour to "low"-efficiency disks. These borderline disks departed from ideal "high"-efficiency behaviour in that they exhibited a small decrease in biotin-binding capacity with time. However, their bound avidin content remained constant with time and the B/A_4 ratio stabilized at a number close to the ideal value of 3. The findings suggest that most avidin molecules are attached via a single site in "high"-efficiency disks, and that few molecules can shift to a two-site binding mode with time owing to the relative dearth of surface-bound biotin. The fact that the avidin plateau is almost the same for both "high"- and "low"-efficiency cellulose disks (Fig. 13A) suggests that the avidin content of the former is as close to saturation as random orientation would allow.

The model outlined above on the basis of this study is probably general and helps to explain phenomena related to time- and concentration-associated activity

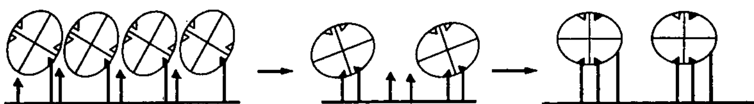


Fig. 14. Time-induced changes in the orientation of surface-bound avidin.

deterioration in affinity systems of high ligand density (see also refs. 23–25). For example, Hammond *et al.*[11] recently showed that affinity purification of recombinant protein A[26] by binding to immobilized IgG gave quantitative protein recoveries only up to a certain immobilized ligand density. At higher densities of column-bound IgG, the recovery of protein A declined almost to zero. Presumably, attachment of protein A to IgG is mainly of a single-site nature when bound IgG levels are low and progressively shifts to a stronger two-site mode of binding as the immobilized ligand content increases. Similarly, the degree of recovery of specific populations of functionally intact cells from immunoadsorbent affinity columns is known to be inversely related to the level of antibody substitution on the matrix[9,10]. Again, poor recovery can be explained by assuming that each cell-immobilized ligand complex will, on average, be more strongly held together by a greater number of contact points as the density of surface-bound ligands is raised.

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Behaviour of amino acids in gel permeation chromatography

Correlation with the effect of Hofmeister solutes on the conformational stability of macromolecules

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ABSTRACT

The gel chromatographic behaviour of the twenty naturally occurring amino acids was investigated. The effect of salts ranking in the salting-out side of the Hofmeister series was studied over a wide range of concentrations. The dependence of ln K_d on salt concentration was utilized to rank the amino acids in a "solvatochromic" scale which could help in predicting the propensity for regions in the polypeptide chain to be exposed to the solvent.

INTRODUCTION

The stability of biological macromolecules is a result of the sum of the interactions that take place in solution both intramolecularly and with other solutes. Since the discovery of the marked effect of neutral salts on protein solubility by Hofmeister[1] in 1888, various phenomena have been linked with this peculiar ranking of the salts not directely linked to discernible chemical or structural features of the ions[2].

The most direct consequence of, or simply relationship with, the stabilizing effect of salting-out salts in polypeptides endowed with catalytic properties is that in their presence enzymes exhibit an increased affinity for their substrates and often a higher V_{max}[3].

Another aspect, developed more recently, concerning the factors that dictate the attainment of a lower energy conformation by a polypeptide chain, is the attribution to single amino acid residues of a relative ranking of a physico-chemical property or parameter to generate a scale, such as the hydrophobicity scale, which may be of use in predicting the general behaviour of an amino acidic sequence in solution or whether

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that sequence would most likely be buried in the interior of the protein or exposed at its surface.

To quantitate hydrophobicity, many scales have been proposed (for a review, see ref. 4). Some of these scales are empirical calculations of the partitioning between the solvent-accessible surface and the buried interior in proteins of known structure. Some other scales are based on solution measurements, generally of distribution coefficients between an aqueous and an organic phase.

This paper deals with the chromatographic behaviour of the naturally occurring amino acids on gel permeation media in the presence of increasing salt concentrations. The results are interpreted in the light of a general framework linking the solvation properties of the single units of a polypeptide chain with the osmolyte composition of the solution and the attainment of an organized folded conformation of the macromolecule. A partial report on this subject was presented elsewhere as a short communication[5].

EXPERIMENTAL

Analytical reagent grade inorganic salts were used without further purification. Amino acids and ovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, U.S.A.). Sephadex G-25 (Superfine, batch No. 6277) was purchased from Pharmacia (Uppsala, Sweden) and Bio-Gel P-2 from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Water-jacketed columns (38 cm × 1 cm I.D.) were prepared from pre-equilibrated, degassed swollen suspensions of gel and the top of the column was fitted with an adjustable piston. Temperature was controlled to within ±0.01°C with a Haake F-3C circulating water thermocryostat. Column parameters were determined with BSA and ammonium sulphate. This salt was preferred to tritiated water to measure the inner volume of the column, because with the latter an overestimate of the volume occurs owing to tritium exchange with the exchangable hydrogens in the matrix[6]. The sample (200 μl) was applied with a standardized procedure using a Pharmacia four-way valve fitted with a 200-μl loop. Each sample was chromatographed in the presence of 10 μl of 10 mg/ml BSA solution.

Elution profiles were determined by continuous monitoring at 206 nm using an LKB 2089 Uvicord III connected to a LKB 2210 potentiometric recorder. Ammonium sulphate was determined nephelometrically as its barium salt at 500 nm. Special care was taken to measure accurately the elution parameters of the column. The distance from the start line of the recording to the peak maximum was utilized, with the appropriate corrections for tubing volume to calculate the elution volume.

RESULTS

The elution positions of the twenty naturally occurring amino acids were bdetermined by aqueous column chromatography on both Bio-Gel P-2 and Sephadex G-25. The chromatographic behaviour of these small molecules, as related to the exclusion limit of the gel pores, is largely independent of classical sieving effects which are the basis of gel permeation chromatography. If a salt ranking high in the Hofmeister series (salting-out) is present in the elution buffer, the elution volume is

generally affected, to a varying extent. Moreover, this effect displays a linear correlation with salt concentration over an extremely wide range, usually limited by the salt solubility.

Relative equilibrium constants for the amino acid–gel interaction could also be calculated from the elution volumes, as it was demonstrated that these values were independent of flow-rate, thus confirming that local diffusional equilibrium was attained. Bio-Gel P-2 and Sephadex G-25 were used in these studies to avoid possible gel permeation effects (steric exclusion of the amino acids from any part of the gel).

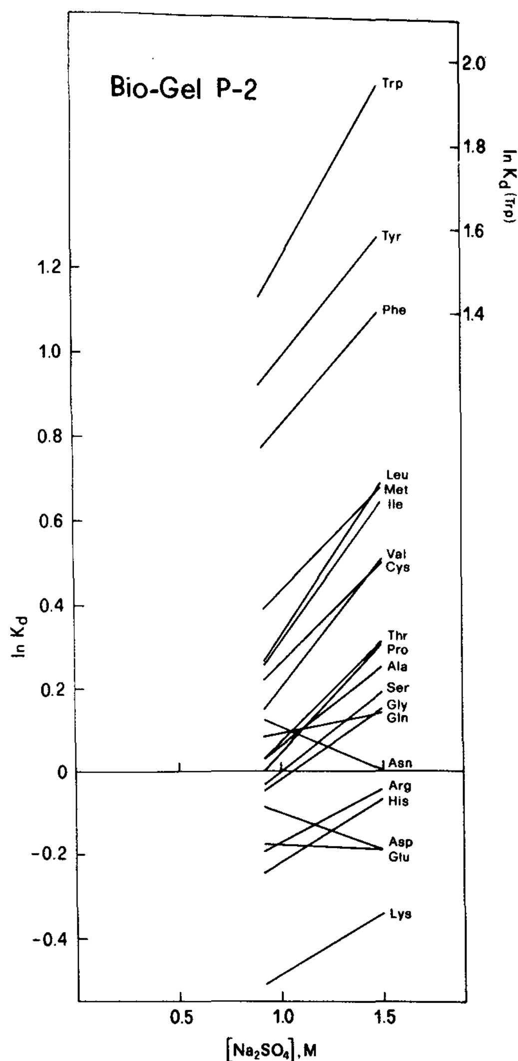

Fig. 1. Salt dependence of the distribution coefficient for the twenty naturally occurring amino acids. Amino acids were chromatographed under the conditions indicated under Experimental using Bio-Gel P-2. Temperature, 20°C.

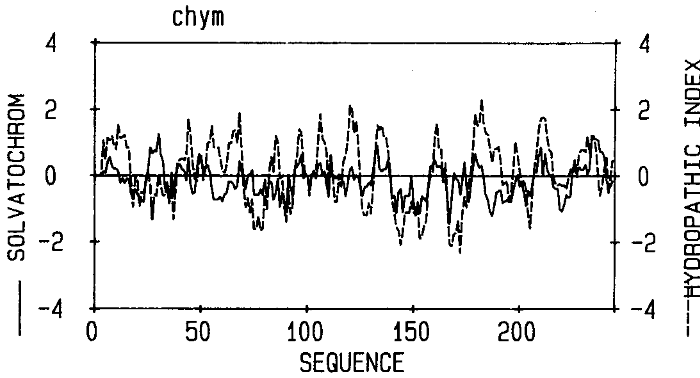

Fig. 2. Hydrophobicity plot for the chymotrypsin sequence, utilizing the Kyte and Doolitle[7] hydropathy scale and the "solvatochromic" scale deduced from the experiments shown in Fig. 1.

Fig. 1 shows the salt dependence of the distribution coefficient for the twenty amino acids when chromatographed on a Bio-Gel P-2 column using sodium sulphate as the Hofmeister solute. The same pattern and ranking were observed using Sephadex G-25. The effect is also independent of the type of salt used, provided that it has a similar ranking in the Hofmeister series. This was tested using both sodium sulphate and potassium phosphate as eluents, for both Bio-Gel and Sephadex matrices.

The ranking of the ln K_d (distribution coefficient) for the amino acids was utilized to create a hydrophobicity scale with arbitrary values ranging from -4 to $+4$ as the hydropathy scale devised by Kyte and Doolittle[7] on the basis of semi-empirical

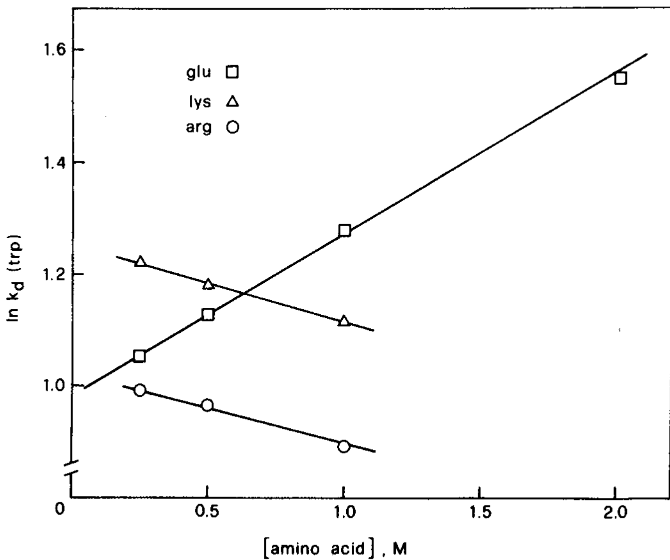

Fig. 3. Plot of ln K_d for tryptophan as a function of the concentration of the charged amino acids: (△) lysine, (○) arginine and (□) glutamic acid.

evaluations. The plot for a model protein (chymotrypsin) is shown in Fig. 2. Some similarities can be observed for certain regions in the polypeptide chain, such as at residues 135–142 or 212–220, but in general the scale displays a different overall pattern.

Among the ln K_d plots for the twenty amino acids as a function of salt concentration, as shown in Fig. 1, the behaviour of charged amino acids was noticeably different, displaying a negative dependence on salt concentration. This behaviour was not uniform among the charged amino acids and, for example, lysine displayed a marked positive correlation with salt concentration.

We therefore tested the ability of some of the charged amino acids to affect, at moderate to high concentrations, the elution position of others amino acids. The charged amino acids were thus utilized as Hofmeister solutes in the gel chromatographic experiments shown in Fig. 3. The test solute in this instance was tryptophan, for which a strong dependence on salt concentration had already been established[8,9]. Interestingly, the charged amino acids did not behave similarly. Lysine and arginine were effective, to different extents, in reducing the affinity of tryptophan for the gel. Glutamic acid, in contrast, displayed a strong positive effect on the distribution coefficient of tryptophan, behaving similarly to salts ranking high in the Hofmeister series.

CONCLUSIONS

Amino acids bind in the presence of highly salting-out salts to the gel permeation media with a rank order that is generally, bu not exclusively, dependent on the ratio of non-polar to polar groups in the compound. A linear dependence on salt concentration is present over a wide range of concentrations, often limited only by the salt solubility, as has previously been shown in the gel chromatography of some aromatic compounds[8]. With aromatic amino acids, the dependence shows a positive correlation with salt concentration. With hydrophobic or apolar amino acids, this dependence is markedly reduced and not exclusively correlated with the degree of hydrophobicity of the amino acid. A negative dependence on salt concentration is observed for a small number of neutral and for the charged amino acids.

The amplification inherent in liquid column chromatography and the enhancement of the above-mentioned effect by the presence of a Hofmeister solute is of importance for the present study. In fact, for most amino acids the expected or retarded elution is not detectable at a low ionic strength or could not be measured easily with methods such as equilibrium dialysis. Also, the adsorptive properties of the amino acids may result in a totally different ranking order when measured in the presence of moderate concentrations of a salt that is not markedly salting-out, such as sodium chloride. Moreover, the effect of different salt covering the entire range of the Hofmeister series will cause various extents of retardation with different dependences on salt concentration[9].

As we are concerned primarily with the application of this method to the development of a scale which could be advantageous in predicting exposed regions of a polypeptide sequence, we shall compare these results with those of previous studies that have dealt with the chromatographic behaviour of Hofmeister solutes.

Gel chromatography of neutral salts had been carried out under various

conditions[10,11] and in certain instances[12] utilized to calculate free energy values accounting for the stabilizing effect of a given solute on macromolecular conformation. Interestingly, the elution order of any of these salts was always found to parallel their position in the Hofmeister series, the salting-out salts being eluted earlier than expected and the chaotropes being eluted later. This effect has recently been more thoroughly investigated and correlated with the structure of the hydration shell of the ion[2].

This study has shown that the most soluble amino acids, considered to be osmolytes, may be evaluated for their effect on the solvation properties of other amino acids and, more interestingly, on the stabilization of a polypeptide chain in a solution of varying osmolyte composition. This experimental system lends itself to versatile applications such as the study of very weak interactions with a number of biologically relevant solutes, chromatographic applications or calculations of thermodynamic parameters to investigate stabilization factors in thermal denaturation processes.

ACKNOWLEDGEMENTS

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CHROM. 22 367

Extended Abstract

Salt-promoted adsorption chromatography

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Molecules surrounded by organized water tend to self-associate and form molecular complexes with other kinds of molecules which also have hydration shells of water that are more organized than in the aqueous bulk phase. Formation constants for such complexes are to a greater or lesser extent governed by the entropic gain accompanying the reorganization of water following complexation. If one of the interacting species, the ligand, is surface bound to the support and the other, the ligate, is present in the surrounding solution, the latter will become adsorbed on the solid support. Three kinds of adsorption or affinity chromatography based on this principle and with interaction strength and capacity thereby promoted by water-structuring (anti-chaotropic) salts were briefly described: (1) HIC (hydrophobic interaction chromatography; (2) EDAC (electron donor–acceptor chromatography including thiophilic affinity chromatography; and (3) TAC and IMAC (immobilized metal ion affinity-based chromatography). The discussion is confined to peptide and protein fractionation.

In a narrow sense, hydrophobic interaction of protein molecules refers to the mutual attraction of alkyl side-chains in water. Intermolecular interactions between alkyl ligands and alkyl ligates are exploited in hydrophobic interaction chromatography.

If an aliphatic ligand is unsaturated, as is the case for propargyl-liganded agarose, another kind of interaction is superimposed on hydrophobicity. Likewise, phenyl or naphthyl ligands attract unsaturated ligates more strongly than can be accounted for by hydrophobicity alone. The additional contribution to the interaction is due to the π-electron system of the ligand. The electron donor or acceptor strength of a ligand can be strongly enhanced by introduction of electron-releasing or -attracting substituents so that the hydrophobic character will play only a secondary role. This is the case if several groups are located adjacent to double bonds or are suitably located in heterocyclic rings. Nitro groups introduced in a benzene nucleus deplete the latter of electrons, making the support an electron acceptor adsorbent. On the other hand, a dimethylamino group enhances the electronegativity of the nucleus. Groups in proteins most likely aromatic side-chains, are the counterparts in the formation of adsorption complexes with these kinds of ligands. EDA adsorption may be further strengthened in the presence of high concentrations of alkaline and ammonium phosphates, sulphates, chlorides and other water-structuring salts.

A non-ionic ligand may interact with a protein without being in a narrow sense hydrophobic, and still the interaction may be strongly salt-promoted. The first ligand of such a kind that we discovered has the simple structure $-OCH_2CH_2SO_2CH_2$-$CH_2SCH_2CH_2OH$. Owing to its sulphur content, we call the ligand thiophilic, and the corresponding agarose gel was named "T-gel", where T represents either thiophilic or thioether. Used with buffer solutions, 0.3–0.65 M in K_2SO_4, the T-gel turned out to be an excellent adsorbent for immunoglobulin isolation. The underlying phenomenon seems to be more general than was at first thought. The ligand may not necessarily contain thioether sulphur. The protein counter-ligands are likely to be the surface-exposed aromatic side-chains, particularly indole groups. The nature of the ligand and solid support and the composition of the solvent medium modulate the strength and selectivity also in the metal–protein attraction and, consequently the specificity and capacity of an IMA adsorbent.

There are a wide variety of methods to promote the desorption of proteins in IMAC, such as including in the eluent additives ethylene glycol, surfactants, affinity-competing solutes etc., and changing the pH and temperature.

Metal ions such as Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} interact chiefly with imidazole, but also with surface-exposed thiol and in certain circumstances to a lesser extent with indole and terminal amino groups. Very efficient high-performance liquid chromatography of peptides and proteins has been achieved using pH and imidazole gradients for elution. The extent of adsorption and desorption depends on the concentration and the kind of water-structuring salts used, but the effects are often the opposite for "soft" and "hard" metal ions.

Preliminary studies on IMAC for the isolation of calcium- and magnesium-binding proteins and phosphoproteins indicate that "hard" metal ions, immobilized or included in the eluent, may offer a great advantage over conventional purification procedures. High selectivity may be achieved in spite of the fact that the interactions, as in ion-exchange chromatography, appears to be only ionic in nature. Presumably the protein counter ions are carboxylic and phosphate ester groups.

It is worth pointing out that the separation of proteins in solutions of water-structuring salts can be made sequentially, without intermittent desalting, using in optional order the affinity principle hydrophobicity, thiophilicity and metal ion specificity. By use of gradients and selective eluting agents, a refinement in the resolution of complex mixtures may be obtained in each chromatographic step. Other advantages are that bacterial growth is prevented by the virtually complete elimination of essential metal ions and the high salt concentration, which also tends to stabilize the tertiary structure of proteins. Adsorption and desorption may be effected at physiological pH and at ambient temperature.

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CHROM. 22 422

Affinity-repulsion chromatography

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ABSTRACT

Affinity-repulsion chromatography is a protein separation process in which the electrostatic charges present, or purposely introduced,on affinity matrices are exploited to allow the elution, by electrostatic repulsion, of proteins carrying electrostatic charges of the same sign as that of the matrix. In this process, proteins are loaded on the affinity matrix in a salt solution and eluted with low ionic strength solutions or deionized water. Examples of protein separations carried out by affinity-repulsion chromatography are presented.

INTRODUCTION

Affinity chromatography[1,2] has become established as one of the most powerful techniques for the isolation and purification of proteins. In this procedure, the protein to be purified from a mixture binds to one of its specific ligands immobilized on an insoluble matrix whereas the other proteins in the mixture do not. The protein retained on the matrix is thereby isolated from the other proteins and can be removed from the matrix by addition of its free ligand at concentrations that allow displacement from the immobilized ligand. As a result, the protein is recovered in solution as a complex with its free ligand, from which it can then be dissociated by dialysis or molecular sieving. Often the above procedure encounters difficulties when the protein binds too strongly to the affinity matrix. In such cases, the protein cannot be readily eluted from the matrix by the addition of its free ligand and more drastic elution conditions need to be applied. These include changes in pH, ionic strength, temperature and polarity of the eluting solvent (to decrease van der Waals interactions), the use of chaotropic salts such as guanidine hydrochloride or urea or the use of detergents which alter the structure of the protein, and the application of electrophoretic desorption[3,4]. The disadvantages of some of these elution conditions are their relatively low yields and the possible loss of biological activity of the eluted proteins.

In this paper, we discuss the principle of affinity-repulsion chromatography[5], which aims at easing the elution process, and describe some of its applications, advantages and disadvantages and consider its suitability as a general procedure for the adsorption–desorption of proteins.

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PRINCIPLE OF AFFINITY-REPULSION CHROMATOGRAPHY

The principle of the method (Fig. 1) is based on the fact that both the affinity chromatographic matrix and the proteins that interact with it possess electrostatic charges that can either repel or attract each other according to their sign and their respective distance. The strength of such electrostatic interactions is not substantial in solutions containing high salt concentrations, but can become considerable in deionized water. Accordingly, if a protein and the affinity matrix carry the same overall net electrostatic charge, the protein will be eluted by deionized water whenever the strength of the electrostatic repulsion between the protein and the matrix exceeds the attractive forces between the protein and the immobilized ligand. Such situations can be artificially devised by appropriate chemical modifications of the affinity matrix so as to increase the charge density and adjust the distance between the protein and the matrix.

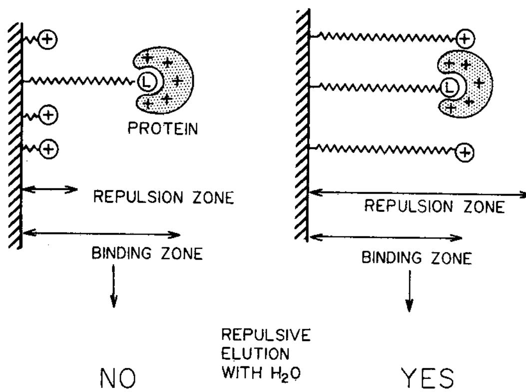

Fig. 1. Principle of affinity-repulsion chromatography. Successful dissociation of the positively charged protein from its immobilized ligand L on elution with deionized water depends on the distance separating the positive charges on the protein from those present on the charge spacers. On the left, the protein electrostatic charges are outside the repulsion zone created by the positive charge spacers and therefore the protein cannot be eluted by deionized water. On the right, the protein binding zone is within the charge repulsion zone and therefore the protein will be eluted either with deionized water only or with the free ligand in deionized water (for proteins with very high ligand binding affinity). From Teichberg *et al.*[5].

EXPERIMENTAL AND RESULTS

Fig. 2 shows the results of the application of peanut agglutinin in a 150 m*M* sodium chloride solution to a lactosyl-Sepharose column. The protein is eluted either with deionized water (upper panel) or lactose in 150 m*M* sodium chloride (lower panel). The elution of the protein with water demonstrates that the strength of the electrostatic repulsion between the lectin and the matrix is greater than the "attraction" between the lectin and its immobilized ligand.

Fig. 3 shows the affinity-repulsion chromatography of peanut agglutinin on a "native" unmodified galactosyl-Sepharose matrix (A and B) and on a negatively charged galactosyl-Sepharose matrix (C). In spite of the presence of these additional

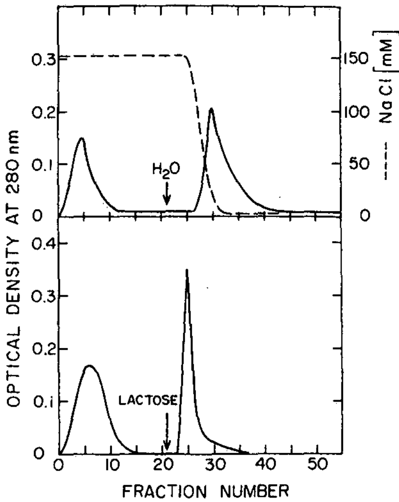

Fig. 2. Affinity chromatography of peanut agglutinin on a lactosyl-Sepharose matrix. The protein was applied in 150 mM NaCl and eluted either with deionized water (upper panel) or 300 mM lactose in 150 mM NaCl (lower panel). From Teichberg *et al.*[5].

negative charges on the matrix, the lectin is not eluted with deionized water more readily from this matrix than from the "native" unmodified matrix. This result indicates that the native Sepharose beads possess an adequate density of negative charges for the electrostatic repulsion of peanut agglutinin. In contrast to its behaviour on a negatively charged matrix, peanut agglutinin cannot be eluted with deionized

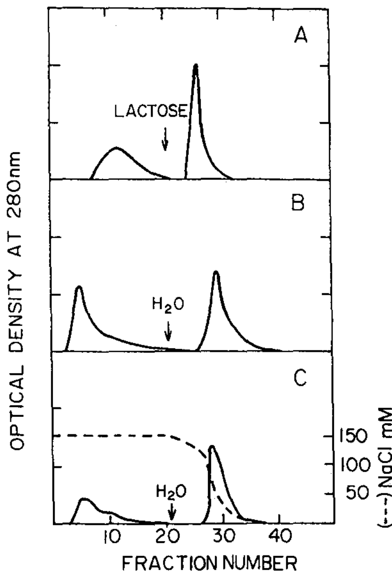

Fig. 3. Affinity chromatography of peanut agglutinin on a galactosyl-Sepharose matrix. A and B, "native" matrix; C, γ-aminobutyric acid-modified matrix. In all instances the protein was applied in 150 mM NaCl and eluted with either 300 mM lactose in water (A) or deionized water (B and C). From Teichberg *et al.*[5].

water when it is affinity chromatographed on a galactosyl-Sepharose matrix to which positively charged residues have been coupled (Fig. 4). However, in spite of this excess of positive charges on the matrix, the protein is eluted wih 300 m*M* lactose in deionized water indicating that the strength of the interaction of the lectin with the free saccharide ligand is stronger than the electrostatic attraction of the negatively charged lectin with the immobilized positive charges of the matrix, possibly because the latter charges are not localized at the optimum Coulombic distance.

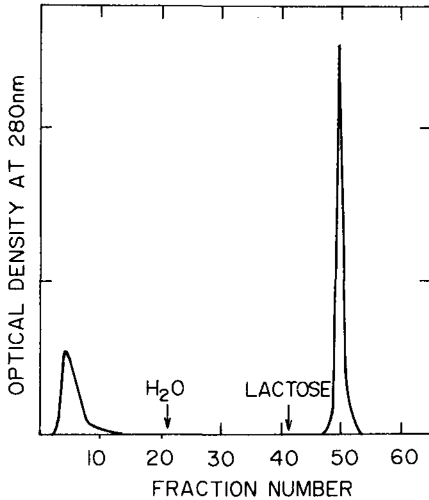

Fig. 4. Affinity chromatography of peanut agglutinin on a galactosyl-Sepharose matrix modified by conjugation with ethylenediamine residues. The protein was applied in 150 m*M* NaCl and eluted with 300 m*M* lactose in water. From Teichberg *et al.*[5].

Fig. 5 illustrates the affinity-repulsion chromatographic patterns of concanavalin A (Con A). Con A, loaded on a maltosyl-Sepharose matrix, cannot be eluted with either deionized water or methyl α-glucoside in water but only with a solution containing methyl α-glucoside, 150 m*M* sodium chloride, 1 m*M* calcium chloride and 1 m*M* manganese chloride (Fig. 5A). However, if Con A is applied on a maltosyl-Sepharose matrix to which ethylenediamine residues have been attached, the protein can be eluted with methyl α-glucoside in water and with deionized water (Fig. 5B and C).

Once applied to a more positive matrix, Con A is eluted with water (Fig. 5C), although the shape of the eluted protein peak is not symmetrical and its size is smaller than expected. The latter result indicates that some of the Con A applied has been retained on the column. Indeed, the application to the column of solutions at pH 8.0 and 3.5 allows the elution of two other protein peaks which, on neutralization, display a methyl α-glucoside specific agglutinin activity and fully account for the amount of Con A loaded on the column.

Interestingly, on rechromatography the protein peak eluted with the pH 3.5 solution emerges in its entirety with water elution at the position of the first peak. The

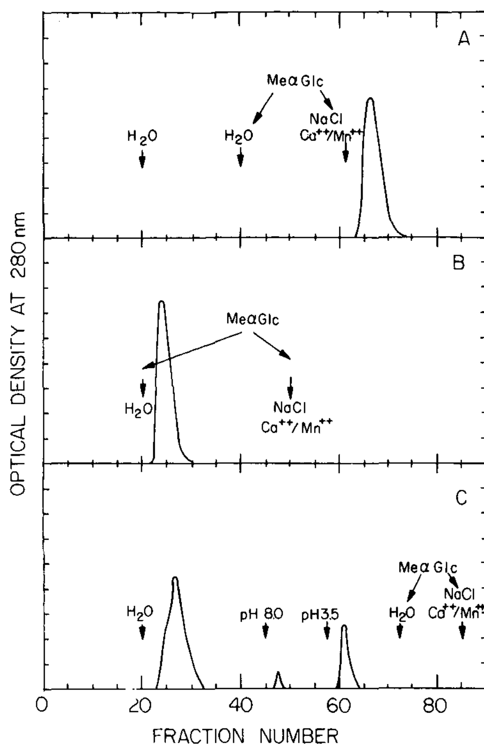

Fig. 5. Affinity chromatography of concanavalin A on a maltosyl-Sepharose matrix. A, "native" maltosyl-Sepharose matrix; B and C, maltosyl-Sepharose matrix modified by conjugation with ethylenediamine residues. Eluent applied at arrows: (A) deionized water, methyl α-glucoside (MeαGlc) in deionized water, methyl α-glucoside in 150 m*M* NaCl; (B) methyl α-glucoside in deionized water, methyl α-glucoside in 150 m*M* NaCl; (C) deionized water (pH 6.5), deionized water (pH 8.0), deionized water (pH 3.5), methyl α-glucoside in deionized water, methyl α-glucoside in 150 m*M* NaCl. All the protein peaks eluted in C display a mannose-specific haemagglutinin activity. From Teichberg *et al.*[5].

finding of two Con A species eluting at neutral and acidic pH is in line with literature data indicating that acidic monomers and neutral dimers of Con A coexist as a non-equilibrium mixture[6].

DISCUSSION

The separation and isolation of proteins by affinity chromatography is based on their reversible binding to specific ligands immobilized on insoluble matrices. Inherent to the technique are the following three requirements: (i) the protein to be isolated should bind in a reversible fashion to its immobilized ligand with an affinity such that it can be retained on the affinity matrix while the other proteins are washed away; (ii) the protein should interact with its immobilized ligand and with the matrix with an affinity allowing its elution, by the free ligand or by other non-denaturating agents; and (iii) accompanying proteins should not bind to the matrix but, if retained, should not be

eluted from it with the same elution conditions applied for the protein specifically bound to the affinity matrix.

As these requirements are not always fulfilled, it is essential to understand and to be in a position to control the forces involved in the specific and non-specific interactions taking place between proteins and affinity matrices.

For all practical purposes, the specific interactions are all those which allow the selective retention of the protein on the affinity matrix. They therefore involve not only the strictly specific non-covalent binding of the protein to its immobilized ligand but also the less specific (hydrophobic and ionic) interactions either with the spacer arm (used to increase the distance separating the matrix and the immobilized ligand so as to prevent steric interferences on binding to the latter) or with the charged residues on the matrix. If the sum of these interactions produces a free energy of binding above 12 kcal/mol (corresponding to a binding affinity above 10^{-9} M), the elution of the native protein from the column may prove difficult and denaturating conditions may be necessary to dissociate it from the matrix. In such cases, the efficacy of the free ligand to cause the dissociation of the protein from the affinity matrix often depends on the mangnitude not only of the strictly specific but also of the non-specific interactions.

By their very nature, the non-specific interactions can affect proteins in an indiscriminate manner and therefore, when they do modify the efficiency of the protein separation process, it is of importance to be able to minimize their effects. The use of buffers with low ionic strength is effective in reducing the amplitude of the hydrophobic interactions whereas buffers with high ionic strength decrease the ionic interactions.

Although the presence of charged groups on the affinity matrix may contribute in some circumstances to the establishment of undesirable non-specific interactions, these groups may play, in other circumstances, a crucial and decisive role either in the binding of a protein to the affinity matrix or in its elution. Indeed, the forces of attraction between electrostatic charges of opposite sign on the matrix and on the protein will increase the overall protein-binding affinity in all the cases when the interactions of the protein with its immobilized ligand are too weak to allow the desired retention on the affinity matrix[3]. In contrast, the forces of repulsion between electrostatic charges of similar sign on the affinity matrix and on the bound protein will facilitate the elution of the protein to the extent that deionized water can be used as the eluent.

We have termed the latter process "affinity-repulsion chromatography" and have established some experimental protocols permitting its successful application[5]. The following discussion deals with some of its theoretical and practical aspects.

The physico-chemical mechanism of affinity-repulsion chromatography is based on the fact that the electrostatic free energy of a charged protein in contact with charged residues on the affinity matrix can be larger than, or at least of the same order of magnitude as the free energy of binding of the protein to its immobilized ligand.

In the successful cases when lectins are eluted with water from charged affinity matrices, the values of the electrostatic free energy must exceed the values of the free energy of lectin–saccharide binding which are of the order of 8.0 kcal/mol (corresponding to a binding affinity of 10^{-6} M) or less[7].

The value of the electrostatic free energy estimated here on the basis of its

counterbalancing relation to the free energy of binding has to be compared with the values available in the literature from theoretical calculation or experimental studies.

Using the Debye–Hückel theory, Tanford[8] calculated the electrostatic free energy of a 40 000-dalton spherical protein in aqueous solutions of various ionic strengths. Depending on the number of net charges on the protein (10–40), the values of the electrostatic free energy obtained varied from 2.5 to 39.6 kcal/mol in a 150 m*M* salt solution and from 6.7 to 108 kcal/mol in a 1 m*M* salt solution. In spite of the simplifying assumptions made in these calculations, the derived values, although approximate, suggest that the electrostatic free energy of a protein can attain a considerable magnitude in deionized water.

Using an experimental approach, Scopes studied, at various pHs, the binding of proteins to carboxymethylcellulose and derived values of the free energy of interaction between single charges on the protein and charges on the matrix[3,9]. Values between 0.14 and 0.95 kcal/mol per charge were obtained, depending on the molecular size of the protein studied. The smaller proteins have the strongest interactions per charge, as is expected from their tighter packing into the pores of the matrix and the shorter average distance that separates the charges on the protein and those on the matrix. Scopes further estimated that a free energy of interaction of 0.5 kcal/mol corresponds to an average distance of 5 nm between charges. As this distance is large with respect to the dimensions of a protein, one can expect the free energy of interaction to reach values higher than 0.5 kcal/mol when the affinity matrix is conjugated with spacers carrying their charges at distances from the bound protein shorter than 5 nm.

If one takes all the above data at face value, it is clear that a relatively small number of electrostatic charges placed on the affinity matrix at optimum interacting distances from those of the bound protein could produce, in salt-free solutions, an electrostatic free energy matching or even in excess of the free energy resulting from the binding of most proteins to their ligands, including the biotin–avidin complex, which displays a free energy of binding of about 20 kcal/mol (corresponding to a binding affinity of about 10^{-15} *M*)!

Evidently, the efficiency of affinity-repulsion chromatography depends on the strength of the Coulombic repulsion (*i.e.*, on the density and distance) between the electrostatic charges on the protein and those on the matrix. The density of charges on the matrix and their distance from the bound protein can be practically controlled by conjugation with charged arms of appropriate length, which we refer to here as "charge spacers".

Some careful thought has to be given to the choice of the charge spacers because, like the ligand spacer arms, they ought not to be hydrophobic. The main reason for avoiding hydrophobic spacers relates to the fact that the conditions of application of affinity-repulsion chromatography are similar to those of hydrophobic chromatography[10,11].

In hydrophobic chromatography, as in affinity-repulsion chromatography, proteins are applied to the hydrophobic matrix in aqueous solutions of relatively high ionic strength, to favour hydrophobic interactions, while they are eluted in low ionic strength solutions.

There are, however, significant quantitative differences between the two chromatographic procedures. In affinity-repulsion chromatography, the proteins are applied to the affinity matrix in monovalent ion solutions at concentrations that ought

not to exceed 80 mM, or may not exceed 20 mM if a divalent ion at 1 mM is also included. In hydrophobic chromatography, the proteins are generally loaded onto the matrix in a 0.5–4 M salt solution and are eluted either by lowering the ionic strength or the polarity of the eluent or by including a detergent in the eluent. In spite of these differences, it is advisible not to use hydrophobic charge spacers so as to minimize the hydrophobic retention of proteins on the affinity matrix.

Does the charged matrix used as support for affinity-repulsion chromatography act also as an ion exchanger? It does indeed, but without affecting the specificity and efficiency of affinity-repulsion chromatography. Once loaded in a salt solution providing counter ions, proteins possessing a net charge similar to that of the charge spacers will not be retained on the affinity matrix (with the exception of the protein that binds to the immobilized ligand) whereas the other proteins will be. The use of deionized water as eluent will increase the ionic interactions of the latter proteins, which will therefore remain tightly bound to the matrix. However, it is possible that these proteins, although not eluted, will neutralize the charge spacers and prevent the latter from playing their expected role in the affinity-repulsion process. It is therefore important to wash the matrix, after protein loading, with a high ionic strength salt solution in order to eliminate by ion exchange all the bound proteins.

Advantages of affinity-repulsion chromatography

The elution process is carried out in low ionic strength solutions or in deionized water. The process is faster than conventional affinity chromatography since the protein eluted does not have to be dialysed in order to remove the eluting free ligand or salts. The process is cheap, as there is no need for eluting ligand. Proteins that cannot be eluted from an affinity matrix because of the strength of their binding to the affinity ligand can be eluted either with deionized water or by the free ligand in deionized water. The process allows the separation of some of the isoelectric forms of proteins.

Disadvantages of affinity-repulsion chromatography

Chemical modifications of the matrix have to be performed in order to introduce onto it appropriate charge spacers. Prior knowledge of the isoelectric points of the protein to be purified or of its behaviour on ion exchangers is required in order to select the appropriate charge spacers. Problems may be encountered in applying the standard procedures for ligand coupling because of the presence of additional sites of reaction. The chemical coupling of the charge spacers subsequent to ligand coupling may be difficult and may require protection of the ligand. The length of the charge spacers may need to be adjusted in order to optimize the efficacy of the elution process. The process may not be effective in all cases and in particular when the immobilized ligand is of proteineous nature or contains multiple charges of opposite sign. In such cases, the application of hypotonic solutions may increase the protein–ligand interactions rather than decrease them.

Affinity-repulsion chromatography as a general process

Several examples can be found in the literature in which either low ionic strength solutions or distilled water were used for the elution of antigens from immunoadsorbents. Svensson *et al.*[12] were the first to apply a low ionic strength elution method in the purification of dipeptidyl-peptidase IV. Vidal *et al.*[13] reported that phospho-

enolpyruvate carboxylase could be eluted with distilled water from a specific immunoadsorbent. A generalization of the latter procedure was then attempted by Bureau and Daussant[14]. They tested six different proteins with their corresponding immobilized immune sera and in all instances achieved elution with water of the adsorbed proteins with an efficiency above 65%. Danielsen *et al.*[15] made similar observations with five different enzymes solubilized from microvillar membranes. In all the above instances, no explanation was provided as to the molecular mechanism of this elution technique. We term it, by analogy, immunoaffinity-repulsion chromatography.

The successful applications of affinity-repulsion chromatography clearly demonstrate that the presence of appropriate charges on the affinity matrix may contribute significantly to the protein desorption process. The interactions between lectins and immobilized electroneutral saccharides present an ideal case, but the success of immunoaffinity-repulsion chromatography indicate that dissociation of a protein–protein complex can also be achieved. Nevertheless, the disadvantages outlined above set clear limits to the field of application of affinity-repulsion chromatography. Hence it does not replace but extends the range of applications of affinity chromatography.

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Application of receptor-affinity chromatography to bioaffinity purification

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ABSTRACT

Receptor-affinity chromatography based upon the receptor–ligand interactions has been utilized for the purification of recombinant human interleukin-2 (rIL-2) from microbial and mammalian sources. The receptor-affinity purification process of rIL-2 is used as a model system to demonstrate the utility of this approach for the purification of recombinant proteins. The receptor-affinity purified biomolecule is shown to be biochemically and biologically more homogeneous than the immunoaffinity purified material.

INTRODUCTION

Affinity chromatography based upon the ability of the macromolecules in solution to bind specifically and reversibly to immobilized ligands is an ideal tool for the purification of biomolecules[1–4]. This versatile technique became well established in the mid-1970's for the purification of proteins such as enzymes, hormones, antibodies, receptors, etc. Immunoaffinity chromatography, which utilizes the specificity of the antigen–antibody interactions, realized its full potential only after the discovery of the monoclonal antibody producing hybrid cell lines by Kohler and Milstein[5] in 1975. In 1987, the development of an alternative affinity method termed receptor-affinity chromatography (RAC) was reported[6], which exploits the biochemical interactions between a matrix-bound receptor and its soluble protein ligand. In general, the receptor is chemically bonded to an inert polymer support, packed into a column and after proper equilibration, the crude ligand preparation is passed through the column. The unadsorbed materials are washed away and the specifically adsorbed ligand is eluted with mild desorbing agents.

In this paper, the receptor-affinity purification process of recombinant human interleukin-2 (rIL-2) is used as a model system to demonstrate the utility of this approach for the purification of recombinant proteins. A systematic approach to the development and optimization of receptor-affinity purification systems is discussed. The receptor-affinity purified biomolecule is shown to be biochemically and biologically more homogeneous than the immunoaffinity purified material.

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EXPERIMENTAL

Materials

Silica-based NuGel P-AF polyaldehyde, NuGel P-DE200 and NuGel P-AF poly-N-hydroxysuccinimide (PNHS) were purchased from Separation Industries (Metuchen, NJ, U.S.A.). Sephacryl S-200 and Sephadex G-50 (superfine) were obtained from Pharmacia (Piscataway, NJ, U.S.A.). Monoclonal antibody 5B1 and rIL-2 were prepared in-house at Hoffmann-La Roche. Mannitol was purchased from ICI (Wilmington, DE, U.S.A.). All other reagents used were of reagent quality.

Construction of receptor-affinity column

Production and purification of interleukin-2 receptor. A recombinantly produced soluble form of the low affinity p^{55} subunit of the human interleukin-2 receptor denoted IL-2RΔNae was engineered and expressed in Chinese hamster ovary cells (CHO) by the gene-linked co-amplification technology[7]. The IL-2RΔNae was purified by employing IL-2 ligand-affinity chromatography as described[8].

Immobilization of IL-2RΔNae. The IL-2RΔNae was immobilized to NuGel P-AF PNHS (500 Å, 40–60 μm) at a coupling density of 1.55 mg/ml gel according to the previously published protocol[6]. The coupling reaction mixture contained equal volumes of the activated gel and a 2-mg/ml IL-2R solution in 0.1 *M* potassium phosphate, pH 7.0, containing 0.1 *M* sodium chloride.

Determination of the IL-2 binding capacity of the receptor column

A known volume (0.5–1.0 ml) of the receptor gel was packed into an Amicon 10 × 1 cm column (Amicon, Danvers, MA, U.S.A.) fitted with two adapters and equilibrated with phosphate-buffered saline buffer (PBS), pH 7.4. The column was then saturated with an excess of purified or crude IL-2 at a flow-rate of 1 ml/min. After washing away the unadsorbed materials, the adsorbed IL-2 was eluted with 0.2 *M* acetic acid containing 0.2 *M* sodium chloride and its protein content determined by the method of Lowry *et al.*[9].

Factors affecting the IL-2 binding capacity of the receptor adsorbent

Effect of coupling pH. A 1-g amount (1.4 ml) of NuGel-PNHS was mixed with 2 ml, 2.5 mg/ml IL-2RΔNae made up in buffers having pH 5, 6, 7, 8 and 9. The IL-2 binding capacities of the resulting affinity sorbents were determined as described in the preceding paragraph.

Effect of IL-2R coupling density. To each of the 2-ml IL-2R solutions having concentrations of 1.0, 2.5, 5.0 and 10 mg/ml, 1 g NuGel-PNHS was added and the coupling reaction was carried out as before. The IL-2R coupling density as well as the IL-2 binding capacities of the affinity sorbents were determined as described earlier.

Recombinant IL-2 production

A synthetic gene for IL-2 was constructed and introduced into *Escherichia coli* with the plasmid RR_1/pRK 248 CI_{ts}/pRC 233 (ref. 10) and grown in appropriate medium in large fermentors. A CHO cell line transfected with the IL-2 gene was the source of mammalian glycosylated rIL-2.

IL-2 bioassay

IL-2 bioactivity was determined by the IL-2 dependent proliferation of murine CTLL cells as determined by the colorimetric determination of lactic acid produced as an end product of glucose metabolism[11]. A unit of activity is defined as the reciprocal of the dilution yielding half-maximal cell growth. A BRMP reference reagent, purified from a human T cell leukemia cell line designated Jurkat-FHCRC, was used as a reference reagent, for human IL-2.

Receptor-affinity purification procedures

The general RAC purification scheme for the production of highly pure rIL-2 from microbial and mammalian sources are given in Fig. 1. All the RAC purification steps were carried out at 2–4°C, unless otherwise noted.

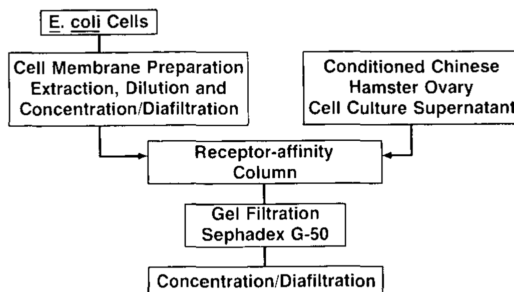

Fig. 1. The overall receptor-affinity purification scheme for rIL-2 from microbial and mammalian sources.

Extraction and solubilization of rIL-2

A 4-ml/g *E. coli* cell suspension was made in buffer A (30 m*M* Tris–HCl, pH 8.0, containing 5 m*M* EDTA). The cell suspension was passed through a Gaulin homogenizer (APV Gaulin, Everet, MA, U.S.A.), two to three times at 7000 p.s.i. The homogenized cells were centrifuged in a Sorvall RC-5 centrifuge with a GSA rotor (DuPont, Wilmington, DE, U.S.A.) at 24 000 *g* for 20 min and the pellet collected. The pellet was washed once with 4 volumes of 1.75 *M* guanidine (Gu) · HCl solution in buffer A, centrifuged and the pellet saved. The pellet was extracted with 7 *M* Gu · HCl in 0.1 *M* Tris–HCl, pH 8.0, containing 1 m*M* EDTA (4 ml/g cells) for 30–60 min and the supernatant collected by centrifugation.

rIL-2 refolding studies

The supernatant from the previous step was diluted 40-fold with PBS buffer and allowed to stand for 0–5 days. The supernatant was carefully decanted and filtered through a 0.8/0.2-μm filter (Sartorius). The clarified extract was used as the starting material for the RAC step. Aliquots of the diluted extract corresponding to 1 g cells were withdrawn each day during the five days of aging, clarified and their rIL-2 content was determined by RAC (5 × 1 cm column) as described for the determination of the saturation binding capacity of the receptor sorbents.

Receptor-affinity chromatography

An Amicon G-44 × 250 column (16.5 × 4.4 cm) was packed with 250 ml receptor gel and equilibrated with PBS buffer, pH 7.4. The clarified extract (17.5 l) derived from 100 g *E. coli* cells was applied to the receptor column at a flow-rate of 60 ml/min. The column effluents were monitored by a Gilson 111B UV detector in conjunction with a Kipp & Zonen recorder (Gilson Medical Electronics, Middletown, WI, U.S.A.). The column was washed with PBS until all the UV absorbing materials were removed. The adsorbed rIL-2 activity was eluted from the column using 1–2 bed volumes of 0.2 *M* acetic acid containing 0.2 *M* sodium chloride. The rIL-2 eluate was concentrated in a stirred-cell Amicon thin-channel concentrator fitted with a YM 5 membrane.

Gel permeation chromatography

A Pharmacia K50/100 column was packed with Sephadex G-50, superfine, to a height of 90 cm. The mobile phase used was 50 m*M* sodium acetate, pH 3.5, containing 200 m*M* sodium chloride and 5 mg/ml mannitol. An amount of 50 ml concentrated rIL-2 was applied to the column at a flow-rate of 1 ml/min. Five-minute fractions were collected on an LKB Ultra RAC-7000 fraction collector (LKB, Bromma, Sweden) and the column effluents were monitored as in the RAC step. The fractions containing the rIL-2 activity were pooled and concentrated to 5–10 mg/ml, sterile filtered with a 0.2-μm filter and stored at 4°C under aseptic conditions.

Purification of mammalian rIL-2

The conditioned CHO medium (18 l) was filtered through a 0.8/0.2-μm filter and applied to the receptor column. The receptor-affinity purified material was also subjected to gel permeation chromatography as described earlier.

Protein determination

The protein content of the rIL-2 samples was determined by the method of Lowry *et al.*[9] and confirmed by quantitative amino acid analysis using a post-column fluorescamine amino acid analyzer[12].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis

The rIL-2 samples were analyzed by sodium dodecyl (lauryl) sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions according to the method of Laemmli[13].

Determination of amino acid composition

Amino acid composition was determined using a post-column fluorescamine amino acid analyzer[12]. Samples were hydrolyzed in 6 *M* hydrochloric acid containing 4% thioglycolic acid at 110°C *in vacuo* for 20–24 h. Proline and cysteine values were determined after performic acid oxidation.

Immunoaffinity purification of microbial rIL-2

A murine monoclonal antibody to rIL-2 designated 5B1 was immobilized to NuGel-PNHS at an antibody loading of 12.8 mg/ml gel, as described for IL-2R. The rIL-2 binding capacity of the immunoadsorbent was determined as described for the

RAC column. The rIL-2 from *E. coli* was immunoaffinity purified and subjected to gel permeation chromatography using the same conditions used in the RAC purification scheme.

RESULTS AND DISCUSSION

IL-2R production, purification and immobilization

The soluble form of the low affinity p^{55} subunit of the human interleukin-2 receptor designated IL-2RΔNae lacks 28 amino acids at the carboxyl terminus and contains the naturally occuring N- and O-linked glycosylation sites[7]. These deletions result in the removal of the presumptive transmembrane and cytoplasmic domains in the IL-2R, thus allowing it to be secreted into the medium by the transfected CHO cells. The IL-2RΔNae was purified from conditioned CHO medium employing IL-2 ligand affinity chromatography[8]. The purified IL-2RΔNae was chemically bonded to the commercially available high-flow, low-pressure, silica-based NuGel-PNHS at a coupling density of 1.55 mg/ml gel with a coupling efficiency of 78%. At this IL-2R loading, the affinity adsorbent had an IL-2 binding capacity of 1.3 mg/ml gel or 68% of the theoretical binding capacity (see Table VI).

Factors affecting the IL-2 binding capacity of the receptor sorbent

Coupling conditions such as pH, activated group density on the matrix and the receptor coupling density affect the coupling efficiency and IL-2 binding capacity of the receptor bead. The IL-2 binding capacities of the receptor adsorbents prepared at various coupling pHs are given in Table I.

Both the coupling efficiency and the IL-2 binding capacity were optimized when the coupling pH was around 7–8. The best coupling efficiency and IL-2 binding capacities were observed when the activated group density was 15–30 μmol/ml gel (data not shown).

The effect of IL-2R coupling density on the IL-2 binding capacity of the receptor-affinity sorbents are summarized in Table II.

As observed for antibodies by Comoglio *et al.*[14], high IL-2R loadings resulted in

TABLE I

EFFECT OF COUPLING pH

Binding capacities were calculated taking into account the molecular weights of IL-2RΔNae (encoded for the polypeptide chain) and rIL-2 as 25 000 and 15 500 dalton, respectively.

Coupling pH	*IL-2R coupled*		*IL-2 binding capacity*		
	mg/ml gel	*nmol/ml gel*	*Calculated (nmol/ml)*[a]	*Observed (nmol/ml)*	*Residual (%)*
5.0	0.900	36	72	14	19
6.0	1.150	46	92	36	39
7.0	1.505	60	120	80	67
8.0	1.575	63	126	88	70
9.0	1.625	65	130	84	65

[a] Two IL-2 binding sites (equivalents) were assumed for the IL-2RΔNae (unpublished observation).

TABLE II

EFFECT OF COUPLING DENSITY

The IL-2R coupling and the IL-2 binding capacity determinations were carried out as described in the text.

IL-2R coupled		*IL-2 binding capacity*		
mg/ml gel	*nmol/ml gel*	*Calculated (nmol/ml)*	*Observed (nmol/ml)*	*Residual (%)*
0.830	33	66	45	68
1.505	60	120	80	67
3.610	144	288	128	44
7.780	311	622	174	28

lower binding capacities, possibly due to steric hindrance. The maximal IL-2 binding capacities were observed when the receptor coupling density was 1–2 mg/ml gel.

Receptor-affinity purification procedures

Extraction, solubilization and renaturation of rIL-2. The rIL-2 was expressed in high concentrations in *E. coli* in an insoluble form within the inclusion bodies. The 1.75 *M* Gu · HCl wash of the pellet, prior to 7 *M* Gu · HCl extraction, removed soluble cellular materials. The extraction of the rIL-2 from inclusion bodies required strong denaturants like 7 *M* Gu · HCl. The solubilized rIL-2 under these conditions was denatured. It was diluted 40-fold and allowed to age for varying periods of time. The results of such a refolding study are shown in Table III. Approximately 3–4 days of aging were needed to renature the Gu · HCl extracted rIL-2.

In the case of conditioned CHO medium, in general, no special treatments were necessary before application to the receptor column.

Adsorption, washing and elution. The adsorption is one of the critical aspects of RAC. The 40-fold dilution of the 7 *M* Gu · HCl extract rendered the Gu · HCl concentration low enough to allow efficient adsorption of the rIL-2 to the receptor column. In order to take advantage of the ability of the receptor sorbent to

TABLE III

rIL-2 REFOLDING STUDIES

The diluted extract was aged for varying periods of time. See text for details.

Age of extract (days)	*Amount of rIL-2 recovered (mg/g cells)*
0	1.536
1	1.918
2	2.300
3	2.480
4	2.604
5	2.572

preferentially bind the fully renatured soluble form of rIL-2 from a heterogeneous population, the column was operated at or above saturating binding conditions. Up to 20 ml/min (maximum flow-rate tried), the capture efficiency was unaffected and no pressure increase or compression of the column bed was observed. The high-flow NuGel affinity support allowed the processing of large volumes of diluted extract and conditioned medium in a short period of time.

Immediately after adsorption, the column was washed extensively with PBS to remove unadsorbed and non-specifically adsorbed materials from within and surrounding the beads. The efficient elution of the adsorbed rIL-2 from the receptor column was achieved by dissociating the receptor–ligand complex with the low pH buffer (pH 2.8) of 0.2 *M* acetic acid containing 0.2 *M* sodium chloride.

Sizing-column, concentration and storage. Gel permeation chromatography was a final step in the purification scheme. It was a convenient way of preparing the rIL-2 free of high-molecular-weight contaminants such as oligomers, trace unwanted proteins of microbial or mammalian origin, pyrogens, etc., as well as low-molecular-weight contaminants like fragments and buffer reagents. This step also exchanged the rIL-2 into its final storage buffer. When kept under aseptic conditions in the acetate–mannitol buffer at pH 3.5, the rIL-2 did not lose any activity, at least for two years.

Purification results

The purification results are summarized in Table IV. During RAC, 63% or 246 mg of the rIL-2 activity in the starting material adsorbed to the column and 39% of the activity was found in the flow-through material. The unadsorbed rIL-2 activity was partly due to the operation of the column above its saturation binding capacity and might be also due to the presence of not fully renatured rIL-2 molecules which might not bind to the RAC column. In the case of mammalian glycosylated rIL-2, 90% or 86 mg bound to the column. After the receptor-affinity step, both forms of rIL-2 had a specific activity of $1.8 \cdot 10^7$ U/mg. The final recoveries for the microbial and mammalian rIL-2s after the gel filtration step were 58 and 88%, respectively. Both forms of rIL-2 had a specific activity of $2.0 \cdot 10^7$ U/mg.

TABLE IV

rIL-2 PURIFICATION RESULTS

To a 250-ml receptor column, 17.5 l diluted extract derived from 100 g *E. coli* cells or 18 l conditioned CHO medium were applied at a flow-rate of 20 ml/min. See the text for further details.

Purification step	*Microbial rIL-2*				*Mammalian rIL-2*			
	Protein (mg)	*Activity ($\cdot 10^7$ U)*	*Recovery (%)*	*Specific activity ($\cdot 10^7$ U/mg)*	*Protein (mg)*	*Activity ($\cdot 10^7$ U)*	*Recovery (%)*	*Specific activity ($\cdot 10^7$ U/mg)*
Extract	1925	703	100	–	81 000	171	100	–
Flow-through	1500	274	39	–	80 900	5	3	–
RAC	246	443	63	1.8	86	154	90	1.8
Sephadex G-50	204	408	58	2.0	75	150	88	2.0

Characterization of the RAC purified rIL-2s

The SDS-PAGE profiles of the microbial and mammalian rIL-2s are shown in Fig. 2. Microbial rIL-2 showed a single band (lane 1), whereas the mammalian rIL-2 (lane 2) contained a major slow moving glycosylated form and a minor fast moving non-glycosylated form, identified by Western blotting with appropriate monoclonal antibodies (data not shown).

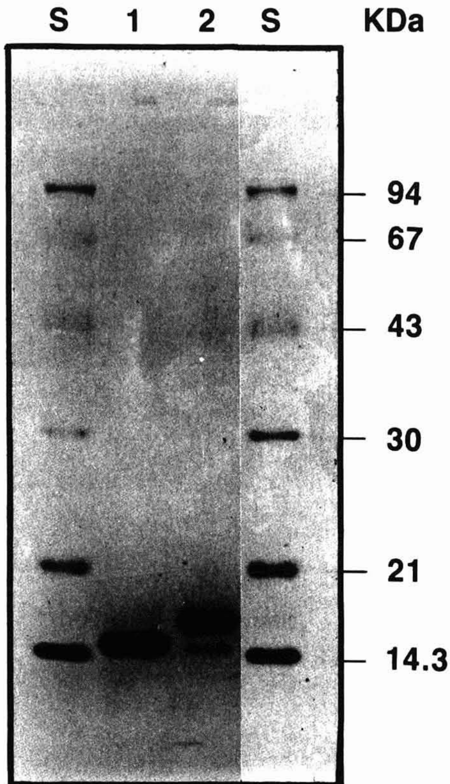

Fig. 2. The SDS-PAGE analysis of receptor-affinity purified rIL-2 from microbial and mammalian sources under non-reducing conditions. Lanes: S = standard molecular weight marker proteins (molecular weights indicated in kilodalton, KDa); 1 = microbial rIL-2; 2 = mammalian glycosylated rIL-2.

The amino acid composition of the both forms of rIL-2 was in good agreement with the expected values (Table V). Structural analysis confirmed the identity of the rIL-2s and the existence of a disulfide linkage between the cysteine residues at positions 58 and 105 (data not shown). The microbial rIL-2 also contained two polypeptide chains, one with an N-terminal Met residue (position 0) and one without, in the ratio of around 9:1 (data not shown).

Comparison of receptor and immunoaffinity purification methods

The rIL-2 binding capacities of the receptor and immunoaffinity adsorbents are given in Table VI.

On a weight-to-weight basis, nine times more antibody was needed per unit

TABLE V

AMINO ACID COMPOSITION OF rIL-2s

Amino acid analyses were performed on a post-column fluorescamine amino acid analyser as described in the text.

Amino acid	*Residues*		
	Microbial	*Mammalian*	*Expected*
Aspartic acid	12.6	11.7	12
Threonine	12.5	12.3	13
Serine	7.2	7.3	8
Glutamic acid	18.5	17.2	18
Proline	5.1	3.9	5
Glycine	2.3	3.0	2
Alanine	5.5	4.7	5
Cysteine	3.0	3.4	3
Valine	4.0	3.7	4
Methionine	4.5	3.9	4 or 5
Isoleucine	8.0	8.1	9
Leucine	22.1	23.4	22
Tyrosine	3.0	3.7	3
Phenylalanine	6.9	6.5	6
Histidine	3.1	3.1	3
Lysine	10.3	11.3	11
Arginine	4.0	4.2	4
Tryptophan	0.9	1.3	1
Total	134	133	133 or 134

volume of immunosorbent to obtain the same amount of IL-2 binding capacity retained by the receptor column. This was partly due to the difference in the molecular weight of the two molecules and also due to the steric hindrance caused by the higher protein loading in the immunosorbent.

As previously shown, the RAC purified rIL-2 was essentially monomeric,

TABLE VI

BINDING CAPACITIES OF RECEPTOR AND IMMUNOSORBENTS

The rIL-2 binding capacities of the receptor and immunosorbents were determined as described in the text. Two binding sites were assumed for the receptor (unpublished observation) and antibody. The molecular weights of the receptor, antibody and rIL-2 were taken as 25 000, 158 000 and 15 500 dalton, respectively.

Adsorbent	*Receptor or antibody coupled (mg/ml gel)*	*IL-2 binding capacity*			
		Calculated (nmol/ml gel)	*Observed*		*Residual (%)*
			mg/ml gel	*nmol/ml gel*	
Receptor	1.55	124	1.3	84	68
5B1 Antibody	12.80	162	1.2	77	48

whereas the immunoaffinity purified material contained significant amounts of oligomeric or aggregated forms of rIL-2, which eluted as a high-molecular-weight fraction during gel permeation[6] along with the desired soluble monomeric form. The specific activities of the rIL-2 purified by both methods are compared in Table VII.

TABLE VII

SPECIFIC ACTIVITIES OF RECEPTOR AND IMMUNOAFFINITY PURIFIED rIL-2s

See the text for experimental details. NA = Not applicable.

Experiment	*Specific activity ($\cdot 10^7$ U/mg)*	
	RAC	*Immunoaffinity*
Affinity step	1.8	0.8
G-50 Peak 1	NA	0.2
G-50 Peak 2	2.0	2.3

Prior to gel permeation chromatography, the specific activities of the RAC and immunoaffinity purified materials were 1.8 and $0.8 \cdot 10^7$ U/mg, respectively. The specific activity of the high-molecular-weight fraction was only one-tenth of that of the soluble monomeric form and caused a two-fold reduction in the specific activity of the immunoaffinity purified total IL-2. These results indicated that in immunoaffinity chromatography, various molecular forms of rIL-2 with varying degrees of biological activity and renaturation state might bind to the antibody column. In contrast, the RAC purified rIL-2 contained essentially the soluble monomeric form.

In summary, RAC has been demonstrated as a viable purification method for rIL-2. The versatility of RAC is such that it is also capable of purifying various rIL-2 muteins, rIL-2 homologues and IL-2 fusion proteins[15,16]. The receptor-affinity sorbents are also stable and performed satisfactorily for at least 500 cycles of operation with no significant impairment in the functionality (data not shown).

The two major areas of concern are the cost-effectiveness and the stringent U.S. FDA regulations[17,18], regarding the quality control requirements for producing recombinant proteins for medicinal use in humans. The advances made in the recombinant DNA technology should enable the large-scale production of soluble receptors economically (*e.g.*, IL-2, IL-1, etc.). The quality control aspects of using RAC for purifying protein biotherapeutics for human use still has to be resolved.

Since biotechnology is on the verge of producing soluble receptors of biomolecules such as IL-1 (ref. 19), γ-interferon, tumor necrosis factor, etc., we strongly believe that it is only a matter of time before RAC becomes an established method for the purification of high-value recombinant proteins.

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Double-coated silica supports for high-performance affinity chromatography of proteins

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ABSTRACT

In order to prepare easily derivatizable supports for high-performance affinity chromatography (HPAC), the advantages of traditional polysaccharide-based supports were combined with the excellent mechanical properties of silica by coating the porous silica beads with a double layer of polysaccharide. The starting material was preliminarily impregnated with dextran or agarose, substituted with a calculated amount of positively charged diethylaminoethyl (DEAE) functions, in order to neutralize the cation-exchange capacity. These silica beads were then recoated by a second coupling with a native dextran or agarose so the DEAE functions introduced by the first coating could be overlayed and the coating state of the silica beads was further improved. The passivation of silica was confirmed by eluting standard proteins on the double-coated silica supports in high-performance size-exclusion chromatography. The elution of an acidic biopolymer, heparin, on different coated silica supports under gradient conditions demonstrated the major improvement of the native polymeric overlayer on the ionic properties of the support. These double-coated silica supports can also easily be activated by classical activation methods and coupled with active ligands (protein A and heparin). The active supports grafted by protein A were used in HPAC of human immunoglobulin G. The double-coated silica supports grafted by heparin were used in HPAC of human α-thrombin and human antithrombin III and for the purification of bovine thrombin from a commercial crude thrombin preparation.

INTRODUCTION

High-performance affinity chromatography (HPAC) combines the great specificity of affinity techniques with the efficiency, sensitivity and speed of operations of high-performance liquid chromatographic (HPLC) techniques. Although significant progress has been made in this area, applications of the technique have been limited owing to the lack of a good general and derivatizable support[1–3]. Such a support for HPAC of proteins requires minimum non-specific adsorption, a hydrophilic character, simple derivatization procedures with a broad range of ligand chemistries and an

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ability to tolerate different solvents and a rapid change in solvent composition necessary for efficient elution of proteins. In particular, the high elution rates used in HPAC require excellent mass-transfer properties and good mechanical properties of the stationary phase.

Polysaccharide-based supports have been successfully used for traditional affinity chromatography becaus of their good chromatographic properties and the simplicity of the activation procedures, but their mechanical instability limits their use in HPAC. In contrast, silica, commonly used for HPLC of proteins[4–7], is mechanically stable but contains acidic silanol groups, among other surface groups, that cause strong and often irreversible non-specific adsorption of proteins in aqueous media[4]. By coating polysaccharide on silica beads, we have combined the advantages of the traditional soft gel affinity supports with the excellent mechanical properties of silica supports.

We have previously reported the preparation of coated silica supports for the HPLC of biopolymers by impregnating silica beads with a polysaccharide (dextran or agarose), substituted by a calculated amount of diethylaminoethyl (DEAE) functions[8,9]. The DEAE-polymer monocoated silica supports present only minimal cation-exchange capacity and have been used in high-performance size-exclusion chromatography (HPSEC)[8] and HPAC of proteins[9]. However, it is difficult to obtain an exact balance between ion-exchange capacities of the DEAE-polymer and the native silica. The neutralization of the silanol functions requires an excess of positive charges and leads to a residual anion-exchange capacity.

In view of the above, we have prepared a new class of double-coated silica supports. After a preliminary coating with a DEAE-polymer, the silica beads are recoated with native dextran or agarose in order to overlay the positive DEAE groups on the surface. The minimization of non-specific adsorptions was investigated by HPSEC. Gradient elution of an acidic polymer, heparin, on the mono- and double-coated silica supports demonstrates the importance of the double coating. Because of their polysaccharidic surface, the techniques used for the immobilization of ligands on polysaccharide-based supports can be easily transferred to these supports. Active ligands (protein A and heparin) have been immobilized with a good yield by classical activation methods[10]. Comparison of the elutions of human IgG on mono- and double-coated silica supports grafted by protein A demonstrates the better performance of the double-coated silica support in HPAC. The elutions of human α-thrombin and human antithrombin III (AT-III) were carried out on the double-coated silica supports, grafted by heparin. Finally, the purification of bovine thrombin from a commercial crude thrombin preparation was achieved on this affinity sorbent by HPAC. The results of the purification are compared with those obtained on the commercial heparin grafted supports.

EXPERIMENTAL

Preparation of coated-silica supports and affinity sorbents

The starting silica beads (silica X015 M and silica X075 M; IBF Biotechnics, Villeneuve La Garenne, France) are mainly spherical (40–100 μm) and have average pore diameters of 1250 and 300 Å, respectively. The polysaccharides used in this study are agaroses (Indubiose A37 HAA and Indubiose A37 N) from IBF-Biotechnics and

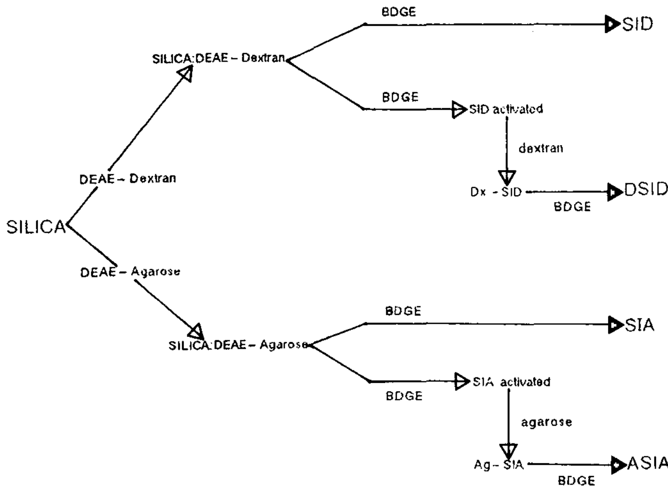


Fig. 1. Preparation of coated silica supports.

dextrans (T40, T70 and T500; Pharmacia France, Bois D'Arcy, France). The preparation of DEAE-polysaccharides and monocoated silica supports have been described previously[8,9].

The preparation of double-coated silica supports is illustrated in Fig. 1. A 10-g amount of the native silica was impregnated with 0.45 g of DEAE-agarose or 1.5 g of DEAE-dextran in 25 ml of doubly distilled water, the pH of which had been adjusted to 11.5, for 30 min at 80°C for DEAE-agarose and at room temperature for DEAE-dextran. The polymeric layer passivate the silica phases and increase the chemical stability of the supports towards the alkaline media under the experimental conditions. The packing was dried for 15 h at 80°C, then added to a solution of 284 mg (2.2 mmol) of 1,4-butanediol diglycidyl ether (BDGE) in 100 ml of diethyl ether. The mixture was stirred for 30 min at 40°C. After evaporation of the solvent, the silica powder was dried for 30 min at 80°C and then coupled with 0.45 g of agarose or 1.5 g of dextran in 30 ml of doubly distilled water (pH adjusted to 11.5) for 2 h at 80°C. After drying for 15 h at 80°C, the surface layer of coated silica was cross-linked with 71 mg (0.55 mmol) of BDGE in 100 ml of diethyl ether for 30 min at 40°C. The amount of polymer covering the material surface was determined by elemental analysis of carbon and expressed as the weight of coating polymer (mg of coating polymer per g of support).

The coated silica beads were actived using 1,1′-carbonyldiimidazole (CDI) or BDGE as activing agent[10]. Immobilization of protein A (IBF Biotechnics) and heparin (101 IU/mg; Institut Choay, Paris, France) was performed using the actived support suspended with the ligand in 0.1 *M* sodium carbonate buffer (pH 8.7) at room temperature. The amount of protein A immobilized was determined by Bradford's method[11] and the amount of heparin was determined by elemental analysis of sulphur. The adsorption capacity of the active supports was determined from the adsorption isotherms.

Chromatographic experiments

The HPLC apparatus was a Merck–Hitachi 655 A-12 gradient system from Lab Merck-Clevenot (Nogent sur Marne, France) with a Rheodyne 7126 injection valve, connected to an LMC UV–visible variable-wavelength monitor and a D2000 integrator. The solutions and the buffers were prepared with doubly distilled water, filtered through a 0.22-μm Millipore HA membrane. All chemicals were of analytical-reagent grade.

The passivation of native silica by the double coating was investigated by testing the performances of the coated silica supports in HPSEC. Molecular weight calibration graphs of standard proteins were obtained as described previously[8]. The size-exclusion retentions of the proteins on the supports were comfirmed by eluting several acidic or basic proteins [human albumin, human immunoglobulin G (IgG), cytochrome *c*, antithrombin III] under gradient conditions. The improvement in the ionic properties of the supports resulting from the double coating was also studied by injecting an acidic biopolymer, heparin, onto the double- and mono-coated silica supports. After washing with the initial buffer, the adsorbed substance was eluted by raising the salt concentration of the eluent.

The different biospecific affinity sorbents prepared from the double-coated silica support were used in chromatographic experiments. Huma IgG (12.5 mg/ml; Sigma), human antithrombin III (3.4 IU/mg; CRTS, Lille, France) and thrombin [1000 NIH.U/mg (*i.e.* activity of thrombin determined by comparison with standard from National Institute of Health, Bethesda, MD, U.S.A.); CNTS, Paris, France] were eluted on their respective affinity sorbents under gradient conditions. A 5-ml volume (23.5 mg of proteins) of crude bovine thrombin sample (Hoffmann-La Roche) was injected onto a column packed with 2.5 ml of heparin affinity sorbent and pre-equilibrated with the initial buffer [0.02 *M* disodium phosphate–0.15 *M* NaCl (pH 7.4)], at room temperature at flow-rate of 60 ml/h. The adsorbed thrombin was then eluted using a salt gradient.

In all chromatographic experiments, the eluted proteins were detected at 280 nm and the chromatographic fractions were collected in order to determine the yield of the separations. The concentrations of proteins were measured by Bradford's method[11]. The biological activity of AT-III was determined using Chromo-Thrombin from Diagnostica Stago (Asniéres-sur-Seine, France)[12]. The thrombin clotting activity of the eluted fractions was measured as described previously[13]. The fractions were also analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 20% gel using the PhastSystem (Pharmacia, Uppsala, Sweden).

RESULTS AND DISCUSSION

Preparation of coated silica support

The purpose of the modification of the silica surface is to minimize the non-specific adsorption of silica supports for biopolymers and to introduce a high concentration of easily activatable groups. This was achieved by coating the inorganic phases with polysaccharide. The cation-exchange capacity of the native silica is minimized by impregnation with dextran and agarose substituted by a calculated amount of positively charged functional DEAE groups[8,9]. The characteristics of the substituted polysaccharides used for the preparation of the different supports are

TABLE I

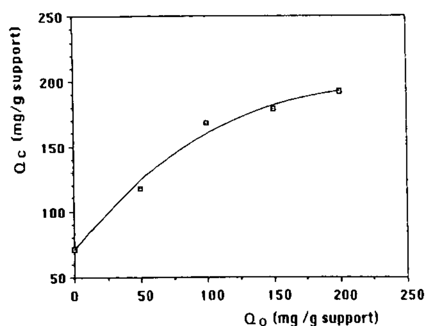
CHARACTERISTICS OF SUBSTITUTED POLYSACCHARIDES

Substituted polysaccharide	*Starting polymer*	*Mol.wt. (g/mol)*	*Percentage of DEAE units*
DDT404	Dextran T40	35 600	4.8
DDT705	Dextran T70	68 000	4.5
DDT50011	Dextran T500	488 000	7.2
DHAA1	Indubiose HAA	—	10.0
DNA1	Indubiose NA	—	10.0

presented in Table I. However, it is difficult to obtain an exact balance between the ion-exchange capacities of the native silica and the DEAE-polymer. Therefore, these silica beads impregnated with DEAE-polysaccharide were recoated by coupling a native dextran or agarose, in order to obtain supports with hydrophilic and non-ionic surfaces. The amount of polysaccharide coupled depends on the amount of polysaccharide used (Fig. 2). The use of a polymer with a high molecular weight is useful for raising the coating capacity (Fig. 3). The characteristics of the mono- and double-coated silica supports used in this study are presented in Table II.

HPSEC performance of the supports

The different coated silicas were tested in HPSEC using several proteins with molecular weight from $6.5 \cdot 10^3$ to $700 \cdot 10^3$ g/mol. Blue dextran ($2 \cdot 10^6$ g/mol), glycyltyrosinamide (434 g/mol) and dinitrophenylalanine (255 g/mol) were used in order to determine the exclusion limits of the support. Most of the proteins were eluted between the exclusion limits of the supports, except cytochrome *c*, which was abnormally retained by the supports prepared from silica X075 M. This effect is probably related to the high p*I* value and the small size of this protein and indicates that some silanol functions inside the pores of the silica with smaller porosity are not



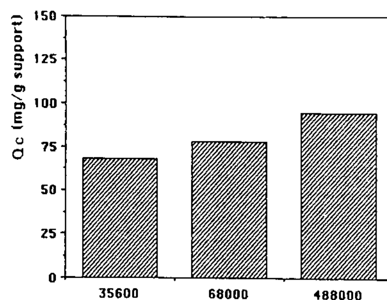


Fig. 2. Variation of the amount of dextran coupled with the initial amount of dextran used. Silica X015 M, dextran T500.

Fig. 3. Influence of the molecular weight (horizontal axis) of dextran used on the amount of coating. Silica X015 M.

TABLE II

CHARACTERISTICS OF COATED SILICA SUPPORTS

Support	*Silica porosity (Å)*	*Coating polymer*		*Amount of polymer fixed (mg/g support)* [a]
		Code	*Mol.wt. (g/mol)*	
SID15705	1250	DDT705	68 000	104
SID1550011	1250	DDT50011	488 000	101
SIA15HAA1	1250	DHAA1	–	45
DSID15M	1250	DDT50011	488 000	72 (1)
		Dextran T500	488 000	94 (2)
DSID75M	300	DDT705	68 000	68 (1)
		Dextran T70	68 000	90 (2)
ASIA15M	1250	DHAA1	–	45 (1)
		Indubiose HAA	–	35 (2)

[a] (1) First coating; (2) second coating.

completely masked. A comparison of the behaviour of two silicas with different porosities but passivated by the same dextran double coating is presented in Fig. 4. The slopes of the calibration graphs indicate that the support prepared with the 300-Å silica beads gives a better resolution for proteins in the range 10–100 kg/mol. However, the molecular weight limits of this support are slightly restricted compared with the exclusion limits of the 1250-Å pore-size unmodified silica packing. Because of their minimal non-specific adsorption and larger exclusion limits, the supports prepared from silica X015 M were chosen as starting materials for the preparation of affinity sorbents.

The normal elution behaviour of several acidic or basic proteins (human albumin, human IgG, human AT-III, cytochrome *c*) on the double-coated silica supports (DSID15M or ASIA15M), even at high ionic strength, demonstrates minimal non-specific adsorptions. Moreover, heparin is strongly adsorbed on the mono-coated silica support at 0.1 *M* sodium chloride (Fig. 5a), but is eluted by the same buffer on the

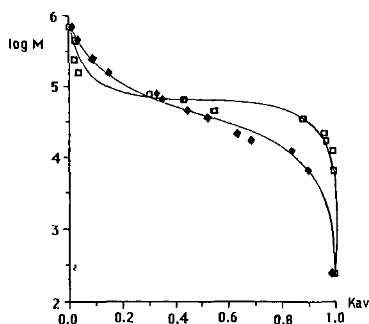


Fig. 4. Molecular weight (*M*) calibration graphs of standard proteins on (□) DSID75M and (◆) DSID15M supports. Column, 25 × 0.7 cm I.D.; flow-rate, 1 ml/min; eluent, 0.02 *M* Tris–HCl–0.15 *M* NaCl (pH 7.4). $K_{av} = (V_e - V_0)/(V_t - V_0)$.

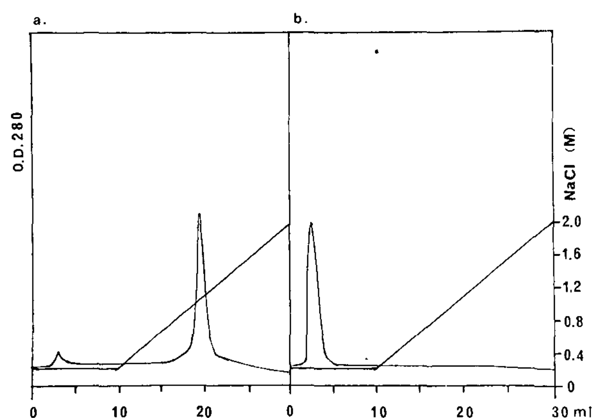


Fig. 5. Elution of 100 μl heparin (3 mg/ml) on (a) SID15M50011 and (b) DSID15M supports. Column, 5 × 0.7 cm I.D.; flow-rate, 1 ml/min; eluent, 0.02 *M* disodium phosphate (pH 7.4).

double-coated silica (Fig. 5b). This result demonstrates that the second coating masks the residual anion-exchange capacity of DEAE groups of the mono-coated phases.

Immobilization of active ligands

The immobilization of a broad range of ligands has been realized on

TABLE III

COUPLING CONDITIONS OF COATED SILICA SUPPORTS

Q_l = amount of ligand used (mg/g support); Q_a = amount of CDI used (mmol/g support).

Support	*Ligand*	Q_l	Q_a	*Time (h)*	*Yield (%)*
SID15M50011	Protein A	2.29	0.615	15	92
DSID15M	Protein A	2.29	0.615	15	93
DSID15M	Heparin	50	1.84	48	68
ASIA15M	Heparin	50	1.84	48	68

TABLE IV

CAPACITY OF THE AFFINITY SORBENTS

$[Q_l]$ = amount of ligand immobilized.

Stationary phase	$[Q_l]$ *(mg/g)*	*Protein adsorbed*	*Capacity (mg/g)*
Protein A–DSID15M	2	IgG	7
Heparin–DSID15M	28	Thrombin	13 000[a]
Heparin–DSID15M	28	AT-III	6.27

[a] NIH.U/g.

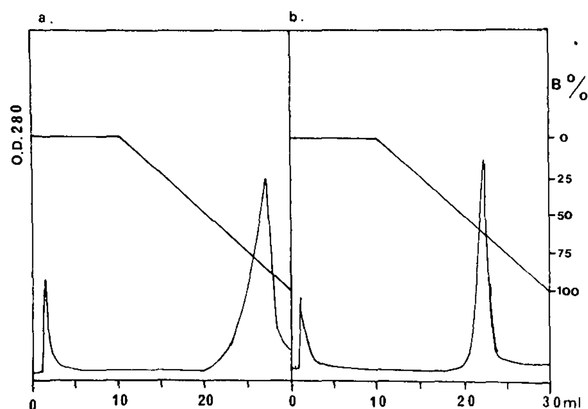


Fig. 6. Elution of 100 μl of human IgG (12.5 mg/ml) on (a) protein A–SID15M50011 and (b) protein A–DSID15M supports. Column, 5 × 0.7 cm I.D.; flow-rate, 1 ml/min; buffer A, 0.02 *M* Tris–HCl–0.15 *M* NaCl (pH 7.4); buffer B, 0.02 *M* glycine–HCl–0.15 *M* NaCl (pH 2.8).

polysaccharide-based supports[10]. These results can be easily adapted to the double-coated silica supports. The immobilization of protein A and heparin was performed with a high yield using CDI and BDGE as activating agents (Table III). The biopolymers immobilized on the double-coated silicas show a considerable adsorption capacity for the corresponding protein in solution (Table IV).

Elutions of proteins in HPAC

In order to study the performances of the supports in HPAC, several human proteins (IgG, AT-III and thrombin) were injected onto their respective affinity sorbents, prepared fom the double-coated silica supports. The conditions used for adsorption and desorption were similar to those used on classical supports, grafted by the same ligand.

A typical chromatogram of human IgG on protein A immobilized on dextran double-coated silica support (protein A–DSI15M) is presented in Fig. 6a. The protein was adsorbed at pH 7.4 (0.02 *M* Tris–HCl–0.15 *M* NaCl) and selectively desorbed by a decreasing pH gradient with 83% protein recovery. The comparison of the elution on dextran mono- and double-coated affinity supports (Fig. 6a and b) shows that IgG is

TABLE V

ELUTION CONDITIONS FOR HPAC OF PROTEINS

Stationary phase	*Protein eluted*	*Condition of desorption*	*Yield (%)*	*Fig.*
Protein A–SID15M	IgG	85% B	83	6a
Protein A–DSID15M	IgG	62% B	83	6b
Heparin–DSID15M	Thrombin	1 *M* NaCl	>80	7a
Heparin–DSID15M	AT-III	1.2 *M* NaCl	82	7b

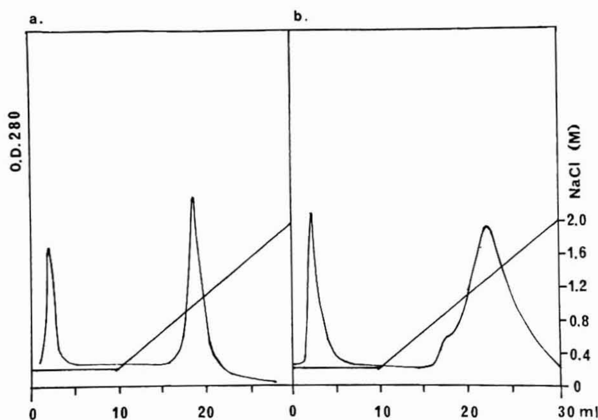


Fig. 7. Elution of (a) 100 μl of human thrombin (1000 NIH.U/ml) and (b) 100 μl of human antithrombin III (34 IU/ml) on heparin–SID15M support. Column, 5 × 0.7 cm I.D.; flow-rate, 1 ml/min; eluent, 0.02 *M* disodium phosphate (pH 7.4).

adsorbed and eluted with the same recovery on both supports (Table V). However, the narrower elution peak and the lower proportion of eluting buffer B at the peak maximum demonstrate the better performance of the double-coated stationary phase. The chromatograms are similar to those obtained on traditional phase, grafted by protein A[14].

HPAC elutions of human thrombin (Fig. 7a) and human AT-III (Fig. 7b) were carried out on heparin immobilized on the dextran double-coated support (heparin–DSID15M). The two proteins were strongly adsorbed at low ionic strength and selectively desorbed by increasing the salt concentration. The eluted fractions were collected and their biological activities were measured, demonstrating a good recovery (>80%) (Table V). The recovery of the enzymatic activity of α-thrombin was reduced because slight inactivation of the enzyme occurs during the separation at room temperature.

A 100-μl volume of AT-III solution was successively injected 20 times onto the

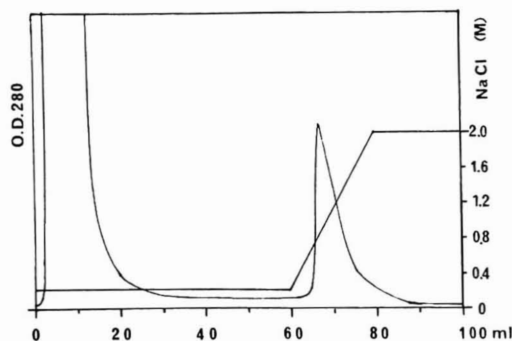


Fig. 8. Purification of bovine thrombin (5 ml) by HPAC on heparin–DSID15M support. Column, 5 × 0.7 cm I.D.; flow-rate, 1 ml/min; eluent, 0.02 *M* disodium phosphate (pH 7.4).

TABLE VI

PURIFICATIOH OF BOVINE THROMBIN

Parameter	*Starting material*	*Desorption peak I*	*Desorption peak II*
Volume (ml)	5	15	12
Protein (mg/ml)	4.7	1.4	0.05
Thrombin (NIH.U/ml)	600	4	104.4
Specific activity (NIH.U/mg)	64	2.9	2130
Yield (%)	–	4	83.5
Purification factor	–	–	33.3

heparin-immobilized column under the same elution conditions; similar chromatograms were obtained and no apparent change in the elution recovery was observable during these elutions. This result demonstrates the excellent chemical stability of the coated-silica supports and the absence of ligand bleeding under these elution conditions.

This support was also used in order to purify bovine thrombin from a commercial crude preparation (Fig. 8). Thrombin is eluted at an ionic strength of about 1 M NaCl as a single, fairly sharp peak. No additional material was eluted at higher

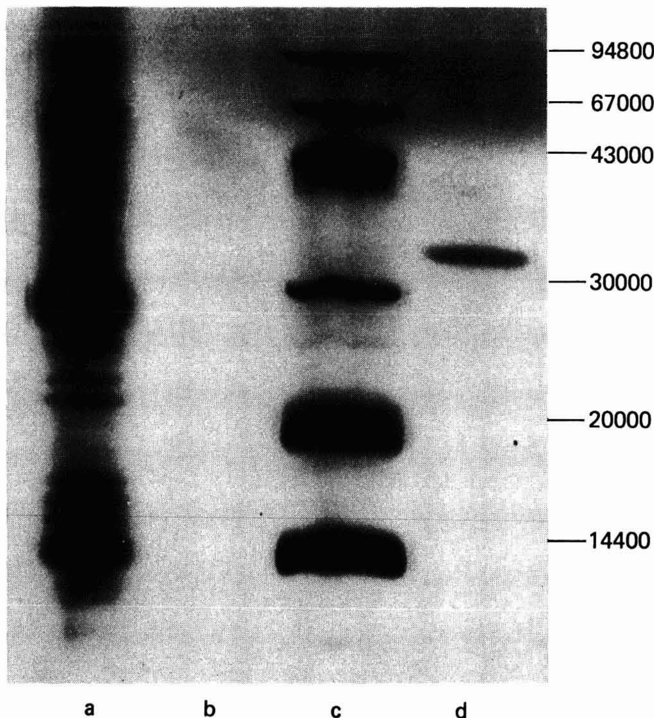


Fig. 9. SDS-PAGE. (a) Crude thrombin; (b) background; (c) standard proteins; (d) purified thrombin.

ionic strength. The enzyme activity, determined by clotting assay, coincides with the protein peak. The results of the different purifications are given in Table VI. The recovery of biological activity in these experiments was about 78% and the total yield of purified thrombin was 0.56 mg, with a specific activity of about 2100 NIH.U/mg. The purity of thrombin was confirmed by SDS-PAGE (Fig. 9). These results are similar to those obtained on commercial heparin-grafted phases[15]. The small difference in recovery is probably due to a slight inactivation of the enzyme during the separation at room temperature.

CONCLUSIONS

The cation-exchange capacity of silica beads can be minimized by a preliminary impregnation of the inorganic material using a hydrophilic polymer with a relatively low percentage of units bearing positively charged DEAE groups. The impregnated silica can be recoated by coupling a native polysaccharide in order to overlay the DEAE units and to improve the coating state. The introduction of a hydrophilic and non-ionic polymeric layer on the silica surface minimizes the non-specific adsorptions and introdues a high concentration of activatable hydroxylic functions. The modified supports can be easily grafted with active ligands by the classical coupling techniques. The affinity sorbents prepared from the double-coated silica supports can be used in HPAC of proteins. In comparison with mono-coated silica supports, these double-coated silicas exhibit better performances in HPAC and HPAC purifications of proteins from a crude material can be carried out on these affinity sorbents. The separations are similar to those obtained on the corresponding traditional matrices. Moreover, because of the mechanical properties of the starting material, these double-coated silica supports can easily be used for scaling up the purification of proteins by HPAC.

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Affinity purification of tissue plasminogen activator using transition-state analogues

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ABSTRACT

The search for a simple affinity ligand to purify tissue plasminogen activator (tPA) was facilitated by a solid-phase synthesis approach. A large variety of tripeptide ligands containing argininal were synthesized on agarose gels containing a spacer with carboxy terminal. The immobilized ligands were easy to test with urokinase, and tPA. While a number of sequence combinations showed initial binding by tPA, only a few resulted in tight binding corresponding to a hemiacetal linkage with the active site serine. Hydrophobic residues, especially aromatics, flanking the N-side of argininal gave rise to ligands which were bound strongly by tPA. A gel containing D-Phe–D-Phe–Argal (an aldehyde derivative of arginine) was very effective in purifying tPA derived from cell culture media at small scale (milligrams) and at large (multi-grams).

INTRODUCTION

Recent clinical studies have shown that thrombolytic rescue of patients undergoing an acute myocardial infarction (AMI) has definite benefit resulting in lowering patient mortality[1]. Among the newer agents showing promise in this regard is tissue plasminogen activator (tPA). Because of the large dosage requirement (100 mg), it is of interest to find more efficient ways to produce and purify this drug. We describe here a novel affinity purification process for tPA using tripeptide transition state analogues as ligands.

Since tPA is a trypsin-like serine protease, we initially surveyed inhibitors well characterized for trypsin. Simple, readily attainable ligands, such as arginine or benzamidine, which had been long shown as inhibitors of trypsin, proved to be weak in binding tPA. The macromolecular (20 000 dalton) ligand, Erythrina trypsin inhibitor (ETI), had been shown to bind tPA with great avidity, and this was verified in our own laboratory. However, its relative scarcity and high cost made it unattractive for consideration in any affinity purification system that was to be directed to large-scale (kilograms) enzyme isolation.

Thus, the challenge was to develop a novel affinity ligand of moderately low

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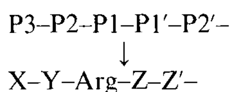
molecular weight but possessing strong binding properties like that of ETI. This led us to evaluating tripeptide structures which could be generally described as follows:



where the "P"residues define parts of the substrate specificity and include the critical arginine the carbamide linkage of which is the scissile bond (indicated by "↓"). Other studies had indicated that lysine in the P1 position was not as good as arginine for tPA, while trypsin seems to bind both residues with more similar avidity.

To further enhance binding potency, we looked to structures that could form transition-state analogues. This could be attained by converting the C-terminal arginine into argininal, which could form a hemi-acetal structure with the active-site serine when the tripeptide ligand was bound to the enzyme. Such structures have been shown by others to bind trypsin-like proteases with great avidity[2].

Given the abundance of trypsin inhibitors from which new tPA inhibitors might be modeled, it was instructive to compare differences (in addition to those already noted above) in substrate specificity between the two enzymes.

Trypsin is capable of attacking a wide range of peptide sequences and even undergoes autolysis. By contrast tPA seems uniquely poised to hydrolyze a specific peptide loop in plasminogen and cause its conversion to plasmin. Owing to this specificity tPA does not autolyze. With its more complex structure comprised of finger, growth factor, and two kringle domains attached to the protease domain, tPA does not function through a zymogen stage, but normally does its task by first binding to a freshly formed fibrin clot which also binds plasminogen. It has been demonstrated that the catalytic activity of tPA, while intrinsically well below that of trypsin[3], is significantly stimulated by fibrin[4]. The generation of plasmin by this process results in efficient dissolution of the fibrin clot. Thus, tPA has gathered much attention as a thrombolytic of great clinical promise.

In our quest for a novel inhibitor selective for tPA binding, we studied the example of ETI[5], which in addition to binding trypsin has unusual avidity towards tPA. TPA binding to ETI may not involve a reversible scissile bond. Indeed, tPA inactivated with diisopropylfluorophosphate (DFP) is still capable of binding to ETI[6]. This suggested that inhibitor–enzyme recognition may involve structural elements some what removed in space from the catalytic triad.

Among the early candidates for a tPA-specific ligand was the tripeptide sequence (Pro558–Gly559–Arg560) neighboring the scissile bond in plasminogen. The structure proved disappointing. In addition, several other structures suggested by known substrates and inhibitors, proved to be equally disappointing. Thus, we were prompted to empirically search for a more avid binding tripeptide, argininal-containing sequences.

Our approach was to synthesize a series of affinity gels containing tripeptide structures bearing a C-terminal argininal moiety. These gels were tested in mini-columns with partially purified tPA preparations for effectiveness in capturing and purifying the enzyme.

Upon identifying a effective ligand, it was necessary to determine the best

conditions for capturing tPA from conditioned media and for its further purificaton. Unlike chemically unreactive affinity ligands, one which contains an argininal moiety has the potential to non-selectively react with primary and secondary amines in buffers or culture media. Beyond these studies, it was of interest to study the process at large-scale with a view to developing a system for meeting the growing needs of tPA in treating acute myocardial infarctions[7].

EXPERIMENTAL

Materials sources

Amino acids and protected derivatives were purchased from Bachem Bioscience (Philadelphia, PA, U.S.A.) Affi-Gel-10 was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Chromogenic enzyme substrates were purchased from Helena Labs. (Beamont, TX, U.S.A.). Bovine thrombin was from Miles Diagnostics (Kankakee, IL, U.S.A.). Urokinase was obtained from Calbiochem (San Diego, CA, U.S.A.) tPA was obtained from in-house souces. Other specialty chemicals were procured from Sigma (St. Louis, MO, U.S.A.). Arginine semicarbazone (ArgSC) was synthesized according to a new method (patent applied for). Alternatively one could prepare the reagent according to Patel and Schultz[8].

Synthesis of ESEP-Cl-6B-EDA-SA gels

This was done according to the epibromohydrin procedure described by Nishikawa and Bailon[9]. Instead of shake flasks a modified stirred resin flask (Kontes Glass, No. K-614012-9006) was used. The epoxy-derivative of Sepharose Cl-6B (obtained from Pharmacia) was reacted with ethylenediamine in water at room temperature overnight. Unreacted ethylenediamine was washed out from the gel with 0.1 *M* acetic acid followed by water. Next the gel was reacted with succinic anhydride in water at pH 6. Finally, the gel was washed with 0.1 *M* sodium carbonate followed by water until washings were neutral. The product gel was rinsed three times with isopropanol and stored as a moist powder.

Synthesis of ESEP-Cl-6B-EDA-SA-X-Y-ArgSCgels

The "X" (P3), "Y" (P2) and ArgSC (P1) groups were coupled sequentially to the succinyl moiety using water-soluble carbodiimide [N-ethyl-N′-(3-dimetylaminopropyl) carbodiimide hydrochloride], triethylamine, and N-hydroxybenztriazole. The X and Y groups were added as methyl ester derivatives and followed by saponification with 0.1 *M* sodium carbonate[10].

Synthesis of Affi-Gel-10-X-Y-ArgSCgels

The chemically reactive Affi-Gel-10 gels were coupled directly at pH 7 to X–Y groups already preformed as dipeptides. The ArgSC moiety was coupled to the Affi-Gel-10–X–Y using water-soluble carbodiimide (procedure as above). The semicarbazide protecting group was removed by treatment with formaldehyde in dilute acetic acid.

Testing of sorbents

Usually a 5 × 1 cm column was packed and pre-equilibrated with a pH

6 imidazole buffer, except with tPA where 0.1 *M* sodium acetate pH 4.5 (plus sodium chloride) was used. After loading enzyme, the column was washed with various buffers to remove contaminants. The enzyme could be recovered with either 0.1 *M* acetic acid or 0.1 *M* semicarbazide. Occasionally 0.2 *M* ammonium hydroxide as used.

Assay of enzyme activity

Samples were assayed with appropriate chromogenic substrates at room temperature. Increases in absorbance (at appropriate wavelength) were recorded in a spectrophotometer for 300 s. Samples were diluted so that linear absorbance tracings were obtained during the enzyme reactions.

Trypsin. *A* 247 nm, 900 μl 0.1 *M* Tris–0.01% PEG-3400 pH 8.1 buffer, 100 μl 0.01 *M* tosylarginine methyl ester in water, 100 μl of sample.

tPA. *A* 405 nm, 900 μl Tris buffer (see trypsin), 50 μl 0.01 *M* D-Ile–Pro–Arg–*p*-nitroanilide (pNA) · HCl (S-2288), 50 μl of sample; also assayed by S-2251 (ref. 11).

Urokinase. *A* 405 nm, 800 μl Tris buffer (see trypsin), 50 μl 0.003 *M* pyroglutamate (Pyr–Glu)–Gly–Arg–pNA · HCl (S-2444), 200 μl of sample.

Thrombin. *A* 405 nm, 900 μl 0.1 *M* Tris–0.025 *M* $CaCl_2$–0.01% PEG-3400 pH 8.1 buffer, 50 μl 0.002 *M* D-Phe–pipecolic acid (Pip)–Arg–pNA (S-2238), 50 μl of sample.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis

General method was according to Laemmli[12]. Stacking gel was 4% acrylamide and 5% cross-linker. Separating gel was 10% acrylamide and 5% cross-linker. Dithiothreitol was used to reduce proteins.

RESULTS AND DISCUSSION

Inhibitor studies

ETI. Among the serine protease inhibitors (serpins) described in the literature, ETI[5] stands out as being uniquely selective for tPA (in addition to reacting with trypsin). Heussen *et al.*[6] had shown that it was capable of avidly binding even active-site blocked inhibited tPA. This suggested that a considerable part of the binding of tPA to ETI migh involve macroscopic structural domains in the two proteins (as depicted in Fig. 1). But since trypsin (which is similar in size to the protease domain of tPA), appears to avidly recognize and bind the sequence: Arg61–Leu62–Arg63 in ETI, we decided to test an oligopeptide containing this sequence as an affinity ligand for tPA.

Affi-Gel-10–Arg–Leu–Argal was prepared as described above and tested with tPA. The results are shown in Table I. While tPA appears to adsorb initially to the gel,

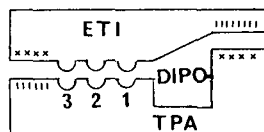


Fig. 1. Model depicting ETI binding to tPA as diisopropylphosphoryl (DIPO) derivative.

TABLE I

BINDING STUDY OF tPA WITH AFFINITY MATRIX (Affi-Gel-10–Arg–Leu–Argal) CONTAINING HOMOLOGOUS SEQUENCE TO ETI ACTIVE SITE SEQUENCE

Test sample	*Enzyme activity (%)* *t-PA*
Load	100
Flow through	0
0.2 *M* sodium chloride/buffer wash	49
1.0 *M* sodium chloride/buffer wash	53
0.2 *M* Ammonium hydroxide elution	8

it is readily desorbed by buffer containing salts to simply disrupt ionic interactions between enzyme and ligand. The results point to the importance of macro-surface recognition features (even though not yet known) in ETI which cause tight binding to tPA.

The plasminogen loop. Owing to the efficiency with which tPA can cleave the scissile Arg560 bond in plasminogen, we decided to explore an oligopeptide containing the sequence: Pro558–Gly559–Arg560 as a possible affinity ligand.

A gel bearing Pro–Gly–Argal was tested with tPA and urokinase (UK) another well established plasminogen activator. The results seen in Table II indicate that both enzymes initially bind to the affinity sorbent. tPA is readily desorbed with buffer containing salts to disrupt ionic interactions. While a large portion of UK desorbs readily with salt-containing buffers, a smaller but significant amount requires low pH to disrupt the hemiacetal linkage and allow release of the enzyme. This agrees with other studies involving amidolysis of oligopeptide substrates bearing the Pro–Gly–Arg sequence[3]. Perhaps the low avidity of tPA to this linear sequence is not surprising. The Pro–Gly sequence is typically found in β-turns and this tripeptide sequence is part of a small disulfide enclosed loop in plasminogen[13]. Thus one might expect that the scissile bond in the zymogen might be present in a highly accesible conformation to the tPA which has been activated by the attachment of fibrin.

TABLE II

BINDING STUDY OF tPA AND UROKINASE WITH AFFINITY MATRIX [Sepharose–ETHYLENEDIAMINE (EDA)–SUCCINIC ACID (SA)–Pro–Gly–Argal] CONTAINING PLASMINOGEN LOOP SEQUENCE HOMOLOGY

Test sample	*Enzyme activity (%)*	
	tPA	*Urokinase*
Load	100	100
Flow through	1	0
0.1 *M* Sodium chloride/buffer wash	81	48
1.0 *M* Sodium chloride/buffer wash	7	9
0.1*M* Acetic acid	6	21

TABLE III

BINDING OF tPA-1 (ONE CHAIN) TO KNOWN PEPTIDE INHIBITOR SEQUENCES

Affi–E–G–Argal = Affi–Glu–Gly–Argal; Affi–Q–G–Argal = Affi–Gln–Gly–Argal.

Test sample	*Enzyme activity (%)*	
	Affi–E–G–Argal	*Affi–Q–G–Argal*
Load	100	100
Flow through	0	3
Low salt wash	84	74
High salt wash	6	13

Dansyl-Glu–Gly–Arg–chloromethyl ketone. As a serine protease, tPA was studied sometime ago with active-site directed alkylating agents bearing chloromethyl ketones. One such reagent comprised of dansylated Glu–Gly–Arg was found to have particular avidity for tPA[14]. Without the dansyl group, the reagent was about 100-fold less potent but still appreciable.

Hence, we prepared an agarose gel containing Glu–Gly–Argal for testing. In addition, a Gln–Gly–Argal gel was prepared to assess the charged group effect of the γ-carboxyl of glutamate. The results seen in Table III indicate that while tPA binds to the gel but no apparent hemiacetal is formed. To rule out interference by low-molecular-weight amine compounds in the sample (conditioned media), the binding study was repeated with diafiltered enzyme (data not shown), which also bound but then eluted with a salt wash. The results with a gel containing Gln instead of Glu (Table III) indicate that loss of a negative charge at the P3 position of the ligand has little consequence and the tPA still desorbs easily with low salt buffer.

PPACK. D-Phe–Pro–Arg–chloromethyl ketone (PPACK) was originally developed as a thrombin inhibitor[15]. It was found that this compound has to have good activity against tPA bu a weak effect on plasmin[16]. This has prompted its use as an agent for preserving blood samples following fibrinolytic therapy[16].

Owing to this effectiveness we decided to explore an affinity gel containing

TABLE IV

BINDING STUDY OF tPA WITH AFFINITY MATRIX (Sepharose–EDA–SA–Phe–Pro–Argal) CONTAINING PPACK ANALOGUE

Test sample	*Enzyme activity (%)*	
	tPA-1[a]	*t-PA-2*[b]
Load	100	100
Flow through	1	0
Low salt wash	33	4
High salt wash	2	1
0.1 *M* Acetic acid	74	84

[a] One chain.

[b] Two chains.

TABLE V

BINDING STUDY OF MYELOMA tPA (ONE CHAIN) WITH VARIOUS AFFINITY MATRICES WITH X–Y–Argal AS LIGANDS

Ligand, X–Y–Argal	*Enzyme activity (%)*		
	Unbound	*Wash*	*Recovered*
D-Phe–L-Ala–Argal	0	24	54
L-Ile–L-Ala–Argal	0	73	3
L-Ile–D-Phe–Argal	0	98	8
L-Val–D-Phe–Argal	0	92	10
L-Ala–D-Phe–Argal	0	64	34
L-Tyr–L-Ala–Argal	1	55	31
L-Tyr–L-Val–Argal	0	87	25

D-Phe–Pro–Argal for purifying tPA. The results shown in Table IV suggest that this ligand is fairly effective in capturing and purifying tPA. However, this depended on the chain content of tPA. Preparations where the cell line and/or culture conditions yielded primarily single-chain enzyme, showed as slightly weaker binding in that some activity was lost on low salt elution (experimental error in activity determinations is about 10%). This unexpected finding was further explored (see below) with ligands which appeared to better capture both forms of tPA.

Search for better ligands

After we examined ligands based on analogy to substrates and inhibitors of historical precedence and found them wanting, we concluded that an empirical screening for structure was an appropriate endeavor. The prospect was not that of a totally random screen, however. There were clues that hydrophobic residues in the P3

TABLE VI

BINDING STUDY OF MYELOMA tPA (ONE CHAIN) WITH VARIOUS AFFINITY MATRICES WITH X–Y–Argal AS LIGANDS

Hydrophobic residues in P2 and P3.

Ligand, X–Y–Argal	*Enzyme activity (%)*		
	Unbound	*Wash*	*Recovered*
D-Phe–D-Phe–Argal	1	0	72
L-Phe–D-Phe–Argal	1	0	58
L-Phe–L-Phe–Argal	0	0	74
D-Phe–L-Phe–Argal	0	0	53
D-Phe–L-Trp–Argal	1	0	64
D-Phe–L-Val–Argal	0	3	80
D-Phe–L-Ile–Argal	0	0	59
D-Phe–L-Tyr–Argal	0	0	63
L-Tyr–D-Phe–Argal	0	1	78
L-Trp–D-Phe–Argal	0	0	50

and P2 positions of a ligand could enhance binding. Table V summarizes the ligand structures which were screened and found deficient. Table VI list the structures with hydrophobic moieties and branching on the beta carbon which were found to be most effective in binding tPA. The D- or L-enantiomers seemed by and large equally effective. In view of this and to take advantage of resistance to proteolytic cleavage by D-amino acid moieties, we focused attention on D-Phe–D-Phe–ArgSc sequence.

Chain content of tPA preparations

Two-chain tPA appears to arise from one-chain molecules by the action of proteases contaminating the culture media. Since the two forms seem to act similarly *in vivo*, it was of interest to harvest both equally in any purification scheme. With the gel containing D-Phe–D-Phe–Argal the problem of capturing both forms was solved. Although slight differences in mobility could be noted during sequential elution, the bulk of the tPA was recovered in the acetic acid eluent.

Small-scale affinity purification

At various times in the search for a better affinity ligand for tPA, samples of the enzyme expressed in conditioned media were tested directly, *i.e.* without prior fractionation or concentration. For reasons that are not entirely clear, the results were irregular and rather unpromising. In conjunction with an ongoing in-house project for large-scale purification of the enzyme, we examined partially purified samples of tPA. These turned out to be predictable and reproducible. Hence, most of the chromatographic tests in the preceding sections were done with partially purified samples of tPA.

Recombinant human tPA was expressed in CHO or myeloma cel lines. The enzyme was harvested from conditioned media by capture onto a zinc chelate gel[17]. The tPA recovered from this column was then used for further affinity purification studies.

tPA can bind to arginine-containing ligands over a wide pH range (usefully about 3 through 9). But owing to its marginal solubility about pH 7, we opted to work

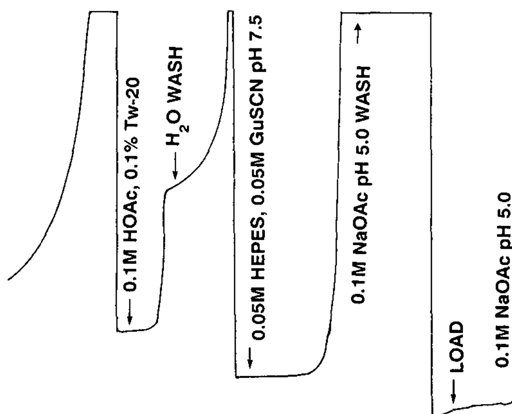


Fig. 2. Small-scale affinity purification of tPA. HOAc = Acetic acid; NaOAc = sodium acetate; TW-20 = Tween-20.

TABLE VII

SMALL-SCALE AFFINITY PURIFICATION OF tPA

Step	*Enzyme activity (%)*		
	Volume (ml)	*S-2288*	*S-2251*
Load	50	100	100
Flow through	45	6.7	–
Buffer wash	40	1.0	–
Chaotrope wash	20	23.0	–
Water wash	40	–	–
Recovery (%)	30	44.41	87

with the enzyme in acidic pH. Partially purified tPA could be conveniently handled in 0.1 *M* sodium acetate buffer pH 4.5 containing 0.6 *M* sodium chloride for loading onto a gel of D-Phe–D-Phe–Argal. At pH 3 at least 0.3 *M* sodium chloride was required to allow enzyme binding to the affinity sorbent. Here the tPA could then be recovered by using pH 3 buffer *without* salt. We surmise that at these low pH conditions, moderate concentrations of electrolytes are necessary to overcome electrostatic repulsions between protein and ligand in order to allow binding.

A chromatographic tracing of one small-scale preparation is shown in Fig. 2.

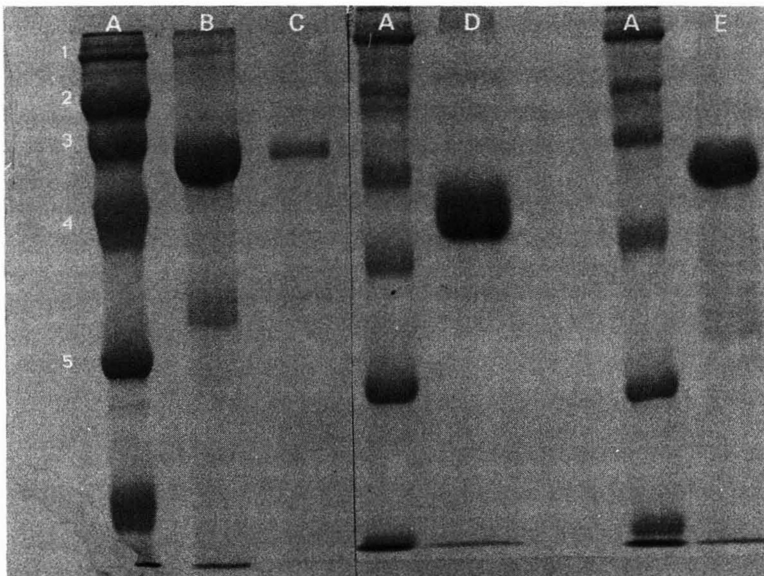


Fig. 3. SDS-PAGE analysis of affinity-purified tPA. Lane A: molecular weight markers; 1 = 200 000 dalton; 2 = 92 500 dalton; 3 = 69 000 dalton; 4 = 46 000 dalton; 5 = 30 000 dalton. Lane B: 5 μl column load (reduced). Lane C: 3.25 μl (2.5 μg) eluted band (reduced). Lane D: 20 μl eluted band (high load) (non-reduced). Lane E: 20 μg eluted band (high load) (reduced).

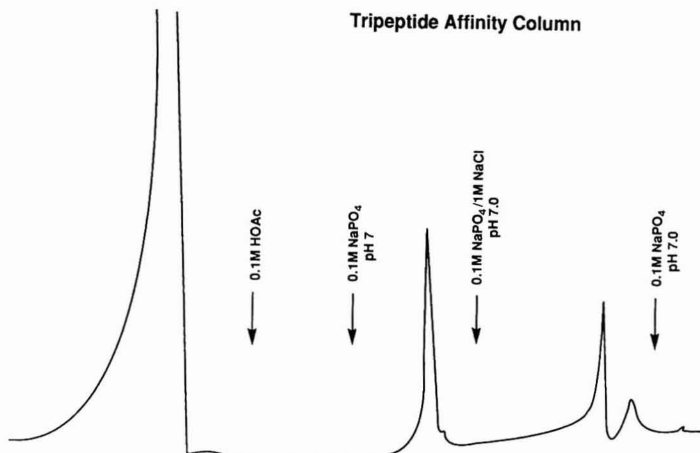


Fig. 4. Large-scale affinity purification of tPA.

The sample of partially purified tPA[17] equivalent to 11.25 · 10^6 I.U. (S-2251) was loaded on a 5 × 1 cm affinity column at pH 5. After various washes of the column, the enzyme was recovered with 0.1 *M* acetic acid–0.1% Tween-20 solution. Fractions were assayed using S-2288, a general substrate for trypsin-like proteases and S-2251, a specific tPA-Plasmin linked assay. Results summarized in Table VII indicate an 87% recovery based on assay with S-2251 substrate assay. The lower apparent recovery seen with S-2288 substrate may be due to proteases other than tPA in the starting sample.

The significant clean-up achieved in this affinity step is seen in the SDS-PAGE gel shown in Fig. 3. Akey accomplishment was the removal of serum albumin, which was a frequent contaminant carried over from the culture media.

Large-scale affinity purification

There was interest to develop a large-scale affinity process, which could be used to prepare tPA for clinical studies. Conditions for convenient handling and loading of partially purified tPA were chosen from the data accumulated in the bench-scale studies (noted above). The results from one large-scale purification study are shown in Fig. 4. The tPA had been captured from conditioned media on a zinc chelate column. After recovery from the column, the enzyme was concentrated by isoelectric precipitation. This protein (3.4 g) was dissolved in 0.1 *M* acetic acid–0.3 *M* sodium chloride pH 4.5 buffer and applied to a 420 ml (20 × 5 cm) pre-equilibrated with 0.1*M* sodium acetate pH 4.5 buffer. The pH 7 buffer containing 1 *M* sodium chloride served to wash out serum albumin contaminant. This was followed by a dilute pH 7 buffer to wash out the salt, which would interfere with the recovery of tPA by 0.1 *M* acetic acid. Yields were typically 95% for this step, and protein purity routinely exceeded 95%.

CONCLUSION

By adopting a solid-phase synthesis approach to prepare oligopeptide affinity resins, were were able to conveniently prepare a wide variety of sorbents that could be

effective in purifying tPA. Thus we were able to readily explore sequences known from the literature, as well to screen for novel ones. This enabled the discovery of Phe–Phe–Argal peptide wich have good avidity for tPA. We found that tripeptide ligands containing Argal can be readily used in large-scale affinity purification of tPA.

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CHROM. 22 332

High-performance affinity chromatography of concanavalin A

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ABSTRACT

D-Glucose was immobilized as a form of maltamyl group on Asahipak GS-520, a synthetic polymer-type gel, and employed as an affinity adsorbent for high-performance affinity chromatography. The method proved useful for the rapid fractionation of tetravalent and divalent molecular species contained in the usual preparations of concanavalin A. It also afforded an adequate means for the efficient purification of a monovalent derivative of concanavalin A produced by a photochemical reaction.

INTRODUCTION

Even in a conventional form, affinity chromatography shows high efficiency for the rapid purification of substances that can interact with a certain biospecific affinity adsorbent from non-interacting impurities. However, when the purpose is to fractionate a series of substances with similar but slightly different affinities toward the same adsorbent, the use of high-performance affinity chromatography (HPAC) has many merits. This paper deals with the application of HPAC to the fractionation of tetravalent and divalent molecular species of concanavalin A (Con A), a lectin with specific binding ability toward the α-D-manno- or α-D-glucopyranoside residues of polysaccharides. The utility of HPAC is further illustrated in the isolation of a monovalent Con A derivative from a complex mixture produced from native Con A by photochemical reaction.

EXPERIMENTAL

Affinity adsorbent for HPAC

The matrix employed for the preparation of the adsorbent was Asahipak GS-520 (particle size 9 $\pm$ 0.5 μm), a hard gel of a synthetic polymer type developed for high-performance gel permeation chromatography by Asahi Chemical (Tokyo, Japan). D-Glucose was immobilized on the matrix mainly by the method of Matsumoto *et al.*[1], as follows. Asahipak GS-520 (2 g of dry powder) was added to a mixture of epichlorohydrin (1.1 ml), 2 *M* sodium hydroxide solution (4.7 ml) and

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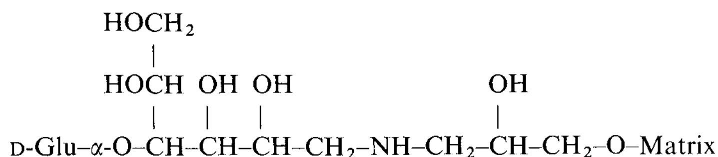
dimethyl sulphoxide (10.9 ml) and shaken for 15 h at 30°C. The activated gel was washed with water. Amino groups were introduced to the gel by shaking in 28% ammonia solution (10 ml) for 1.5 h at 40°C. Amino-Asahipak was then treated with maltose (189 mg) and $NaBH_3CN$ (92.4 mg) in 0.2 M K_2HPO_4 (5.44 ml) for 33 days at room temperature. Maltose was immobilized on the gel as a form of maltamyl group with the following structure:



Remaining amino groups on the gel were blocked by acetylation with acetic anhydride. Maltamyl-Asahipak GS-520 thus prepared was found to contain 35–40 μmol of immobilized glucose per gram of dry gel, when assayed by the Park and Johnson method[2] after hydrolysis in 0.5 M sulphuric acid at 100°C for 4 h. It was later found that maltamyl-Asahipak with almost the same glucose content could be obtained in about 20 h if amino-Asahipak was allowed to react at 80°C with maltose and $NaBH_3CN$.

Maltamyl-Asahipak was suspended in 0.01 M Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl, 2 mM $MnCl_2$ and 2 mM $CaCl_2$ and packed in a stainless-steel column, which was then connected to a Tosoh HLC-803D high-performance liquid chromatograph equipped with a GE-4 gradient unit and a UV-8 Model II spectrophotometer.

Concanavalin A

Con A was extracted from finely ground powder of jack bean seeds (Sigma, St. Louis, MO, U.S.A.) and purified according to the method of Agrawal and Goldstein[3]. This preparation is called whole Con A. Tetravalent and divalent molecular species (α-Con A and β-Con A, respectively) were separated from whole Con A by affinity chromatography on Sephadex G-100 as described previously[4] or on maltamyl-Asahipak as described below.

Photochemical reaction to produce monovalent Con A

The reaction was carried out as reported previously[5]. Briefly, α-Con A (100 mg), chloroacetamide (468 mg) and methyl-α-D-mannopyranoside (180 mg) were dissolved in 100 ml of 0.01 M Tris–HCl buffer (pH 7.4) containing 1.0 M NaCl, 10 mM $MnCl_2$ and 10 mM $CaCl_2$. The solution was irradiated with a 200-W high-pressure mercury lamp through a filter of 1.0% $CuSO_4$ (1.2 cm pass) for a defined period under continuous bubbling with argon.

Amino acid composition analysis

Proteins were hydrolysed with 4 M methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole at 115°C for 24 h in evacuated tubes and subjected to composition analysis on a Hitachi 835 amino acid analyser.

RESULTS AND DISCUSSION

Con A, a typical lection, has been widely used as a probe for cell surface analyses and as a modulator of cell functions. Most of the cell biological studies were carried out with commercial Con A specimens. All the specimens, including that prepared by the method of Agrawal and Goldstein[3], are mixtures of at least two molecular species with different sugar-binding valencies[6,7] and the mixing ratio of the two is variable from one preparation to the other. The first Con A species is a tetravalent tetramer composed of homologous "intact subunit", with a molecular weight of 102 000 at physiological pH[8]; we call it α-Con A. The second species, β-Con A, consists of two pairs of "split subunits". These subunits look as if they have been formed by a single cleavage at a central portion of the polypeptide chain of the intact subunit. The molecular weight and the sugar-binding valency of β-Con A are half those of α-Con A. Many studies have suggested that the effect of lectins on various cell functions alters depending on the binding valency of lectin molecules toward cell surface receptors[9–13]. Therefore, the use of homogeneous Con A preparations with the defined sugar-binding valency may be strongly recommended for future studies in cell biology and other fields.

Separation of α-Con A and β-Con A

Fractionation of α- and β-Con A in a whole Con A preparation has usually been carried out by selective elution with a concentration gradient of D-glucose from a column of Sephadex G-100, which has D-glucose residues at the non-reducing termini, as a specific bioaffinity adsorbent[4]. However, this affinity chromatography is very time consuming because Sephadex is a soft gel and does not tolerate high pressure to allow rapid elution.

HPAC on maltamyl-Asahipak was therefore tried in order to achieve an effective fractionation of the Con A species. A whole Con A preparation was applied to a column (7.5 × 0.75 cm I.D.) of this adsorbent and eluted at a flow-rate of 1 ml/min by a linear gradient of D-glucose concentration from 0 to 0.1 *M* under the conditions described in the legend of Fig. 1. As shown in Fig. 1, the divalent and tetravalent Con A were well separated from each other within only 14 min. The efficiency of the column is in marked contrast to that of a conventional Sephadex column, which requires more than 24 h to separate the two species with the same resolution as in Fig. 1. The sample size used in this experiment was only 50 μg, but even 10 mg of an α- and β-Con A mixture were found to be similarly well separated with the same maltamyl-Asahipak column.

It was thought at first that high-performance gel permeation chromatography should be utilizable for the separation of α-Con A from β-Con A, because there is a large molecular-weight difference between them. However, the two Con A species were eluted closely together, either from an Asahipak GS-510 column (Asahi Chemical) (50 × 0.76 cm I.D.) or from a TSKgel G-3000SW column (Tosoh) (60 × 0.75 cm I.D.).

The ability of these columns to act as high-performance molecular sieves seems to have been adversely affected by some interaction of the column matrices with Con A. Thus HPAC by maltamyl-Asahipak appears to be the best means of separating Con A species at least for analytical purposes.

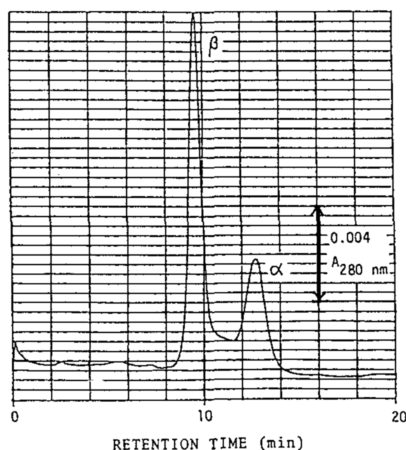


Fig. 1. Separation of α-Con A and β-Con A by affinity chromatography on maltamyl-Asahipak. The sample dissolved in 1 ml of 0.01 *M* Tris–HCl buffer (pH 7.4) containing 0.1 *M* NaCl, 2 m*M* $MnCl_2$ and 2 m*M* $CaCl_2$ was loaded on a column (7.5 × 0.75 cm I.D.) of the adsorbent equilibrated with the same buffer and eluted at a flow-rate of 1 ml/min by increasing the concentration of D-glucose in the same buffer linearly from 0 to 0.1 *M* in 15 min. The column operation was carried out at room temperature.

HPAC separation of monovalent Con A

The utility of the maltamyl-Asahipak column is further demonstrated by the following example of a more complicated system. We have previously reported that α-Con A can be converted into a monomeric monovalent form (m-Con A) by a light-induced alkylation reaction with chloroacetamide[5]. The reaction affords a complex mixture involving unfavourable products. In the previous work, m-Con A present in the reaction mixture was isolated by affinity chromatography on a column of Sephadex G-100 from various side-reaction products by a glucose-concentration gradient elution for 3 days (see Fig. 1 in ref. 5). The m-Con A thus purified has been widely used in cell biological studies as a valuable tool which can bind to a cell surface receptor but does not make a cross-link between them[9–13].

HPAC on maltamyl-Asahipak was successfully applied to the rapid isolation of m-Con A. Fig. 2 indicates the results of this experiment, in which two columns of the adsorbent connected in tandem were used. Elution was carried out by raising the glucose concentration from 0 to 0.1 *M* within 30 min at a flow-rate of 0.5 ml/min. Fig. 2A shows the chromatogram for the reaction product obtained by mercury-lamp irradiation of α-Con A in the presence of chloroacetamide for 20 min, and Fig. 2B shows that for the product obtained by 80-min irradiation. In both instances the reaction mixtures were separated into five peaks within 40 min. The material in peak I was identical with the desired Con A derivative, which was confirmed to have monovalent binding features and monomeric molecular weight. The molecular weight was determined by the combined use of high-performance gel permeation chromatography (on a column of Asahipak GS-510) and the low-angle laser light-scattering technique according to Takagi[14]. Peaks 0, II, III and IV seem to consist of inactivated, divalent, trivalent and tetravalent materials, respectively. The presence of the trivalent material was not observed in our previous work with chromatography on a Sephadex G-100 column[5], indicating the superior resolution of the maltamyl-Asahipak column.

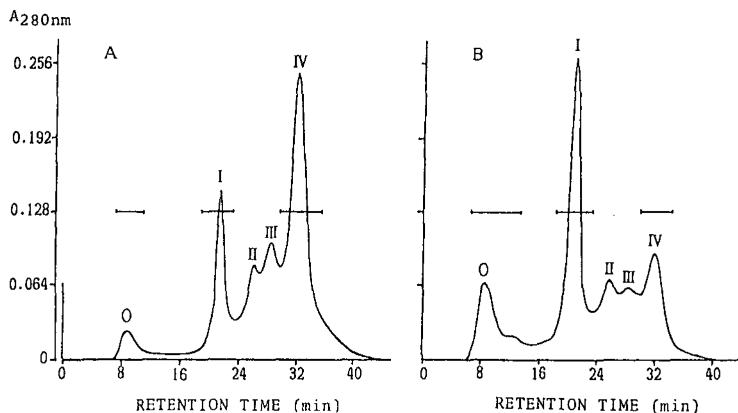


Fig. 2. Separation of photoalkylation products from α-Con A by affinity chromatography on maltamyl-Asahipak. α-Con A (0.8 mg in 1 ml) after irradiation with chloroacetamide under the conditions described under Experimental for (A) 20 min or (B) 80 min was loaded on the adsorbent, which was packed in two columns (each 7.5 × 0.75 cm I.D.) connected tandem. Elution was carried out by raising the glucose concentration linearly from 0 to 0.1 *M* in 30 min at a flow-rate of 0.5 ml/min. Other chromatographic conditions as in Fig. 1. Peaks 0, I, II, III and IV consist of inactivated, monovalent, divalent, trivalent and tetravalent materials, respectively, derived from α-Con A.

The HPAC method was also useful for a quick survey of the reaction conditions most suitable for the formation of m-Con A and for the characterization of this derivative. The yields of m-Con A and other products were calculated from the chromatogram and plotted against the reaction times (Fig. 3). The results suggest that a 60-min reaction is sufficient for the formation of m-Con A. The amino acid compositions of m-Con A and a tetravalent material isolated from the reaction mixtures obtained after various irradiation times were compared with that of native α-Con A. The differences in the compositions, which are regarded as the contents of modified amino acid residues in the products, are plotted in Fig. 4. Comparison of these contents clearly indicates that excessive modification of the polypeptide chain at

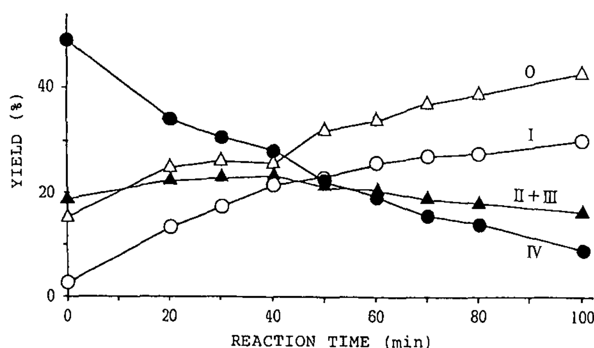


Fig. 3. Yields of tetravalent, di- + trivalent, monovalent and inactivated materials separated by affinity chromatography on maltamyl-Asahipak, as shown in Fig. 2, from α-Con A after photoreaction with chloroacetamide for various times.

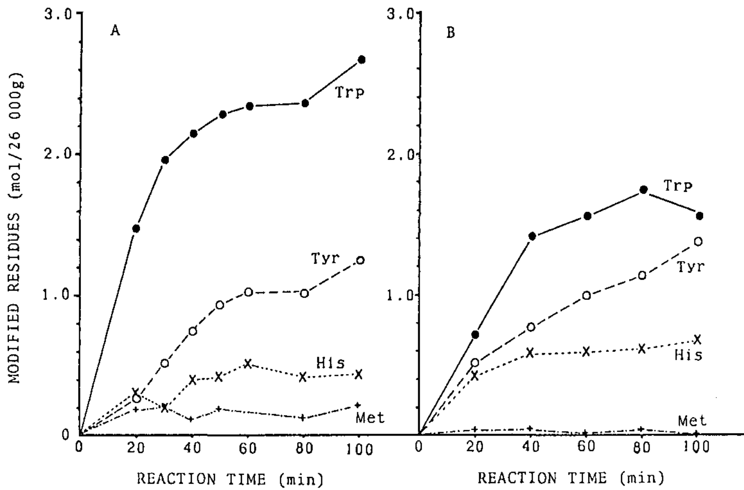


Fig. 4. Amounts of modified amino acids determined in (A) a monovalent material (= m-Con A) and (B) a tetravalent material isolated as shown in Fig. 2 from α-Con A after photoreaction for various times.

one tryptophan residue may be responsible for the conversion of α-Con A into the monomeric form. This result is consistent with our previous proposal[12] on the importance of a tryptophan residue for monomer–monomer interactions in Con A.

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Tresyl-activated support for high-performance affinity chromatography

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ABSTRACT

A new activated support TSKgel Tresyl-5PW was evaluated for the coupling of antibodies, which was found to occur easily under mild conditions with high yields. Optimum coupling conditions were a 2-h reaction at 25°C in 1 *M* phosphate buffer (pH 7.5) when 2–3 mg antibody/ml support is to be coupled and a 6–7-h reaction when *ca.* 10 mg antibody/ml support is to be coupled. When antibodies were coupled under these conditions, antibody coupling yields >80% and antigen binding efficiencies of 70–80% were achieved, probably owing to a selective attachment of the F_c region of the antibodies. Antigens (human serum proteins) could be separated rapidly without denaturation on antibody-coupled Tresyl-5PW.

INTRODUCTION

The cyanogen bromide activation method reported by Axen *et al.*[1] in 1967 was one of the most important developments in affinity chromatography. Since then, affinity chromatography has progressed considerably and has become widely accepted for the purification of biological substances such as proteins. However, the cyanogen bromide activation method has some shortcomings, *e.g.*, cyanogen bromide is extremely toxic, the activation reaction is complicated and the linkage between the support matrix and the ligand is not very stable. Therefore, other activation methods have also been investigated. The tresyl (2,2,2-trifluoroethanesulphonyl) chloride activation method developed by Nilsson and Mosbach[2] in 1981 has attracted attention recently because the activation reaction is simple and many ligands can be coupled easily under mild conditions via amino, thiol, phenol or imidazole groups.

Some tresyl-activated supports based on agarose or silica became commercially available a few years ago, but there are problems with the mechanical and chemical stability of the base material. However, new tresyl-activated supports based on synthetic hydrophilic resins have become available recently as TSKgel Tresyl-Toyopearl

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650M and Tresyl-5PW (TOSOH, Tokyo, Japan). They are mechanically and chemically stable and therefore seem useful as supports for high-performance affinity chromatography.

We have been evaluating these tresyl-activated supports and have already reported results on the study of the coupling conditions for some proteins[3,4]. As reported there, the optimum coupling conditions were dependent on the proteins. Accordingly, we have investigated further the coupling conditions for antibodies, because they constitute one of the most important ligands in affinity chromatography. We have also examined rapid separations of antigens (human serum proteins) by immunoaffinity chromatography on supports coupled with anti-human serum protein antibodies. The results are reported in this paper.

EXPERIMENTAL

Antibodies were coupled to Tresyl-5PW, which was prepared by introducing tresyl groups at a level of *ca.* 20 μmol/ml support into TSKgel G5000PW of particle diameter 10 μm and pore diameter *ca.* 1000 Å. Potassium phosphate solution (1 *M*) was employed as a coupling buffer throughout all the experiments because it was very effective in the coupling of other proteins[4]. The effects of pH of the coupling buffer, reaction time, temperature and amount of ligand antibody on the antibody coupling yield, antigen binding capacity and antigen binding efficiency were investigated.

The antibody coupling reaction and subsequent evaluation of antibody-coupled supports were performed as follows. A certain amount of antibody was dissolved in 2.2 ml of coupling buffer and 0.25 g dried Tresyl-5PW powder, which gives a volume of 1.0 ml in the swollen state, was added to 2 ml of the antibody solution. The remaining 0.2 ml of the antibody solution was used to measure the UV absorption of the solution at 280 nm, which was necessary for determining the antibody coupling yield. After the mixture had been allowed to stand with gentle shaking at a constant temperature for a certain period of time, 18 ml of distilled water was added and the diluted mixture was filtered through a sintered-glass funnel. This dilution was necessary to prevent adsorption of antibody on the surface of the support without covalent bonding. The UV absorption of the filtrate was measured at 280 nm. After washing the gel in the funnel three times with 10 ml of 0.1 *M* Tris–HCl buffer (pH 8.5), the remaining tresyl groups were blocked by suspending the support in 5 ml of 0.1 *M* Tris–HCl buffer (pH 8.5) for 1 h at 25°C. Then the support was packed into a stainless-steel column (10 mm × 6 mm I.D.).

The column was installed in a high-performance liquid chromatography (HPLC) system and equilibrated with 0.1 *M* phosphate buffer (pH 7.4) at a flow-rate of 0.5 ml/min at 25°C, then 5 mg of antigen dissolved in 0.5 ml of 0.1 *M* phosphate buffer (pH 7.4) was applied to the column. After unbound excess antigen had been completely washed from the column, bound antigen was eluted with 0.1 *M* citric acid of pH 1.6, adjusted with hydrochloric acid. A 5-ml volume of column effluent containing bound antigen was collected and the UV absorption of the fraction was measured at 280 nm. The antibody coupling yield was calculated from the UV absorption of the antibody solutions before and after the coupling reaction. The amount of coupled antibody was calculated from the coupling yield and concentration of antibody solution before the coupling reaction. The antigen binding capacity

was calcualted from the UV absorption of the antigen fraction by assuming $A_{280}^{1\%}$ = 5.8, 11.4, 14.7 and 13.3 for albumin, transferrin, immunoglobulin (Ig) G and IgM, respectively. The antigen binding efficiency, which is defined as the percentage of coupled antibody which is active and can bind antigen, was calculated from the amount of coupled antibody and antigen binding capacity considering the fact that one antibody molecule can bind two antigen molecules. We employed values of 67 000 and 150 000 as molecular weights of albumin and antibody, respectively.

Separation of antigens by immunoaffinity chromatography was carried out on a 10 mm × 6 mm I.D. or 20 mm × 10 mm I.D. column with a step gradient from 0.1 *M* phosphate buffer (pH 7.4) to 0.1 *M* citric acid–hydrochloric acid (pH 1.6) at a flow-rate of 1 or 2 ml/min at 25°C by using an HPLC system consisting of a Model CCPM double-plunger pump, a Model UV-8000 variable-wavelength UV detector operated at 280 nm, and a Model CP-8000 data processor (TOSOH). The fractions collected were tested for purity by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) in slabs of 4–20% polyacrylamide gradient gel (Tefco, Tokyo, Japan). The recovery of activity was also examined in the purification of plasminogen. Plasminogen activity was measured with a Testzyme PLG kit (Daiich Pure Chemicals, Tokyo, Japan).

All antibodies employed were IgG fractions of rabbit polyclonal antibodies purified by ion-exchange chromatography and gel filtration (Dakopatts, Glostrup, Denmark), except one which was affinity-purified goat polyclonal anti-human albumin antibody (Biosys, Compiègne, France). Human albumin, transferrin and plasma were purchased from Sigma (St. Louis, MO, U.S.A.), human IgG and serum from Miles Labs. (Elkhart, IN, U.S.A.), human IgM from Protogen (Laufelfingen, Switzerland) and human serum standards for single radial immunodiffusion analysis from Hoechst Japan (Tokyo, Japan).

RESULTS AND DISCUSSION

Fig. 1 shows the effect of the pH of the coupling buffer. The antibody coupling yield was almost 100% at pH ≥7.0, but it decreased to about 50% at pH 6.5. The

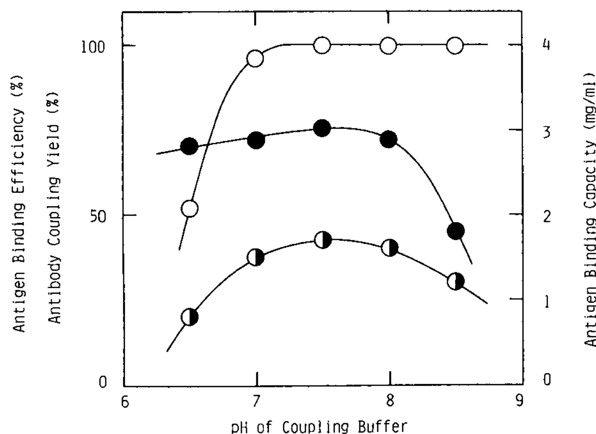

Fig. 1. Effect of pH of coupling buffer on (○) antibody coupling yield, (◑) antigen binding capacity and (●) antigen binding efficiency. Affinity-purified anti-human albumin antibody (2.5 mg) was reacted with 0.25 g of Tresyl-5PW in 2 ml of 1 *M* potassium phosphate buffer of pH 6.5–8.5 at 4°C for 16 h.

antigen binding efficiency was approximately constant at *ca.* 70% at pH $\leqslant$8.0, but it was about 45% at pH 8.5. The antigen binding capacity was maximum at pH *ca.* 7.5. Therefore, the optimum pH of coupling buffer can be said to be *ca.* 7.5. Because the reactivity of tresyl group with amino, thiol, phenol and imidazole groups is higher at higher pH, multi-point attachment of proteins to Tresyl-5PW must occur more easily at higher pH. Multi-point attachment sometimes causes changes in the structure of proteins and results in lower binding efficiencies[5–8]. Accordingly, the multi-point attachment is supposed to be responsible for the lower antigen binding efficiency at pH 8.5. In this study, the amount of antibody was fairly small, 2.5 mg/ml support. A similar study was also performed with a larger amount of antibody, *ca.* 10 mg/ml support, and the results are shown in Fig. 2.

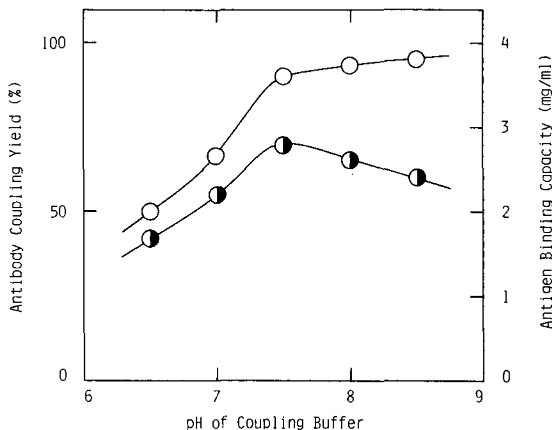

Fig. 2. Effect of pH of coupling buffer on (○) antibody coupling yield and (◑) antigen binding capacity. IgG fraction of anti-human albumin antibody (10.7 mg) was reacted with 0.25 g of Tresyl-5PW in 2 ml of 1 *M* potassium phosphate buffer of pH 6.5–8.5 at 25°C for 16 h.

The antibody coupling yield was more than 90% at pH $\geqslant$7.5, but it decreased considerably when the pH decreased below 7.0. The antigen binding capacity was maximum at pH 7.5. At higher pH, the antigen binding capacity decreased slightly although the antibody coupling yield was slightly higher. This slight decrease in antigen binding capacity at pH 8.0 and 8.5 may be due to a change in antibody structure due to multi-point attachment. Steric hindrance to antigen binding is also assumed to be responsible because the amount of coupled antibody is almost half the total antibody coupling capacity of Tresyl-5PW (15-20 mg/ml support). Accordingly, a pH of *ca.* 7.5 is also optimum in the coupling of large amounts of antibody.

Fig. 3 shows the effect of the amount of antibody reacted. The antibody coupling yield was 100% for amounts of antibody less than 2.5 mg and decreased to 94% with 5 mg. The antigen binding efficiency was very high (>80%) when the amount of antibody was small ($\leqslant$1 mg) and it gradually decreased with increasing amount of antibody. Steric hindrance to the binding of antigen to antibody is assumed to be responsible for the decrease in antigen binding efficiency with increase in the amount

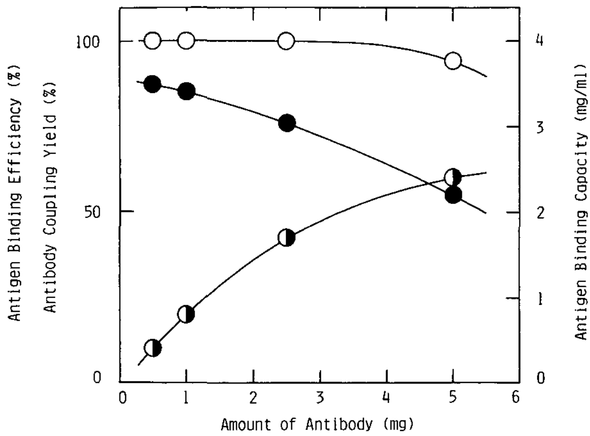

Fig. 3. Effect of amount of reacted antibody on (○) antibody coupling yield, (◑) antigen binding capacity and (●) antigen binding efficiency. Affinity-purified anti-human albumin antibody (0.5–5 mg) was reacted with 0.25 g of Tresyl-5PW in 2 ml of 1 *M* potassium phosphate buffer (pH 7.5) at 4°C for 16 h.

of antibody. On the other hand, the antigen binding capacity increased continuously with increase in the amount of antibody up to 5 mg. Consequently, when it is desirable to couple antibody as effectively as possible, the amount of antibody should be less than 2–3 mg/ml support, whereas more antibody should be reacted when a high antigen binding capacity is required.

Fig. 4. shows the effect of reaction time on the coupling of antibody of 2.5 mg/ml support at 25°C. The coupling reaction was fast. The antibody coupling yield was nearly 90% after 1 h and almost 100% after 2 h. The antigen binding capacity and efficiency also reached a maximum after 2 h but decreased afterwards. Therefore, reaction times of *ca.* 2 h are best in the coupling of small amounts of antibody at 25°C.

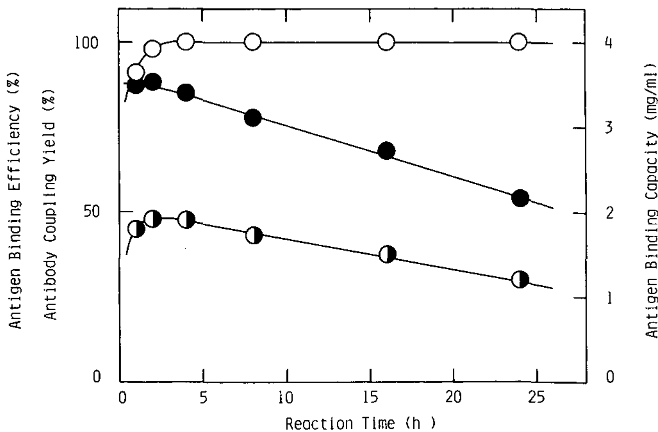

Fig. 4. Effect of reaction time on (○) antibody coupling yield, (◑) antigen binding capacity and (●) antigen binding efficiency. Affinity-purified anti-human albumin antibody (2.5 mg) was reacted with 0.25 g of Tresyl-5PW in 2 ml of 1 *M* potassium phosphate buffer (pH 7.5) at 25°C.

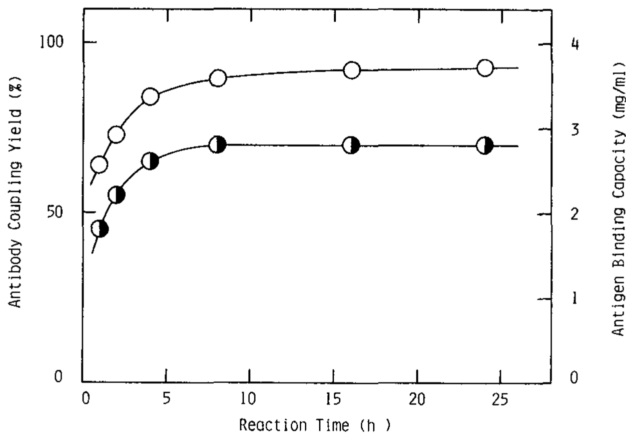

Fig. 5. Effect of reaction time on (○) antibody coupling yield and (◑) antigen binding capacity. IgG fraction of anti-human albumin antibody (10.7 mg) was reacted with 0.25 g of Tresyl-5PW in 2 ml of 1 *M* potassium phosphate buffer (pH 7.5) at 25°C.

In this case, longer reaction times are not preferable because multi-point attachment, resulting in a decrease in antigen binding capacity and efficiency, seems to occur. Fig. 5 shows the effect of reaction time observed with a larger amount of antibody of *ca.* 10 mg/ml support. The coupling reaction was not as fast as in Fig. 4. The antibody coupling yield was *ca.* 65% after 1 h and reached *ca.* 90% after 6–7 h, but hardly increased afterwards. A similar pattern was also observed with the antigen binding capacity, which reached a maximum after 6–7 and then remained almost constant. Accordingly, a reaction time of 6–7 h is sufficient to achieve a maximum antibody coupling yield and antigen binding capacity in the coupling of a large amount of antibody, *e.g.*, 10 mg/ml support. In this instance, however, longer reaction times do not seem to cause multi-point attachment and changes in the structure of the antibody. The effect of reaction time was also studied in the coupling of a small amount of antibody at 4°C (data not shown). The coupling reaction was slow at this low temperature even with a small amount of antibody. Reaction for more than 16 h was neces-

TABLE I

RESULTS OF COUPLING OF VARIOUS ANTIBODIES TO Tresyl-5PW

Anti-human serum protein antibodies (5–12 mg) were reacted with 0.25 g of Tresyl-5PW in 1 *M* potassium phosphate buffer (pH 7.5) at 25°C for 8 h. Antigen binding capacity was not determined for anti-human orosomucoid antibody and anti-human plasminogen antibody.

Antibody	*Amount of antibody reacted (mg)*	*Coupling yield (%)*	*Antigen binding capacity (mg/ml)*
Anti-human albumin antibody	9.0	89	2.8
Anti-human transferrin antibody	7.5	89	2.0
Anti-human IgG (γ-chains) antibody	5.5	96	2.5
Anti-human IgM (μ-chains) antibody	5.0	90	2.5
Anti-human orosomucoid antibody	12.0	83	–
Anti-human plasminogen antibody	5.5	90	–

sary to achieve an antibody coupling yield of more than 90%. However, multi-point attachment seemed to occur even at 4°C, although very slowly. Accordingly, there is no advantage in coupling at low temperature.

Table I shows the results of coupling of various antibodies under conditions which seemed optimum from the data given above. The coupling yield was more than 80% for all the antibodies examined. The antigen binding capacity was 2–3 mg/ml support. A similar binding capacity was obtained even for a large molecule such as IgM. This is probably due to the large pore size of Tresyl-5PW.

Rapid separations of human serum proteins by using antibody-coupled Tresyl-5PW were tried. The elution conditions were examined first by using Tresyl-5PW coupled with anti-human albumin antibody. The results are shown in Fig. 6. Almost no albumin was eluted at pH 3.0. Although albumin was eluted at pH 2.0, the recovery was still low (55%). However, albumin was recovered quantitatively at pH 1.6. We then employed 0.1 *M* citric acid (pH 1.6) for the elution of bound antigens from antibody-coupled Tresyl-5PW. However, because these conditions seemed severe, the stability of antibody-coupled Tresyl-5 PW was examined. The same separation as in Fig. 6C was repeated 100 times and the albumin binding capacities before and after the separations were compared.

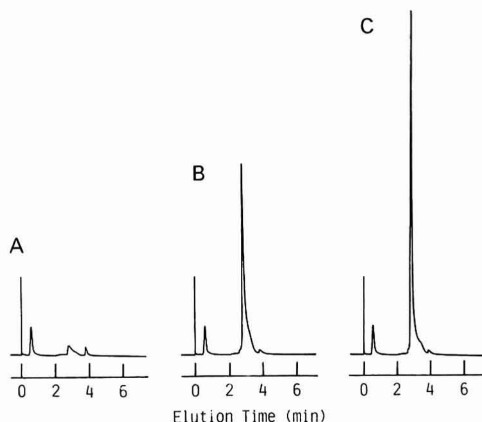

Fig. 6. Effect of eluent pH on the elution of antigen from antibody-coupled Tresyl-5PW. Human serum albumin was applied to a column (20 mm × 10 mm I.D.) of Tresyl-5PW coupled with anti-human albumin antibody in 0.1 *M* phosphate buffer (pH 7.4) at a flow-rate of 2 ml/min, and 2 min after the sample injection the eluent was changed stepwise to 0.1 *M* citric acid the pH of which was adjusted to (A) 3.0, (B) 2.0 or (C) 1.6 with NaOH or HCl.

The albumin binding capacity decreased by 10%, which does not seem much in comparison with reported values observed under other conditions[9,10].

Examples of the purification of human serum and plasma proteins are shown in Figs. 7–11. Very pure transferrin and albumin were obtained in less than 5 min, as indicated in Figs. 7 and 8. Only a single band corresponding to transferrin and albumin is seen in the SDS-PAGE pattern of the bound fraction. Several bands are seen in the SDS-PAGE patterns of bound fractions in the purification of IgG and IgM. Therefore, the IgG and IgM obtained are not very pure, although two main bands must be heavy and light chains of IgG and IgM. However, these results indicate that

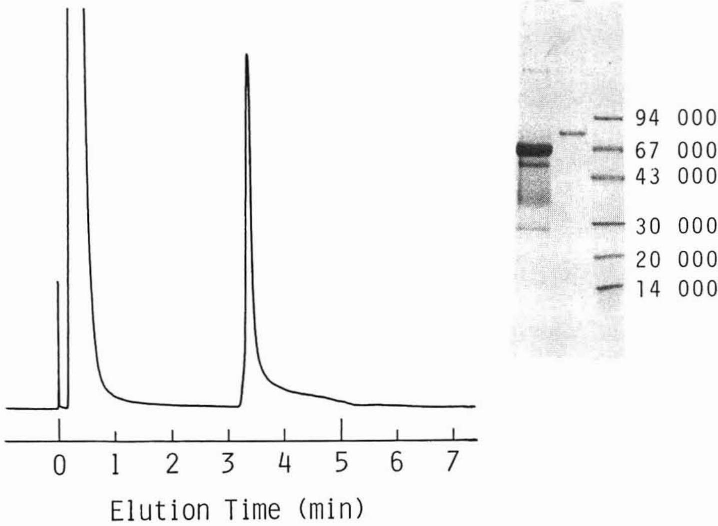

Fig. 7. Purification of transferrin by high-performance affinity chromatography. Human serum (20 μl) was applied to a column (10 mm × 6 mm I.D.) of Tresyl-5PW coupled with anti-human transferrin antibody at a flow-rate of 1 ml/min at 25°C. The pH of eluent was changed stepwise from 7.4 to 1.6 3 min after the sample injection. Bound and unbound peaks were collected and subjected to SDS-PAGE. The results of SDS-PAGE are included; left, center and right lanes represent patterns of unbound fraction, bound fraction and molecular weight markers, respectively.

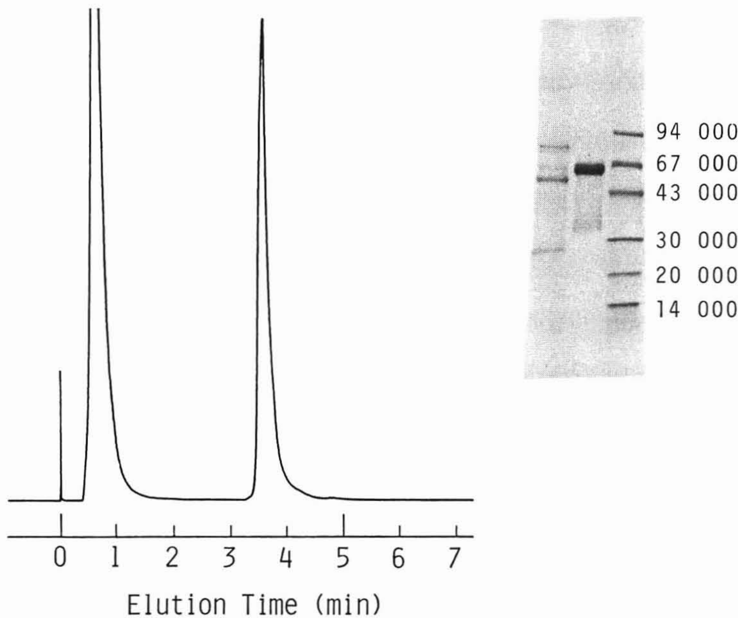

Fig. 8. Purification of albumin by high-performance affinity chromatography. Human serum (50 μl) was applied to a column (20 mm × 10 mm I.D.) of Tresyl-5PW coupled with anti-human albumin antibody at a flow-rate of 2 ml/min at 25°C. The elution of bound albumin, collection of peaks and purity test were performed as for transferrin in Fig. 7.

antibody-coupled Tresyl-5PW can be applied to large molecules. Purified plasminogen also contained small amounts of impurities. A few faint bands are seen in addition to the main band corresponding to plasminogen in the SDS-PAGE pattern of the bound fraction. The recovery of plasminogen activity was 90%. Although 0.1 *M* citric acid of pH 1.6 was used for the elution of bound antigen proteins here, the separation was very rapid and antigen proteins were exposed to the harsh conditions for only about 1 min. Therefore, the possibility of denaturation of antigen proteins during elution seems low. This is one of advantages of high-speed affinity chromatography.

Rapid analyses of human serum proteins were also examined. Fig. 12 shows a separation of IgG in human serum. The separation was repeated 300 times, the sample was injected at intervals of 4 min and the IgG content was calculated from the height of bound peak. The separation was very reproducible. IgG contents observed in the first and the 300th analyses were the same within experimental error (the relative standard deviation was 3%). A good linear correlation was observed between

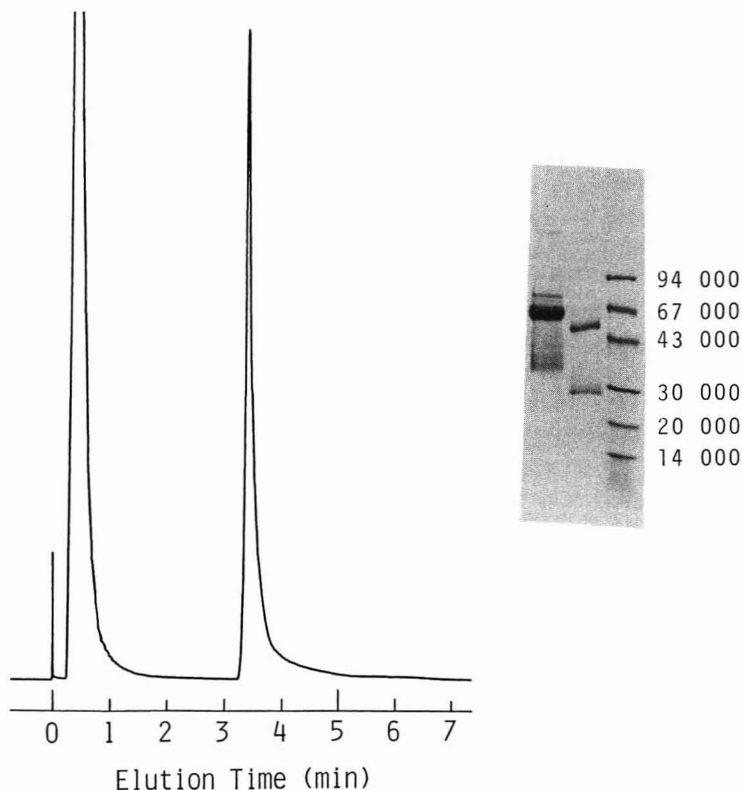

Fig. 9. Purification of IgG by high-performance affinity chromatography. Experimental procedure as for transferrin in Fig. 7, except that 20 μl of human serum were applied to a column of Tresyl-5PW coupled with anti-human IgG antibody.

the height of the bound peak and the amount of IgG injected. The linearity extended up to 160 μg of IgG. Commercial human serum standards with different IgG contents for use in single radial immunodiffusion analysis were analysed. The IgG contents determined by high-performance affinity chromatography are plotted against IgG contents determined by single radial immunodiffusion in Fig. 13. A good linear correlation was obtained between the IgG contents determined by the two methods. Consequently, high-performance affinity chromatography can be a good alternative to existing methods for analysing some serum proteins because it is rapid, reproducible, easy to automate, etc.

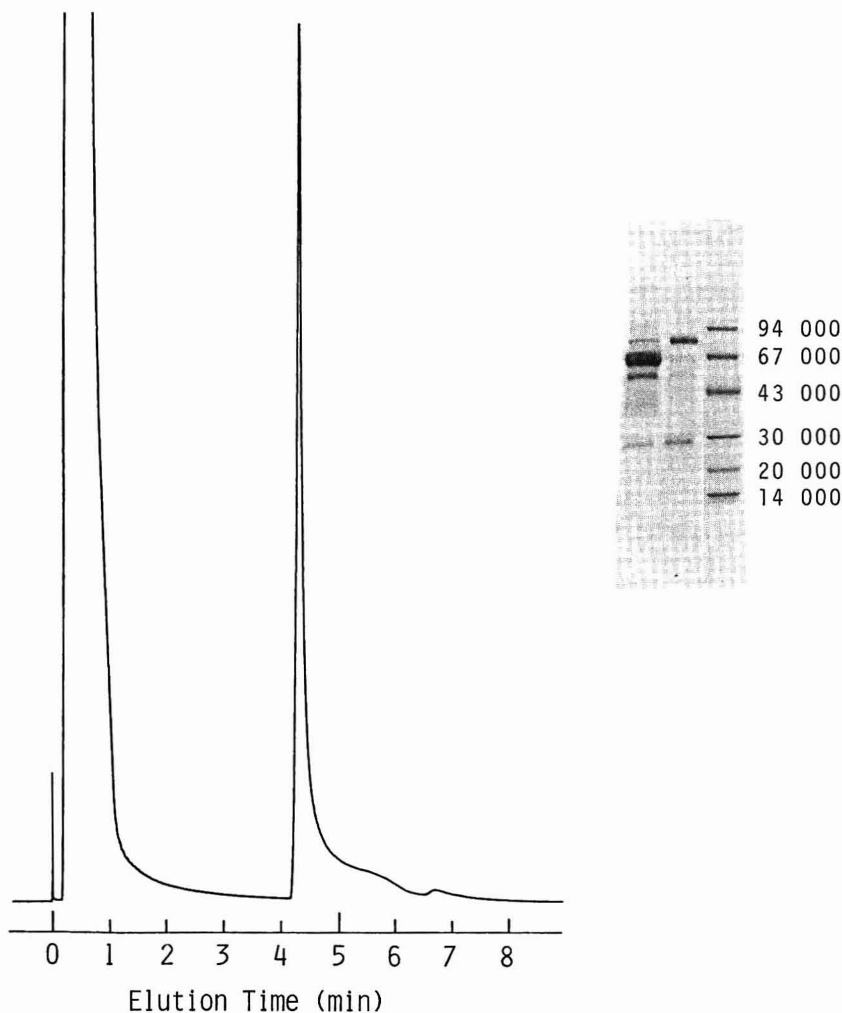

Fig. 10. Purification of IgM by high-performance affinity chromatography. Experimental procedure as for transferrin in Fig. 7, except that 100 μl of human serum were applied to a column of Tresyl-5PW coupled with anti-human IgM antibody and the pH of eluent was changed 4 min after the sample injection.

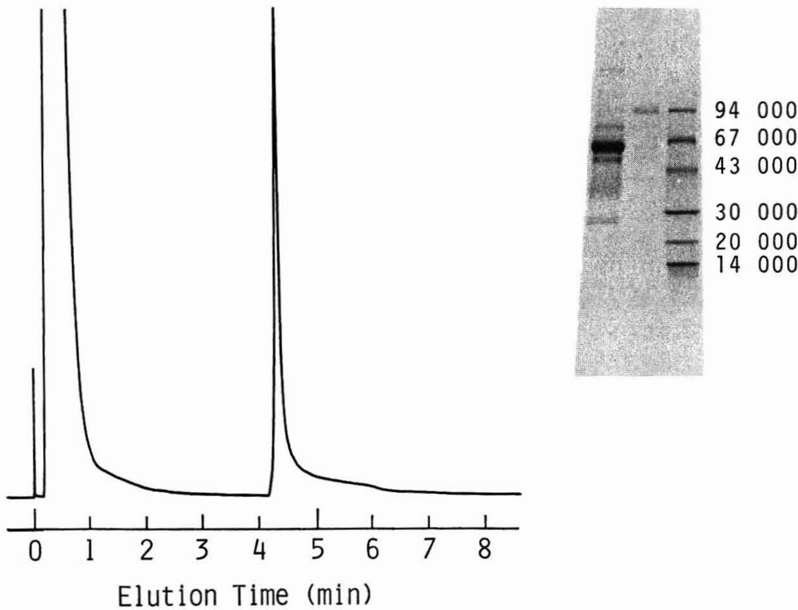

Fig. 11. Purification of plasminogen by high-performance affinity chromatography. Experimental procedure as for transferrin in Fig. 7, except that 50 μl of human plasma were applied to a column of Tresyl-5PW coupled with anti-human plasminogen antibody and the pH of the eluent was changed 4 min after the sample injection.

In conclusion, TSKgel Tresyl-5PW is a suitable activated support for high-performance immunoaffinity chromatography. Antibodies can be coupled easily in high yield under mild conditions. Antibody coupling yields of >80% and antigen-binding efficiencies of 70–80% are achieved when antibodies are coupled under suit-

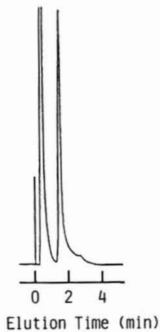

Fig. 12. Analysis of IgG in human serum by high-performance affinity chromatography. A 1-μl volume of human serum diluted 10-fold with 0.1 *M* phosphate buffer (pH 7.4) was applied to a column (10 mm × 6 mm I.D.) of Tresyl-5PW coupled with anti-human IgG antibody at a flow-rate of 1 ml/min at 25°C. Bound IgG was eluted by pulse injection of 2 ml of 0.1 *M* citric acid (pH 1.6) 1 min after the sample injection.

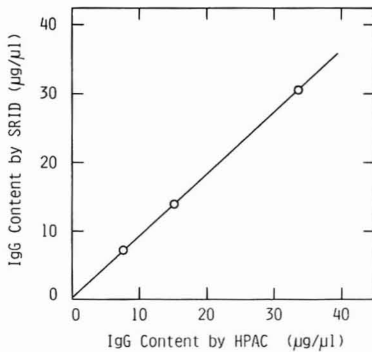

Fig. 13. Relationship between IgG contents in serum determined by single radial immunodiffusion (SRID) and by high-performance affinity chromatography (HPAC). Three human serum standards (1 μl) of different IgG contents were analysed in the same way as in Fig. 12.

able conditions. Directions for the proper selection of antibody coupling conditions are as follows. Phosphate buffer (1 *M*) is very effective as a coupling buffer. The optimum pH is *ca.* 7.5. A temperature of *ca.* 25°C is better than lower temperatures such as 4°C in order to save time without any disadvantages. The reaction time must be properly selected according to the amount of antibody. When a small amount of antibody, *e.g.*, 2.5 mg/ml support, is to be coupled, *ca.* 2 h is the optimum time, but when large amounts of antibody, *e.g.*, 10 mg/ml support, are to be coupled, 6–7 h are optimum. Antigen can be separated rapidly without denaturation on antibody-coupled Tresyl-5PW. Accordingly, antibody-coupled Tresyl-5PW is useful for both purification and analysis of antigen.

Phosphate buffer (1 *M*) was particularly effective for the coupling of antibody. Antibody molecules are assumed to be forced to come near the surface of the Tresyl-5PW matrix owing to the salting-out effect of 1 *M* phosphate buffer and the coupling reaction between the antibody and the tresyl group occurs more easily. This situation is the same as for other proteins. However, more conveniently in the case of antibody, the F_c region rather than the F_{ab} region is expected to approach the surface of the Tresyl-5PW matrix because the former region is more hydrophobic than the latter. Therefore, there is a possibility of selective attachment of the F_c region of antibody without special conditions when 1 *M* phosphate buffer is used as a coupling buffer. We guess that the high antigen binding efficiency achieved in 1 *M* phosphate buffer is due to the selective attachment of the F_c region of antibody. Other buffers having a similar salting-out effect to 1 *M* phosphate buffer may also be effective for the coupling of antibody.

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Novel media for chromatography and immobilization using a radiation grafting technique

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ABSTRACT

Radiation grafting onto polyamide and poly(vinyl alcohol) was performed using different vinyl monomers. This grafting technique permits the synthesis of carrier media with a wide range of physical and chemical properties. A number of immobilization tests with antibodies and enzymes *e.g.*, penicillin acylase, glucose isomerase and formate dehydrogenase, are described, exhibiting binding capacities which are distinctly higher than those achieved with commercial media. The epoxy- and isocyanate-activated grafted copolymers were used for the affinity chromatographic separation of insulin, factor VIII and human serum albumin using antibodies as affinity ligands. The radiation-modified media allow a high antibody coupling, thus overcoming drawbacks of currently available commercial media. The separation of blood group antibodies can be performed by using novel antibody-specific oligosaccharide ligands, which permit a much more specific separation than protein G coupled media.

INTRODUCTION

The interest in and demand for biospecific molecules (enzymes, antibodies and glycoproteins) in biotechnology, biochemistry and medicine have contributed to an increased exploitation of affinity chromatography. As affinity chromatography represents by far the most powerful separation method, great efforts have been made in the whole field of chromatography to extend beyond the laboratory scale and to adapt this technology to large-scale industrial production. Essential requirements for transfer to a technical level are physical and chemical stability, hydrophilicity, appropriate ligand-binding capacity and suitable activation and coupling procedures. If one takes into account the technical usage, price and reusability must then also be considered.

Despite tremendous efforts, none of the currently available commercial media fulfil all of these criteria. Ineffective and laborious coupling techniques, low binding capacities and high costs are the main drawbacks among current supports[1]. In this respect large-scale affinity chromatography must be regarded as being in a preliminary stage.

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In this present study, radiation-grafted polyamide-6 (Biograft) and poly(vinyl alcohol) (PVA) as media for affinity chromatography (AC), enzyme immobilization and haemoperfusion are described. As a result of grafting, a molecular structure is formed which enables high ligand–protein binding densities well above those achieved using established commercial matrices. Simple coupling techniques via epoxy- (Biograft) and isocyanateactivated carriers (PVA) contribute to a high binding and separation performance.

EXPERIMENTAL

Materials

The materials were purched as follows: Affi-Gel Hz, Bio-Rad Labs. (Munich, F.R.G.); Sepharose media, Pharmacia–LKB (Freiburg, F.R.G.); Fractogel media, Merck (Darmstadt, F.R.G.); Eupergit C, Röhm Pharma (Darmstadt, F.R.G.); VA-Epoxy, Riedel-de Haën (Seelze, F.R.G.); Synsorb A, blood group A oligosaccharide, Chembiomed (Edmonton, Canada); polygalacturonase, formate dehydrogenase, penicillin acylase, Braunschweiger Biotechnologie (Braunschweig, F.R.G.); horseradish peroxidase, Sigma (Deisenhofen, F.R.G.); glucose isomerase, CPC/ Europe (Vilvoorde, Belgium); anti-human serum albumin (anti-HSA), anti-human growth hormone (anti-HGH), anti-insulin, Sorin (Saluggia, Italy); and insulin, HSA, Behring Werke (Berlin, F.R.G.). All other chemicals were obtained from Fluka (Neu-Ulm, F.R.G.).

Preparation of affinity and immobilization carriers

Radiation grafting of polyamide-6 and PVA with 2-hydroxyethyl methacrylate (HEMA), N-vinylpyrrolidone (NVP), acrylamide (AA) and 2-dimethylaminoethyl methacrylate (DAEM) was conducted using a simultaneous grafting technique as described elsewhere[2–4]. Such a grafting procedure is performed as follows: 5 ml of HEMA, 0.5 ml of NVP, 3 ml of AA (20% aqueous solution), 1 ml of DAEM, 1 ml of formamide, 4 ml of tetrahydrofuran, 7 ml of methanol and 2 ml of water were added to 5 g of polyamide powder with a bead size of *ca.* 150 μm. The mixture was exposed to a cobalt γ-radiation source for 1 h, receiving a total dose of 0.18 Mrad. After washing with methanol, the graft uptake (weight per cent increase of the starting polyamide powder) was 62%. A 1-g amount of the grafted and dried resin was activated with 3 ml of epichlorohydrin with addition of 10 ml of 0.5 *M* NaOH for 2 h at 55°C. After intensive washing with water, the oxirane content of the dried carrier was determined according to Sundberg and Porath[5], amounting to 800–1000 μmol/g resin. For a detailed description of the protein coupling procedures to the epoxy-activated carriers, see the legends of the figures and tables. Isocyanate activation per gram of PVA was carried out with addition of 10 ml of dimethyl sulphoxide containing 2 ml of 1,6-hexamethylene diisocyanate at 40°C for 60 min, followed by thorough washing with acetone.

Activation of Fractogel TSK HW-75F with 2-fluoro-1-methylpyridinium toluene sulphonate (FMP) and coupling of ligands to this carrier were conducted following the method described by Ngo[6]. Antibodies were coupled to cyanogen bromide (CNBr)–Sepharose ad Affi-Gel Hz according to the manufacturer's instructions[7,8]. For the coupling of the blood-group hapten to isocyanate-activated PVA,

2 mg of hapten were dissolved in 5 ml of dimethyl sulphoxide and incubated with 1 g of carrier for 5 h at ambient temperature.

Procedures for affinity chromatography

All affinity chromatographic tests were carried out as a batch process. The affinity media coupled with the ligand were placed in a 2-ml plastic syringe equipped with a 0.2-μm pore size sterile filter unit. After the protein solution to be separated had been incubated for 12 min, the solution was pressed through the filter and the remaining protein content in the filtrate was analysed according to Lowry *et al.*[9]. For further details, see the figure and table legends.

Analytical methods

The amount of protein immobilized was calculated from the difference between the initial amount of protein applied, determined by Lowry *et al.*'s method[9], and the amount in the supernatant after coupling. The blood-group A antibody was determined using an agglutination test, each with 20 μl of undiluted serum and freshly prepared erythrocyte suspension. Using increasing serum dilutions (1:1, 1:2, 1:5 and 1:10), the agglutination times were measured and served as calibration values for the quantitative detection of antibodies. The enzymatic tests were carried out following the Merck[10] and Boehringer Biochemical[11] standard test methods.

RESULTS AND DISCUSSION

Some carrier manufacturers offer affinity ligands that include an attached affinity ligand, *e.g.*, Cibacon Blue-, heparin-, protein A-, concanavalin A- and benzamidine-coupled media. Despite the frequent use of these affinity media, they only express group specificity and do not show specificity towards a single compound. Owing to the lack of specific ligands, an increased usage of antibodies and in particular monoclonal antibodies as affinity ligands has been observed in the last few years. This trend has been enhanced by hybridoma technology.

In this study, a number of antibody immobilization tests on different carriers were conducted in order to assess the performance of the grafted media in comparison with established matrices.

Fig. 1 shows a plain anti-HGH coupling on four epoxy-activated carriers. The

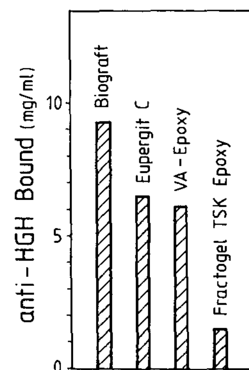

Fig. 1. Immobilization of anti-HGH on different epoxy-activated carriers. Coupling conditions: 3 mg of protein per 100 mg of carrier in 1 *M* potassium phosphate (pH 8.0) for 24 h at room temperature.

coupling of the grafted sample (Biograft) lies well above that of the commercial media. With the introduction of high-molecular-weight affinity ligands, the drawback regarding accessibility increases, as is evident on comparing the HSA and insulin recoveries on different anti-HSA and anti-insulin affinity supports (see Figs. 2 and 3). The coupling differences between Biograft and Affi-Gel Hz (a hydrazide-activated gel) and CNBr-activated Sepharose respectively are large. This is in accordance with recent practical experience, namely that CNBr is an ineffective coupling agent[12]. These media were therefore not considered suitable for further tests. In contrast, coupling via epoxy groups clearly shows a better performance, as can be demonstrated by the results obtained using Eupergit and VA-Epoxy, both oxirane-containing carriers.

Based on the present and recent studies, it could be shown that coupling via epoxy and isocyanate groups is superior, from the methodological and kinetic point of view, to currently used methods[2,13]; this also applies to the excellent stability of the bond formed. Recently described methods recommending tresyl chloride[14], 1,1'-carbonyldiimidazole[15] and FMP[6] are too expensive and the techniques too laborious for technical application. Further, these agents split off organic moieties during the coupling procedure, which could be detrimental to the biological activity of a bound biomolecule (see enzyme immobilization tests in Table I); interference with UV detection is another crucial aspect of these methods.

Coupling, however, is only one aspect contributing to the overall performance, as was found when correlating the anti-insulin loading degree and insulin recoveries (Fig. 3). From these results, one can conclude that steric aspects play an important role regarding the accessibility of the affinity ligands when their immobilization density is increased. This trend applies to both VA-Epoxy and Biograft carriers; however, the protein recovery obtained with Biograft remains a factor 2 higher than that obtained with VA-Epoxy. The reason for this difference lies in the unique molecular structure of the grafted sample. Long tentacle-like spacer arms are introduced into the matrix

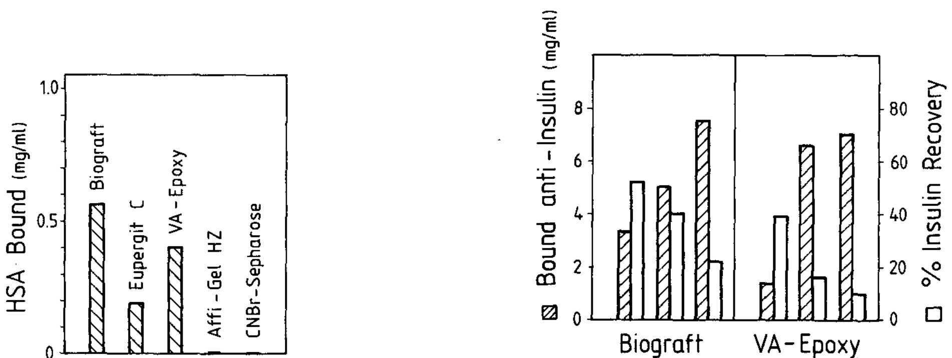

Fig. 2. HSA recoveries on different anti-HSA carriers. Coupling conditions: 3.6 mg of antiserum per 100 mg of carrier in 1 *M* potassium phosphate buffer (pH 7.5) for 24 h at room temperature; amount of HSA applied, 0.5 mg in 25 m*M* phosphate buffer (pH 7.5); incubation period, 12 min; eluent, 8 *M* aqueous urea.

Fig. 3. Immobilization of anti-insulin and insulin recoveries on Biograft and VA-Epoxy resins. Amount antiserum applied, 4 mg per 100 mg of carrier in 1 *M* potassium phosphate buffer (pH 7.5), for 24 h at room temperature; amount of insulin applied, 1.5 mg in 25 m*M* phosphate buffer (pH 7.5); incubation and elution as in Fig. 2.

TABLE I

IMMOBILIZATION OF ENZYMES AND HEPARIN TO DIFFERENT SUPPORTS (% ACTIVITY RETENTION)

Initial enzyme concentration for coupling; 0.5–3 mg per 100 mg of dry carrier.

Immobilized biomolecule	*Support*						
	Biograft	*Eupergit*	*Sepharose 6B Epoxy*	*Sepharose 4B Tresyl*	*VA-Epoxy*	*Fractogel TSK Epoxy*	*FMP-Fractogel TSK HW-75F*
HR Peroxidase	77	36	7	nd[a]	32	8	nd
Penicillin acylase	69	39	8	12	34	10	15
Polygalacturonase	49	<5	<5	nd	<5	<5	nd
Glucose isomerase	74	50	10	16	nd	nd	18
Formate dehydrogenase	78	37	nd	nd	41	8	11
Heparin (mg/g)	26	14	<1	nd	10	<1	<1
Bead size (m)	150	150	70	70	110	50	50

[a] nd = Not determined.

during grafting, thus rendering the ligands more accessible to high-molecular-weight components. There is further evidence that the enlarged surface structure rather than the overall porosity plays a decisive role in the separation performance among grafted carriers[2]. The immunopurification of factor VIII on different supports which are depicted in Table II confirms the above test results.

Affinity chromatographic data obtained with the grafted matrices in comparison with commercial media were substantiated by a number of enzyme immobilizations (Table I). The activity retentions are expressed in per cent; his represents the residual enzymatic activity after immobilization. The activity of the enzyme in solution is set at 100% (blind test). With the same amount of enzyme used for the blind test, the immobilization with a defined amount of carrier is performed. This correlation permits a more realistic evaluation of the immobilized enzyme than relating the activity to the actual bound enzyme, as is frequently done[16].

Based on these results and the excellent physico-chemical stability of Biograft (treatments in 4 *M* NaOH and also magnetic stirring are possible without detectable destruction) a pilot study is currently being done to evaluate the feasibility of the technical production of matrix-bound penicillin acylase. Apart from applications in chromatography, efforts have been made in the last few years to extend the principle of affinity chromatography to the medical field. One promising approach is the development of media for hemoperfusion. The main aim is the removal of blood-group antibodies or toxins from blood using appropriate affinity media[17].

Using specific oligosaccharde blood-group determinants, novel media on the basis of PVA have been developed[18,19]. The optimum matrix for hemoperfusion is PVA, as it exhibits the highest blood compatibilities among all common matrices including dextrans and agarose[20]. In the tests described here, the oligosaccharide α-GalNAc(1–3)-β-Gal-α-Fuc(1–2) specific to blood-group A antibodies was bound to different matrices to assess the antibody clearance performance (Table III).

The efficient antibody-binding properties of isocyanate-activated PVA and

TABLE II

IMMUNOAFFINITY PURIFICATION OF FACTOR VIII ON DIFFERENT ANTI-FACTOR VIII IMMUNOGLOBULIN G (IgG) (MOUSE) COUPLED CARRIER MEDIA

Test results were kindly supplied by Dr. E. Rauenbusch, Bayer, Wuppertal, F.R.G. Coupling conditions: 10 ml of 1 *M* potassium phosphate buffer (pH 7.5) containing 3 mg of IgG per 0.5 g of carrier ; remaining oxirane groups were blocked by incubation with 2 ml of 1 *M* ethanolamine (pH 8.0) for 24 h at room temperatures; sample applied, 1 ml of crude protein solution containing *ca.* 100 μg of protein.

Carrier	*IgG immobilization yield (%)*	*Factor VIII recovery (%)*
Biograft A[a]	99	21
Biograft B[a]	78	39
Biograft C[a]	100	22
Controlled-pore glass	90	17
Eupergit	100	31
VA-Epoxy	95	5

[a] Biograft A, 45% graft uptake; Biograft B, 88% graft uptake; Biograft C, 62% graft uptake.

TABLE III

REMOVAL OF ANTI-A ANTIBODIES FROM HUMAN SERUM TYPE 0

Concentration of hapten in coupling solution, 2 mg per gram of carrier; 400 μl of pooled human serum were incubated for 10 min at room temperature; the antibody content was determined by the agglutination test (for details, see text).

Carrier	*Sample taken (mg)*	*Bead size (μm)*	*Antibody bound (%)*
PVA	50	200–500	>90
Synsorb A	50	200–500	>90
Eupergit	50	150	>90
Fractogel TSK HW-75F[a]	30	50	<50
Protein G Sepharose 4[b]	30	70	<30

[a] Activated with FMP.
[b] Used as supplied by the manufacturer without blood-group hapten.

Synsorb A are excellent, considering the large bead sizes. The high specificity of the oligosaccharide hapten is notable in comparison with the antibody binding performance of protein G, which is recommended as an affinity ligand for immunoglobulins[21].

The described affinity chromatographic and immobilization tests have demonstrated the benefits of grafted polymers in chromatography. The high performance of these carriers in conjunction with their high chemical and physical stability indicate that these supports may be useful alternatives to the currently used media for laboratory applications and provide new prospects with regard to technical usage.

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CHROM. 22 406

Specific adsorption of serine proteases on coated silica beads substituted with amidine derivatives

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ABSTRACT

Amidine derivatives interact with serine proteases, the inhibition being due to interactions between amidine functions and the active sites of the enzymes. Five differents types of amidine (substituted or unsubstituted) were coupled to coated silica beads, which had previously been coated with DEAE-dextran to minimize the non-specific interactions due to silanol groups. Coated silica functionalized with substituted amidines shows a strong affinity towards human plasmin. This affinity is probably due to hydrophobic interactions between the substituted amidine and the human plasmin structure. Coated silica grafted by *p*-aminobenzamide gives a specific interaction with human plasmin. The importance of ionic strength and the steric conformation of the ligand is discussed. This support was used to purify thrombin from crude preparations by high-performance affinity chromatography.

INTRODUCTION

Activation of coagulation factors is the central feature of blood coagulation, fibrinolysis, complement activation system and proteolytic digestion. The resulting serine proteases are able to hydrolyse specifically protein substrates. This hydrolysis generally begins by the interaction of the enzyme with the substrate implying an aspartic acid residue of the enzyme and the arginine residue of the substrate.

Amidine functions mimic the reactive binding site (arginine) of the substrate. Consequently, amidine derivatives are excellent inhibitors of serine proteases. Several studies have reported that benzamidine derivatives inhibit serine porteases in solution[1–3]. The primary step governing the binding of benzamidine to these enzymes is an ionic interaction of the cationic amidine moiety with an anionic amino acid side-

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chain in the enzyme structure, *e.g.*, carboxylic groups of an aspartic acid, leading to a reversible interaction[4]. Benzamidine derivatives have been immobilized on solid supports in order to prepare stationary phases for affinity chromatography[3,5]. On such supports, serine porteases such as thrombin, trypsin kallikrein and urokinase can be purified[5–7] by affinity chromatography.

To assess the relationship between amidine structure and affinity of enzymes, we prepared passivated dextran-coated silica supports bearing differents types of amidines (N,N'-substituted or unsubstituted) (Fig. 1). The affinity constants of plasmin and thrombin for these solide supports were determined using adsorption isotherms.

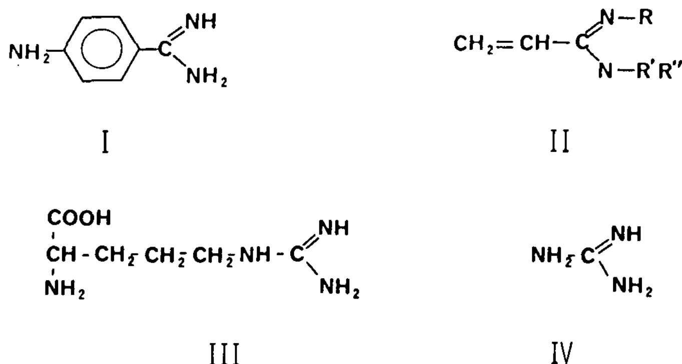

Fig. 1. Structure of amidine derivatives coupled on passivated silica beads. I = *p*-Aminobenzamidine. II = A_x; A_1 (N-*tert*.-butyl-N'-isopropylpropenamidine): R = $C(CH_3)_3$, R' = H, R'' = $CH(CH_3)_2$; A_3 (N^1,N^2-triisopropylpropenamidine): R = R' = R'' = $CH(CH_3)_2$. III = L-Arginine. IV = Guanidine.

The influence of the substitution of the amidine function on the affinity of plasmin was investigated by comparison of the affinity constants of the different supports, in order to observe the steric hindrance of the substituent during the complex formation between the amidine and the enzyme. The affinity of these supports for plasminogen was used to study the specific interaction between the amidine function and the protein in solution. Finally, the supports were used in order to purify thrombin from commercial preparations by high-performance affinity chromatography (HPAC).

EXPERIMENTAL

Reagents

p-Aminobenzamidine (pABA) and L-arginine monohydrochloride were provided from Fluka (Buchs, Switzerland). 1,1'-Carbonyldiimidazole (CDI), used as a coupling agent, was obtained from Sigma (St. Louis, MO, U.S.A.). N-*tert*.-Butyl-N'-isopropylpropenamidine (A_1) and N-triisopropylpropenamidine (A_3) were prepared as described previously[8]. 1,4-Butanediol diglycidyl ether (BDGE) was purchased from Polysciences (Warrington, PA, U.S.A.). Silica beads, obtained from IBF Biotechnics (Villeneuve la Garenne, France), were in the size range 40–100 μm and the pore diameter was about 1250 Å. Dextran T40, batch No. 24512 (weight-average

molecular weight, M_w = 42 000; number-average molecular weight, M_n = 24 700; polydispersity index, $I = \bar{M}_w/\bar{M}_n$ = 1.70) was purchased from Pharmacia-France (Bois d'Arcy, France), human plasmin, human thrombin and the chromothrombin substrate from Diagnostica Stago (Asnières, France), the S 2251 substrate from Kabi-Vitrum (Stockholm, Sweden) and bovine thrombin [batch B 0941, 64 NIH.U/mg (*i.e.* activity of thrombin determined by comparison with standard from National Institute of Health, Bethesda, MD, U.S.A.)] from Hoffman-La Roche Diagnostica (Basle, Switzerland). Human plasminogen was purified from plasma by affinity chromatography on lysine-Sepharose according to the method of Deutsch and Mertz[9].

Preparation of affinity supports

Passivated silica beads (SID). In order to neutralize the anionic silanol groups on the silica surface, silica beads were coated with diethylaminoethyl-dextran as described previously[10]. The degree of substitution of the functionalized dextran polymer is 4% and the extent of polymeric coverage determined by elemental analysis corresponds to 3.6 g of carbon per 100 g of dry support. Passivated silica beads were coupled with several amidines as follows.

p-Aminobenzamidine (SID–pABA). A 2-g amount of SID was suspended in 20 ml of 1,4-dioxane and mixed with 1 g of CDI. The gel suspension was gently shaken at room temperature for 2 h, then the activated support was washed successively with 200 ml of 1,4-dioxane and 200 ml of 0.1 *M* carbonate buffer (pH 10.5) and resuspended in 20 ml of 0.1 *M* carbonate buffer containing 500 mg of pABA. The mixture was gently stirred at room temperature for 48 h. The solid support was filtered and washed successively with 200 ml of 0.1 *M* carbonate buffer (pH 10.5) and 200 ml of 0.05 *M* phosphate buffer (pH 7.5). The excess of activated groups was neutralized by suspending the supports in 0.1 *M* ethanolamine solution for 3 h. The final support was filtered and washed extensively with 200 ml of 0.05 *M* phosphate buffer (pH 7.5), filtred and dried under vacuum at 60°C.

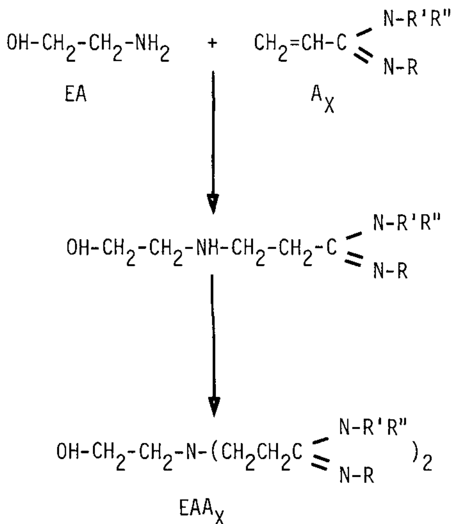

Fig. 2. Reaction of ethanolamine with substituted amidine.

Substituted amidine A1 (SID-B–A_1). The active support (SID-B–A_1) was prepared in two successive steps. The first step (Fig. 2) is the reaction of ethanolamine to A_1, as reported previously[11]. Secondly, the reaction product of addition of ethanolamine to A_1, EAA_1, was coupled to SID support using the following procedure: 2 g of SID were suspended in 20 ml of diethyl ether and 2 ml of BDGE were added. The suspension was gently stirred for 15 h at room temperature and the activated support was washed successively with 200 ml of diethyl ether and 200 ml of methylene chloride. The activated support was resuspended in 20 ml of methylene chloride containing 1 g of EAA_1 and the mixture was gently stirred at room temperature for 48 h. Then the support was washed successively with 200 ml of methylene chloride and 200 ml of 0.05 *M* phosphate buffer (pH 7.5). The excess of BDGE was inactivated in 0.1 *M* ethanolamine solution. Finally, the support was filtered and washed with 300 ml of 0.05 *M* phosphate buffer (pH 7.5), then isolated by filtering and dried under vacuum at 60°C.

Substituted amidine A_3 (SID-B–A_3). The coupling procedure was similar to that used for the SID-B–A_1 support.

p-Aminobenzamidine, using a spacer arm (SID-B–pABA). The step of support activation was similar to those described for SID-B–A_1 and SID-B–A_3 supports. The activated support was washed with 200 ml of 0.1 *M* carbonate buffer (pH 10.5). After filtration it was suspended in 20 ml of 0.1 *M* carbonate buffer (pH 10.5) containing 256 mg of pABA. The mixture was stirred at room temperature for 48 h. The final support was washed and filtered according to the method used for the activated support SID–pABA.

L-*Arginine (SID–Arg).* The protocol of the fixation procedure was similar to that for the SID–pABA support. The activated support was reacted with 500 mg of L-arginine in solution.

Guanidine (SID-B–Gua). This support was prepared in two successive steps. First, 2 g of SID were activated by 1 g of CDI as described above for the SID–pABA support, then the activated support was suspended in a solution of 20 ml of 0.1 *M* carbonate buffer (pH 10.5) containing 1.5 g of hexamethyldiamine (HMD). The mixture was gently stirred at room temperature for 48 h then the support was washed successively with carbonate buffer and 1,4-dioxane. The amine functions of HMD fixed on the support were reactivated with CDI (1 g of CDI per 2 g of support), then the activated support was suspended with 1 g of guanidinium carbonate dissolved in 20 ml of 0.1 *M* carbonate buffer (pH 10.5). The mixture was stirred at room temperature for 48 h. Finally, the support was washed with 0.05 *M* phosphate buffer and dried under vacuum at 60°C.

Characterization of the coated silica beads. The extent of substitution of the different supports by amidine derivatives was evaluated by elemental analysis (Service Central d'Analyse CNRS, Vernaison, France).

Adsorption isotherms and determination of affinity constants

Preliminary experiments were performed from a kinetic study to ascertain that human plasmin and thrombin were adsorbed at equilibrium after contact with silica beads for 30 and 20 min, respectively.

Adsorption of human plasmin. Isotherms were established from measurement of plasmin adsorption using the following procedure: 100 μl of support suspension (30–

100 mg/ml) were incubated with 900 μl of human plasmin solution at various concentrations (3–0.18 nKat/ml) in a polystyrene tube for 30 min at room temperature (1 nKat is the enzymatic activity, observed using CBS.3308, which releases 1 nmol of *p*-nitroaniline per second, under standard conditions). After sedimentation, the amount of residual enzyme was determined by taking 200 μl of supernatant and adding 620 μl of 0.05 *M* Tris–imidazole buffer (pH 7.5). Finally, 80 μl of chromogenic substrate S2251 were added and the mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 100 μl of pure acetic acid. The absorbance of the solution was read at 405 nm. The amount of adsorbed plasmin corresponds to the difference between the control and the remaining concentration of active enzyme in the supernatant.

Adsorption of human thrombin. A 100-μl volume of support suspension (5 mg/ml) was incubated with 500 μl of thrombin solution (concentration varying from 3 to 0.312 NIH.U/ml) for 20 min at room temperature. After sedimentation, the amount of residual thrombin was determined by adding 100 μl of supernatant to a mixture of 700 μl of 0.05 *M* phosphate buffer (0.1 *M* sodium chloride, pH 7.5) and 100 μl of the chromothrombin substrate. Time was measured after adding thrombin. The reaction was stopped after incubation for 3 min at 37°C, by adding 100 μl of pure acetic acid. The amount of adsorbed thrombin corresponds to the difference between the control and the ramaining concentration of active thrombin in the supernatant, determined by the absorbance at 405 nm.

Affinity constants were calculated from the initial slope and plateau of the isotherm on the basis of the Langmuir and Tempkin equations[12]. A computer program is able to establish the isotherm and to calculate the affinity constant of active supports for proteins.

Chromatographic procedure

The column (12.5 cm × 0.4 cm I.D.) was packed with 1 g of SID–pABA support using the slurry method. The liquid chromatographic apparatus consisted of a three-head (120°), chromatographic pump (Merck LC 21B) connected to a Rheodyne 7126 injection valve with a 100-μl sample loop. A variable-wavelength UV–visible detector (Merck-LC 313) and the gradient system are connected to an Epson QX-10 computer. The chromatographic signal is monitored, integrated and stored by the computer. All the equipment was provided by Merck-Clevenot (Nogent-sur-Marne, France). A volume of 50 μl (112.5 μg) of human plasminogen was injected onto the column of SID–pABA support. After adsorption of plasminogen, elution was achieved by competitive elution of arginine, aminocaproic acid or sodium chloride at varying molarity. The amount of plasminogen desorbed was determined from the surface area of the peak given by the computer. Elution of bovine thrombin was achieved as follows: 100 μl of crude thrombin (128 NIH.U) were injected onto the column in the initial buffer (0.05 *M* phosphate, pH 7.5). The column was washed with 20 ml of the initial eluent and the thrombin adsorbed was eluted by competitive elution using a 0.5 *M* arginine solution in the same buffer. The collected fractions were passed to a Sephadex G-25 column in order to separate arginine and thrombin. The specific activity of the eluted fraction was determined by clotting methods as reported previously[13].

All eluents were prepared from high-purity water (ELGA, Villeurbanne,

France), degassed and filtered through a Millipore (Velizy, France) HA 0.22-μm membrane.

RESULTS AND DISCUSSION

Adsorption of plasmin and plasminogen

Native silica beads were coated with dextran bearing a calculated amount of positively charged DEAE units in order to neutralize the negative charges of the silanol groups of native silica[10]. These coated silica supports exhibit two advantages: first, they supports have a minimum non-specific ion-exchange capacity, and second, ligands can easily be immobilized via the hydroxylic functions of the polysaccharide coating using conventional coupling methods. Unsubstituted amidines (*p*-aminobenzamidine, L-arginine and guanidine) and substituted amidines A_1 and A_3 (Fig. 1) were coupled on the coated silica supports which had previously been activated with CDI or BGDE (Fig. 3).

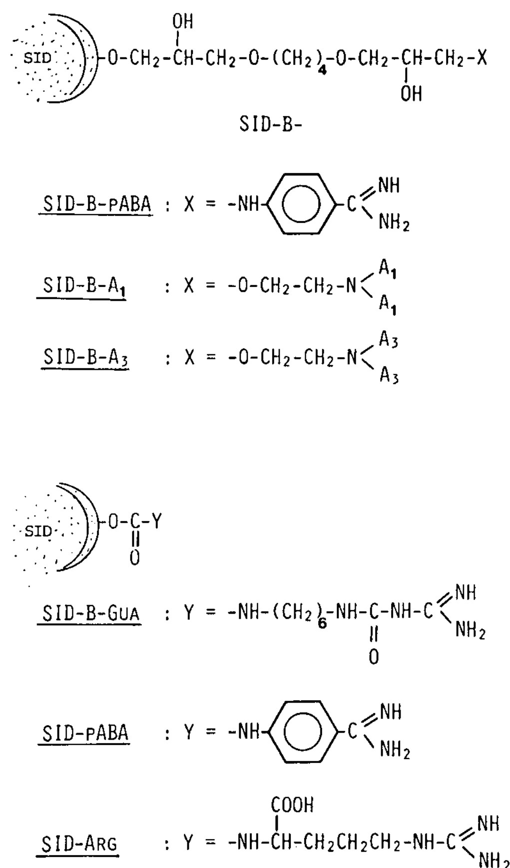

Fig. 3. Structure of the six active supports used in the adsorption and chromatographic studies.

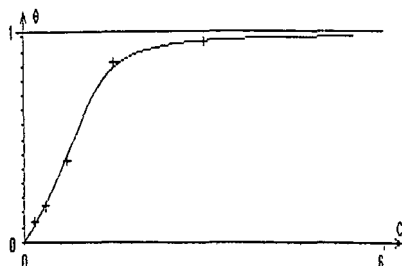

Fig. 4. Example of adsorption isotherm (Tempkin) from the computer program. C = Initial concentration of protein (U/ml); Γ = adsorbed protein[12]. $\theta = \Gamma/\Gamma_{\infty}$, where Γ_{∞} is the concentration of adsorbed protein at saturation.

The affinity of these supports for human plasmin was studied by determining the affinity constants from Langmuir or Tempkin adsorption isotherms (Fig. 4). The affinity constants of the six supports tested are presented in Table I, and are between about 10^7 and 10^8 l/mol. These values demonstrate the strong affinity of all the supports for human plasmin. Comparison of the affinity constants of supports bearing an unsubstituted amidine (SID–pABA, SIB-B–pABA, SID–Arg and SID-B–Gua) shows that, in spite of the presence of the spacer arm, SID–pABA and SID-B–pABA supports have almost the same affinity constant (10^8 l/mol). The spacer arm has only a small influence on the adsorption process of human plasmin. SID-Arg and SID-B–Gua supports exhibit affinity constants in the same range. However the SID–pABA support is the most efficient adsorbent. Indeed, this support possesses the higher binding capacity and affinity constant. This difference in binding capacity is probably related to a better efficiency of the coupling reactions.

The affinity constants of SID-B–A_1 and SID-B–A_3 supports (Table I) show that the substitution of two or three hydrogens of the amidine function by R, R′ and

TABLE I

AFFINITY CONSTANTS AND BINDING CAPACITIES OF PASSIVATED SILICA BEADS FUNCTIONALIZED BY AMIDINE DERIVATIVES TO HUMAN PLASMIN

Material[a]	*Affinity constant ($l\ mol^{-1}$)*	*Binding capacity (M/g)*
SID–pABA (T)	$1.8 \cdot 10^8$	$2.9 \cdot 10^{-9}$
SID-B–pABA (T)	$9.5 \cdot 10^7$	$2.3 \cdot 10^{-9}$
SID–Arg (L)	$6.4 \cdot 10^7$	$1.2 \cdot 10^{-9}$
SID-B–Gua (L)	$1.1 \cdot 10^8$	$1.2 \cdot 10^{-9}$
SID-B–A_1 (L)	$3.2 \cdot 10^7$	$1.5 \cdot 10^{-9}$
SID-B–A_3 (T)	$4.2 \cdot 10^7$	$1.8 \cdot 10^{-9}$

[a] T = Tempkin isotherm; L = Langmuir isotherm.

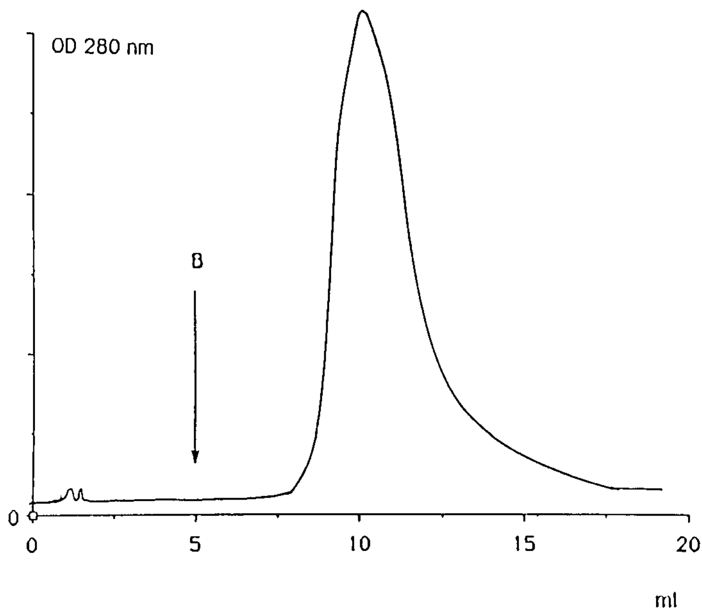

Fig. 5. HPAC profile of human plasminogen on SID–pABA support. Elution conditions: column, 12.5 × 0.4 cm I.D.; eluent 0.05 *M* phosphate buffer–0.1 *M* NaCl (pH 7.5); flow-rate, 1 ml/min; B = 0.5 *M* arginine in initial buffer.

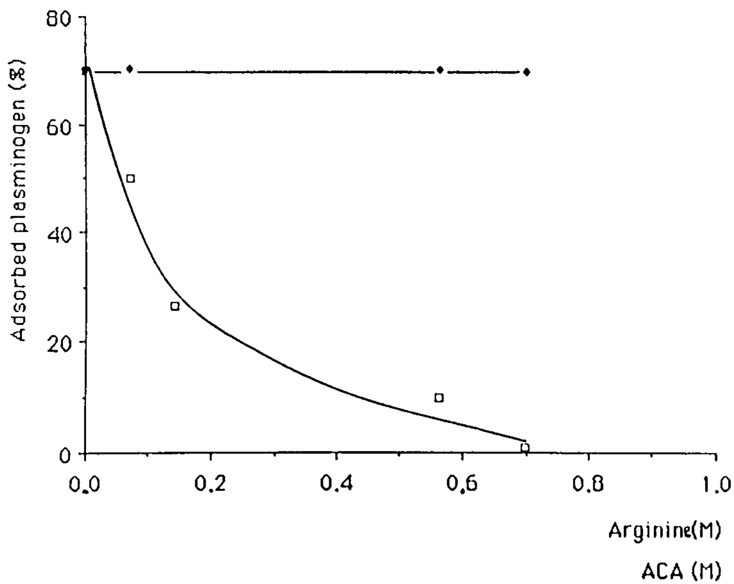

Fig. 6. Elution of human plasminogen on SID–pABA support, with (□) arginine and (●) aminocaproic acid (ACA) solutions.

R″ groups (see Fig. 1) decreases slightly the affinity of the supports towards plasmin. However, this affinity is still strong (K_{aff} = 10^7 $lmol^{-1}$). The hydrophobic character of the substituents (isopropyl, *tert.*-butyl) can explain this high affinity. The interactions between supports such SID-B–A_1 or SID-B–A_3 for plasmin are probably due to hydrophobic interactions taking place between the substituted amidines and the enzyme. Moreover, the presence of hydrophobic sites adjacent to the ionic binding site of serine proteases has been reported by Andrews *et al.*[4].

Results of the chromatographic elution of human plasminogen on SID–pABA support are presented in Figs. 5 and 6. Human plasminogen is adsorbed at low ionic strength and can be desorbed in a single peak with 0.5 *M* arginine solution (Fig. 5). The elution of plasminogen with arginine solutions at different molarity is presented in Fig. 6. Competition between arginine in solution and the coupled amidine allows the desorption of the plasminogen from the stationary phase. In addition, an increase in the ionic strength of the eluent is unable to desorb the plasminogen. These results demonstrate that the affinity between the protein and the support is strong and not due only to ionic interactions. This specific interaction probably requires the cooperation of hydrophobic (aromatic rings of p-ABA) and hydrogen interactions. Finally, the strong affinity of human plasminogen for SID–pABA support, cannot be broken by the competitive elution of aminocaproic acid, which has unsubstituted amine groups (Fig. 6), indicating that the steric conformation of the amidine function is very important in the interaction between the *p*-aminobenzamidine groups fixed on the support and the protein.

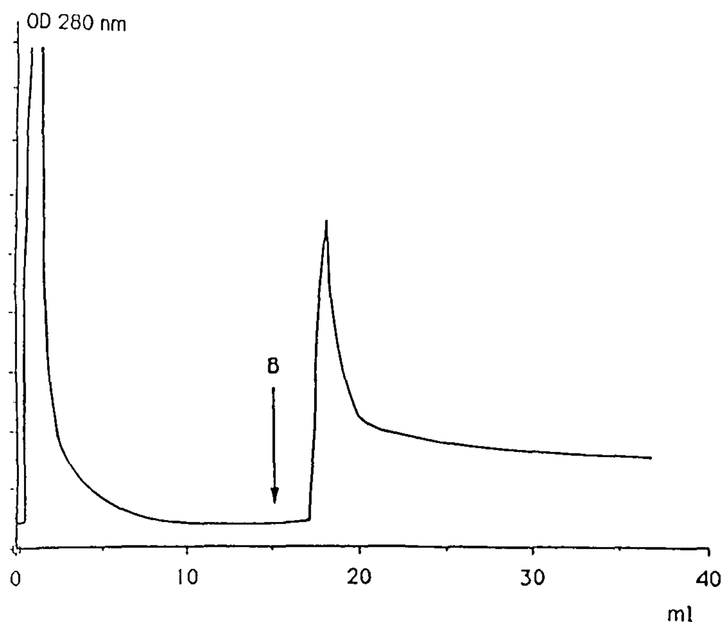

Fig. 7. Purification of bovine thrombin by HPAC on SID–pABA. Elution conditions: column, 12.5 × 0.4 cm I.D.; sample, 100 μg of crude thrombin (128 NIH.U); eluent 0.05 *M* phosphate buffer–0.1 *M* NaCl (pH 7.5); flow-rate, 1 ml/min; B = 0.5 *M* arginine in initial buffer.

Adsorption and separation of bovine thrombin

The affinity of thrombin for the SID–pABA support was evaluated from the adsorption isotherms. The calculated affinity constant is about $1.7 \cdot 10^8$ l mol^{-1}. This active support was used in HPAC in order to purify thrombin. Crude thrombin preparations were eluted on a column packed with SID–pABA support. As shown in Fig. 7, thrombin is adsorbed at low ionic strength and desorbed specifically by competitive elution using 0.5 *M* arginine solution. The specific activity of the purified thrombin is about 1200 NIH.U/mg. The yield of this separation is about 75%.

CONCLUSION

Amidine derivatives can be coupled on silica beads previously passivated with DEAE-dextran. These solid supports exhibit minimum non-specific ionic interactions with proteins in solution. As expected, such coated silica supports functionalized by amidine derivatives have a strong affinity for human plasmin. In addition, substituted amidines (A1, A3) immobilized on coated silica beads have a strong affinity for human plasmin. This affinity is probably due to hydrophobic interactions between the substituted amidine residues and the human plasmin in solution. The specific interaction between *p*-aminobenzamidine and the serine protease is probably the result of several types of interactions. SID–pABA is a good stationary phase for separating and purifying different serine proteases such as thrombin, trypsin and tissue plasminogen activator (tPPA) by HPAC. The good mechanical properties of these supports, particularly the excellent resistance to hydrostatic pressure, allow their use in HPAC and the separation process can easily be scaled up.

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CHROM. 22 378

New ion exchangers for the chromatography of biopolymers

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ABSTRACT

A group of new ion exchangers for the separation of biopolymers is described in which the ionic groups are exclusively located on linear polymer chains grafted on the support surface. This arrangement markedly reduces the contact between the analyte and the matrix, thus suppressing unspecific side activities of the support. Further, this "tentacle-like" arrangement of the ionic groups allows ionic interactions between the ion exchanger and the analyte which are impossible with standard-type exchangers for steric reasons. These changes in the interaction mode manifest themselves in appreciable differences in the selectivities of the two types of ion exchangers. In addition, the new type of arrangement of the binding groups avoids distorsions of the analyte which may occur on interacting with the conventional type.

INTRODUCTION

Ion exchangers suitable for the chromatography of functionally active biopolymers consist in general of natural or synthetic polymers or inorganic materials such as silica, which carry defined ionic groups on their surface. Such materials play the most important role in the isolation and purification of biopolymers, especially proteins with and without catalytic properties. Their basic function may be described as an electrostatic binding of counter-ionic analytes and their release at distinct concentrations of a competing salt. Despite this simple concept, a comprehensive formal treatment, *e.g.*, relating the eluting salt concentration to molecular parameters, is not easy.

The importance of ion-exchange matrices in downstream processing and special fractionations of proteins and nucleic acids is uncontested and they are widely accepted as very potent tools in research and production. However, in order to exert their full potential, they should possess the following properties: (i) the basic support material should be highly inert, *i.e.*, essentially free from non-specific side affinities; (ii) the support should be highly porous with pore sizes large enough to allow larger analytes to freely penetrate the support particles; the internal volume and the surface area accessible to the analyte should be large enough to provide an acceptable binding capacity; and (iii) the support should be rigid enough to allow its application at

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flow-rates high enough to avoid substantial degradations by proteases and nucleases. Although these demands seem reasonable, there are very few materials available that rigorously fulfil all three requirements.

In this paper it is shown that, apart from the basic properties listed above, the arrangement of the ionic groups on or in the ion exchanger seems to be more important than generally expected. In a standard ion exchanger the ionic groups are fixed via short arms on the support surface, thus forming a rigid array of binding sites for the poly-counter-ionic analyte. This implies that the analyte may be distorted during the process of maximizing the number of ion pairs formed between the ion exchanger and the analyte. It is obvious that this process may be of special relevance at low ionic strength at which the electrostatic effects are sufficiently large, *i.e.*, under conditions under which the analyte is bound. The extent to which such a distortion is reversible or able to induce denaturation or irreversible binding by turning hydrophobic areas to the outside is difficult to establish. One can avoid this effect, however, by fixing the ionic groups on linear polyelectrolytes bound to the support surface. This is partially realized in ion exchanger consisting of cross-linked polysaccharide strands to which the ionic groups are bound, or in silica particles that are coated with ionic polymers and cross-linked in an additional step[1,2].

In both instances the true mobility of the groups as provided by the flexibility of the cross-linked polymer chains can hardly be estimated. A maximum motional freedom of the groups is guaranteed for the ion exchangers described in this paper because the polymer chains carrying the charges are grafted on the support surface in the absence of cross-linkers. Preliminary notes on these materials have been presented recently[3,4].

EXPERIMENTAL

All buffer components were analytical-reagent grade materials obtained from E. Merck (Darmstadt, F.R.G.). The water used was deionized with a Milli-Q system equipped with an additional UF filter (Millipore).

The test proteins used were as follows: chymotrypsinogen A (E. Merck; Cat. No. 2306), cytochrome *c* (E. Merck; Cat. No. 24804), lysozyme (E. Merck; Cat. No. 5281), conalbumin (ovotransferrin) (Serva, Heidelberg, F.R.G.; Cat. No. 17466), ovalbumin (Sigma, St. Louis, MO, U.S.A.; Grade VI, Cat. No. A-2512) and human serum albumin (Behring-Werke, Marburg, F.R.G.; ORHA 20/21).

The plasmid digest pBR 322 × HpaII was obtained from E. Merck (Cat. No. 6117); the mixture of the 2.99 kb (kilobase pairs) and 7.8 kb DNA fragments was a gift from Dr. Reiffen (E. Merck).

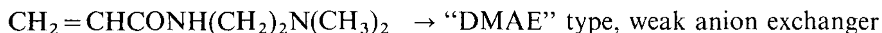
A fast protein liquid chromatographic (FPLC) system (Pharmacia, Uppsala, Sweden) and a high-performance liquid chromatographic (HPLC) system (Merck–Hitachi, Inert Version, E. Merck, Darmstadt, F.R.G.) were used.

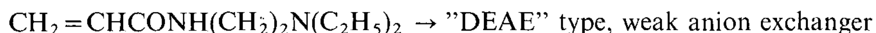
"Conventional" ion exchangers of the Fractogel TSK type were obtained from E. Merck. The "tentacle"-type ion exchangers were prepared as described elsewhere[5]. All these tentacle types [based on Fractogel, EMD types or on silica (LiChrosphers)] are available from E. Merck.

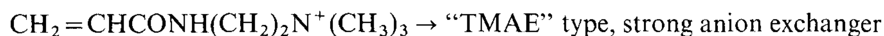
RESULTS AND DISCUSSION

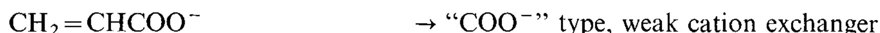
Grafting charged monomers on polymers or modified silica to provide a family of ion exchangers

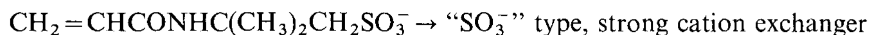
When a porous chromatographic support consists of a basic material on which vinyl polymer chains may be grafted in sufficient lateral density, ion exchangers of the following types may be formed from the monomers listed:


$CH_2=CHCONH(CH_2)_2N(CH_3)_2$ → "DMAE" type, weak anion exchanger


$CH_2=CHCONH(CH_2)_2N(C_2H_5)_2$ → "DEAE" type, weak anion exchanger


$CH_2=CHCONH(CH_2)_2N^+(CH_3)_3$ → "TMAE" type, strong anion exchanger


$CH_2=CHCOO^-$ → "COO^-" type, weak cation exchanger


$CH_2=CHCONHC(CH_3)_2CH_2SO_3^-$ → "SO_3^-" type, strong cation exchanger

The grafting reaction requires primary or secondary aliphatic hydroxyl functions as initiation sites and uses Ce^{IV} ions as the catalyst; it was developed by Mino and Kaizerman[6] for grafting polymers in a homogeneous phase. It is obvious that the density of the hydroxyl groups on the support surface and the amount of catalyst used determine the density of the grafting. Similarly, the amount of monomer present governs the mean length of the grafted chains. Indirect figures and microscopic studies indicate that chains consisting of a few monomers only are equally accessible as long chains, forming brush-like layers up to 50 μm in thickness. The chain length optimally suited for the functioning of an ion-exchange process will definitely depend on the type of analyte used; according to present experience, chains of 5–50 monomers seem to function properly for proteins and nucleic acids. The fact that 50 monomers correspond to a length of about 10 nm in the fully extended state emphasizes the importance of using supports with sufficiently wide pores; materials with 100–500 nm diameter pores have been found to be adequate.

One might expect the following features for materials prepared properly by the procedure described above: (i) the capacity of the ion exchanger no longer depends exclusively on the surface area of the support; (ii) the analyte barely contacts the support surface and is thus prevented from non-specific interactions; (iii) owing to the high flexibility of the uncross-linked polyelectrolyte chains, the charges can easily adopt a configuration that is optimum for their electrostatic interaction with the analyte, exhibiting a tentacle-like function.

Comparative tests of conventional and tentacle-type ion exchangers and special applications

The experimental verification of the statements made above was possible owing to the availability of ion exchangers prepared from a vinyl polymer (Fractogel) in the conventional way and the polymer-grafting process described above.

Comparative data for the capacities of the two types of ion exchangers are given in Table I. The data were obtained by passing solutions of the test proteins through

TABLE I

BINDING CAPACITIES OF CONVENTIONAL AND "TENTACLE-TYPE" ION EXCHANGERS FOR CERTAIN STANDARD PROTEINS

Ion exchanger	*Protein (buffer)*	*Capacity (mg/ml)*
Fractogel TSK 650(s) DEAE (conventional)	Bovine serum albumin (50 m*M* Tris–HCl, pH 8.0)	30
Fractogel EMD 650(s) DEAE ("tentacle")	As above	70–140
Fractogel TSK 650(s) SP (conventional)	Lysozyme (20 m*M* phosphate, pH 7.0)	55
Fractogel EMD 650(s) SO_3^- ("tentacle")	As above	70–140
Fractogel TSK 650(s) CM (conventional)	Haemoglobin (10 m*M* acetate, pH 5.0)	50
Fractogel EMD 650(s) COO^- ("tentacle")	As above	70–140

small column packings until their saturation became detectable by the breakthrough of the protein front (an increase in the absorbance at 280 nm of more than 0.2). The data indicate that a substantial increase in the capacities may be achieved by the tentacle-type modification.

The reduced non-specific interaction between an analyte and the support resulting from grafting polymer chains on the surface could be observed for two smaller proteins known to stick to gel permeation media owing to their hydrophobicity and basicity.

These proteins, lysozyme and chymotrypsinogen A, elute from a diol-modified support of porous glass (pore size 90 nm) at 1.5 and 0.89 column volumes, respectively, in a medium consisting of 100 m*M* sodium chloride and 10 m*M* phosphate (pH 7). These values exceed the expected values by 95% in the first and by 15% in the second case owing to hydrophobic and ionic interactions of the proteins with the support. After grafting neutral polyacrylamide on the support surface, the elution volumes decreased to 1.2 and 0.78 bed volumes, respectively, which are the correct value for the chymotrypsinogen A and a much improved value for the lysozyme.

The third statement is confirmed by some comparative separations on conventional and tentacle-type ion exchangers. All these separations were performed on ion exchangers prepared from the same support material, the vinyl polymer Fractogel TSK HW 65(s). The conventional (TSK) types carry their ionic groups directly on the surface whereas the tentacle (EMD) types carry them on the grafted polymers only. In all instances columns of 150 × 10 mm I.D. were used.

In Figs. 1 and 2, separations of chymotrypsinogen A, cytochrome *c* and lysozyme (1 mg each) by gradient elution are shown. Fig. 1 shows the separation on strong cation exchangers carrying SO_3 groups. Compared with the conventional type, the tentacle type exhibits a marked increase in selectivity coupled with a clear band sharpening for the lysozyme. When the corresponding pair of weak cation exchangers

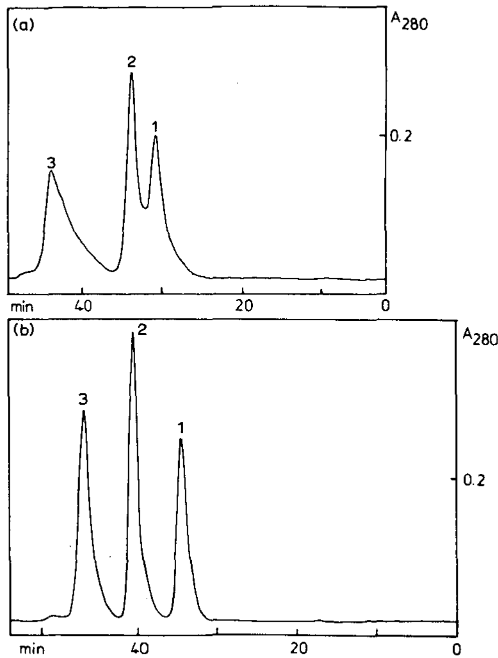


Fig. 1. (a) Fractionation of (1) chymotrypsinogen A, (2) cytochrome *c* and (3) lysozyme on strong cation exchangers. Support, Fractogel TSK 650(s) SP (conventional type); sample, 1 mg each; flow-rate, 1 ml/min; column size, 150 × 10 mm I.D. Solvent A = 20 m*M* phosphate, pH 6.0; solvent B = A + 1 *M* NaCl; gradient, 0–10 min, 0% B; 10–70 min, 0–100% B. Monitor, UV2 (Pharmacia). (b) Fractionation of (1) chymotrypsinogen A, (2) cytochrome *c* and (3) lysozyme on strong cation exchangers. Support, Fractogel EMD 650(s) SO_3^- (tentacle type); conditions as in (a).

with carboxyl groups is used, a very similar effect is observed (Fig. 2); a substantial increase in selectivity is accompanied by a slight band sharpening.

The effects seen with the basic enzymes on cation exchangers are also observed when acidic proteins such as albumins are separated on DEAE-type anion exchangers

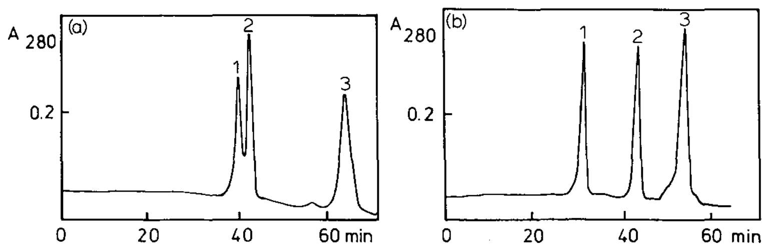


Fig. 2. (a) Fractionation of (1) chymotrypsinogen A, (2) cytochrome *c* and (3) lysozyme on weak cation exchangers. Support, Fractogel TSK 650(s) CM (conventional type); sample, flow-rate and column size as in Fig. 1a. Solvent A = 20 m*M* acetate, pH 5.0; solvent B = A + 1 *M* NaCl; gradient, 0–100% B in 100 min. Monitor, L4200 (Merck–Hitachi). (b) Fractionation of (1) chymotrypsinogen A, (2) cytochrome *c* and (3) lysozyme on weak cation exchangers. Support, Fractogel EMD 650(s) COO^- (tentacle type); conditions as in (a).

by gradient elution. In Fig. 3 the separation of conalbumin, ovalbumin and human serum albumin on a conventional and a tentacle-type anion exchanger is shown. In this instance the selectivity increase due to the tentacle arrangement of the ionic groups is less pronounced; the band sharpening is larger, however.

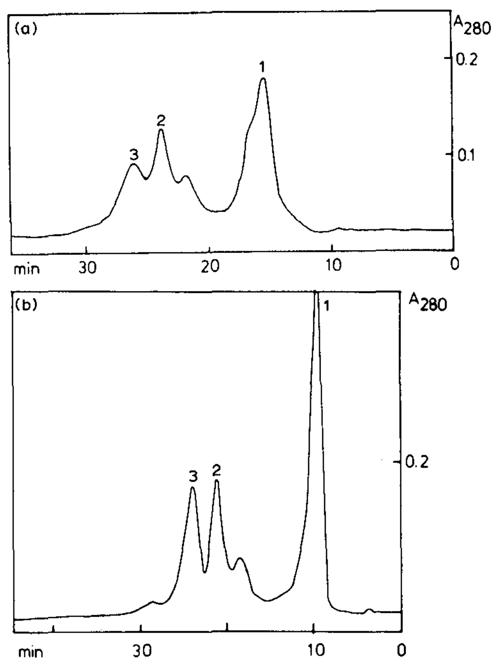
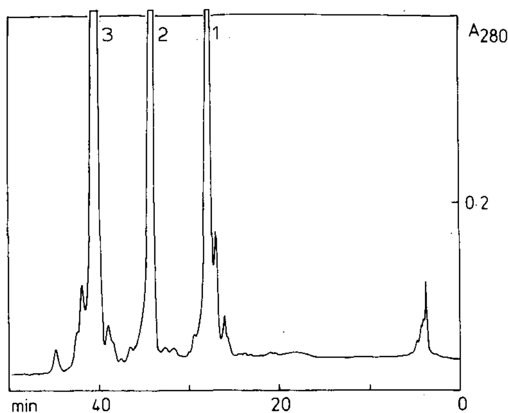


Fig. 3. (a) Fractionation of (1) conalbumin, (2) ovalbumin and (3) human serum albumin on weak anion exchangers. Support, Fractogel TSK 650(s) DEAE (conventional type); flow-rate and column size as in Fig. 1a; sample, 1 mg each. Solvent A = 20 m*M* Tris–HCl, pH 8.0; solvent B = A + 1 *M* NaCl; gradient, 0–100% B in 100 min. Monitor, UV2 (Pharmacia). (b) Fractionation of (1) conalbumin, (2) ovalbumin and (3) human serum albumin on weak anion exchangers. Support, Fractogel EMD 650(s) DEAE (tentacle type); conditions as in (a).

Both effects, sharpening of elution bands and change in selectivity, are most easily explained in terms of the tentacle arrangement of the charges. The band sharpening may be attributed to the reduction of non-specific interactions between the analyte and the support, which eliminates unnecessary kinetic barriers; the change of the selectivity results directly from the fact that tentacles may reach counter-ionic charges in or on the macromolecular analyte, which are not accessible to the surface-fixed charges present in the conventional type of ion exchanger.

The promising results observed for preparative ion exchangers of the tentacle type prompted us to test this structural arrangement of the binding groups also for anaytical materials based on porous silica. In Figs. 4 and 5 two elution profiles with standard test proteins are shown. Comparison of Figs. 1b and 4 reveals that the tentacle-specific selectivity is fully preserved when the matrix is changed.



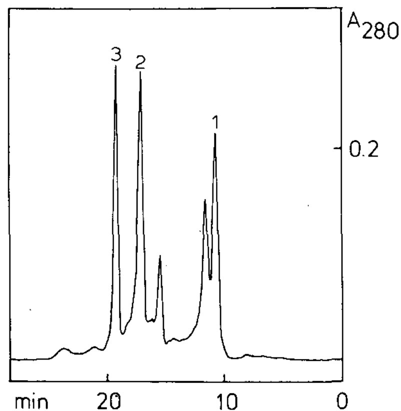
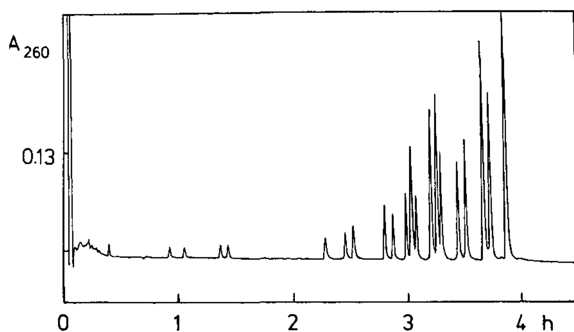


Fig. 4. Fractionation of (1) chymotrypsinogen A, (2) cytochrome *c* and (3) lysozyme (0.5 mg each) on LiChrospher SO_3^- (5 μm) (tentacle type). Column size, 40 × 10 mm I.D.; flow-rate, 1 ml/min; buffers and gradient as in Fig. 1a; monitor, UV1 (Pharmacia).

Fig. 5. Fractionation of (1) conalbumin, (2) ovalbumin and (3) human serum albumin (0.5 mg each) on LiChrospher TMAE (5 μm) (tentacle type). Column size, 40 × 10 mm I.D.; flow-rate, 1 ml/min; buffers and gradient as in Fig. 3a; monitor, UV1 (Pharmacia).



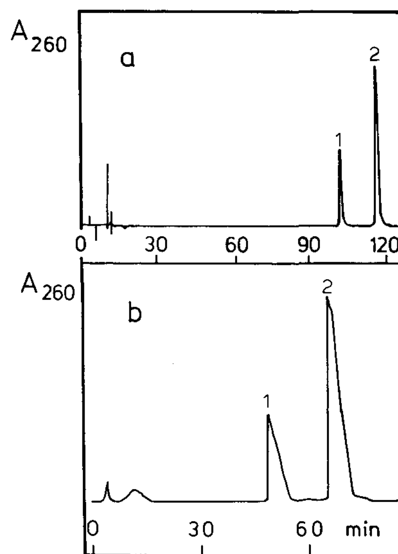


Fig. 6. Separation of DNA restriction fragments on LiChrospher 4000 DMAE (5 μm) (tentacle type). Column size, 40 × 10 mm I.D.; sample, pBR 322 × HpaII (50 μg) containing fragment lengths of 2 × 9, 15, 2 × 26, 2 × 34, 67, 76, 90, 110, 122, 2 × 147, 2 × 160, 180, 190, 201, 217, 238, 242, 309, 404, 527 and 622 base pairs; flow-rate, 1 ml/min; temperature, 23°C. Solvent A = 20 m*M* Tris–HCl + 0.6 *M* NaCl (pH 6.8); solvent B = 20 m*M* Tris–HCl + 1.1 *M* NaCl (pH 6.8); gradient, 400 min, 0–100% B. Monitor, L4200 (Merck–Hitachi).

Fig. 7. Separation of larger DNA fragments on LiChrospher 4000 DMAE (5 μm) (tentacle type). Fragment sizes, (1) 2.99 and (2) 7.8 kb. Solvent: A = 20 m*M* Tris–HCl + 0.4 *M* NaCl (pH 6.8); solvent B = 20 m*M* Tris–HCl + 1.1 *M* NaCl (pH 6.8). Column size, 40 × 10 mm I.D.; flow-rate, 1 ml/min. (a) Analytical run (50-μg sample). Gradient, 0–10 min, 0–57% B; 10–300 min, 57–100% B. (b) Preparative run (450 μg). Gradient, 0–10 min, 0–64% B; 10–250 min, 64–100% B. Monitor, L4200 (Merck–Hitachi).

The most convincing effects were observed, however, when DNA restriction digests were fractionated on a tentacle-type DMAE-LiChrospher (pore size 400 nm, particle size 5 μm). The unexpected degree of resolution shown in Figs. 6 and 7 is not restricted to microgram amounts but also holds for milligram amounts.

CONCLUSIONS

The most interesting properties observed for the tentacle-type ion exchangers are the increased mass transfer rates and the marked changes in selectivity in comparison with the conventional types. The first effect is easy to understand as a result of the reduced non-specific interaction between the analyte and the support matrix, thus providing "cleaner" dissociation kinetics.

The change in selectivity is in perfect agreement with the ion-exchange mechanism for proteins proposed by Kopaciewicz *et al.*[7], according to which an analyte will interact with a conventional ion-exchange matrix only with a certain, distinct area of its surface. In tentacle-type exchangers the flexibility of the charge arrangement allows additional or other electrostatic interactions that should account for the changed selectivity. In this respect, it seems justified to conclude that the action of the tentacle-type ion exchanger involves a new separation parameter, the overall steric distribution of the charges on the analyte.

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High-performance affinity chromatography of basic fibroblast growth factor on polystyrene sulphonate resins modified with serine

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ABSTRACT

Basic fibroblast growth factor (bFGF) is a heparin-binding growth factor, so it was usually purified by affinity chromatography on a heparin-grafted support. It was previously observed that, among several modified polystyrene that exhibit anticoagulant heparin-like properties, insoluble polystyrenes modified by chlorosulphonation substituted with serine (PSSer) develop specific interactions with bFGF in solution. These PSSer resins were used as stationary phases in high-performance affinity chromatography in order to separate the radiolabelled growth factor from a bovine brain crude extract. The growth factor is strongly bound to the solid phase, as demonstrated by the adsorption isotherms. It can be adsorbed on the resin at low ionic strength and be desorbed by raising the salt concentration in the eluent. The effects of flow-rate, of the initial buffer and of the slope of the salt gradient on the adsorption and on the desorption of bFGF were investigated in order to study the thermodynamic and the kinetic parameters of the interactions and to define the optimum conditions for a fast and efficient separation of the growth factor.

INTRODUCTION

Biospecific polymers can be obtained by a chemical modification of synthetic polymers. Hence, when chlorosulphonated polystyrenes are substituted by amino acids, in particular by serine, they develop specific interactions with blood proteins[1,2]. We have previously demonstrated that this type of polymer is able to interact specifically with heparin-binding growth factors (HBGF)[3]. These compounds were

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also named fibroblast growth factors (FGFs), after the early description of their potent mitogenic activity for fibroblast[4]. Two classes of fibroblast growth factors have been described according to their isoelectric point. These closely related polypeptides, acidic and basic FGFs, have about 50% absolute homology and differ in their affinity for heparin–Sepharose[5]. The biological properties of FGFs as angiogenic[6] and wound-healing factors[7] have led to the development of fast and efficient purification methods.

We have previously shown that polystyrene substituted with serine (PSSer) exhibit a strong affinity for the basic form (bFGF). This functionalized polymer was used as a stationary phase in low-pressure affinity chromatography in order to separate and purify this growth factor[3]. Because of its mechanical properties, this support can be used in high-performance affinity chromatography (HPAC)[8,9].

Similar functional supports have also been used in the high-performance liquid affinity chromatography of serine proteases. The specific interactions presented by the active supports can be used in order to separate or purify enzymes. Moreover, HPAC was a good method for studing the interactions between the polymer and the protein in solution and to reveal the importance of kinetic parameters with respect to chromatography[10].

In this work, we determined the affinity constants of purified bFGF for PSSer using adsorption isotherms. The radiolabelled growth factor can also be eluted on the functional support, in HPAC experiments, in order to study the influence of elution parameters on the chromatographic performance of supports.

EXPERIMENTAL

Growth factors bovine brain crude extract and bFGF radiolabelled with iodine-125, were purified and prepared as described previously[3]. Polystyrene substituted with serine was prepared in two successive steps. First, insoluble copolymers styrene–divinylbenzene (2%) 200-400 mesh beads (Bio-Rad Labs., Richmond, CA, U.S.A.) are treated by chlorosulphonic acid[1]. Second, the chlorosulphonated beads are substitued with serine, as described previously[3]. Sulphonate and amino acid sulphamide substitution is determined by potentiometric titration and by elemental analysis. These groups are statistically distributed along the polymer chain. The general structure of the functional polymer is shown in Fig. 1.

Adsorption isotherms

Various amounts of bovine brain crude extract containing 1.2 μg/ml of bFGF were mixed with approximately 1 ng of radiolabelled bFGF with a specific activity of

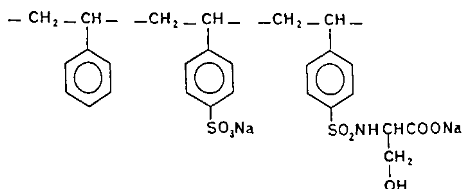

Fig. 1. Structure of functional polymer PSSer. 66% units substituted with sulphonate groups and 14% units by serine sulphamide functions.

70 000 cpm in phosphate-buffered saline (PBS)–0.4 *M* NaCl. The mixture was incubated for 1 h with 4.85 mg of PSSer resin at room temperature in a final volume of 2 ml. After incubation, the supernatant was collected and the residual radioactivity was determined in order to measure the proportion of unbounded growth factor as a function of the initial concentration of bFGF. The affinity constant was obtained using a linearization method as shown in Fig. 2[11].

High-performance affinity chromatography

The modified polystyrene beads were suspended in a 0.15 *M* NaCl–0.01 *M* PBS (pH 7.4) buffer solution and allowed to decant for 3 h. The supernatant containing fine particles was eliminated. This procedure was repeated until the supernatant was free of suspended particles, then 2 g of PSSer resin were packed in a high-performance liquid chromatographic column (5 × 0.7 cm I.D.) using a slurry method. The chromatographic system was described previously[9]. Bovine brain crude extract (1 ml) with radiolabelled bFGF was injected at room temperature using different elution rates. The adsorbed growth factor was desorbed by changing the ionic strength of the eluent. The total protein content and the amount of radiolabelled bFGF of the eluted fractions were determined. After the elution the support was washed with a 2.5 *M* NaCl–0.01 *M* PBS solution and re-equilibrated in the initial buffer.

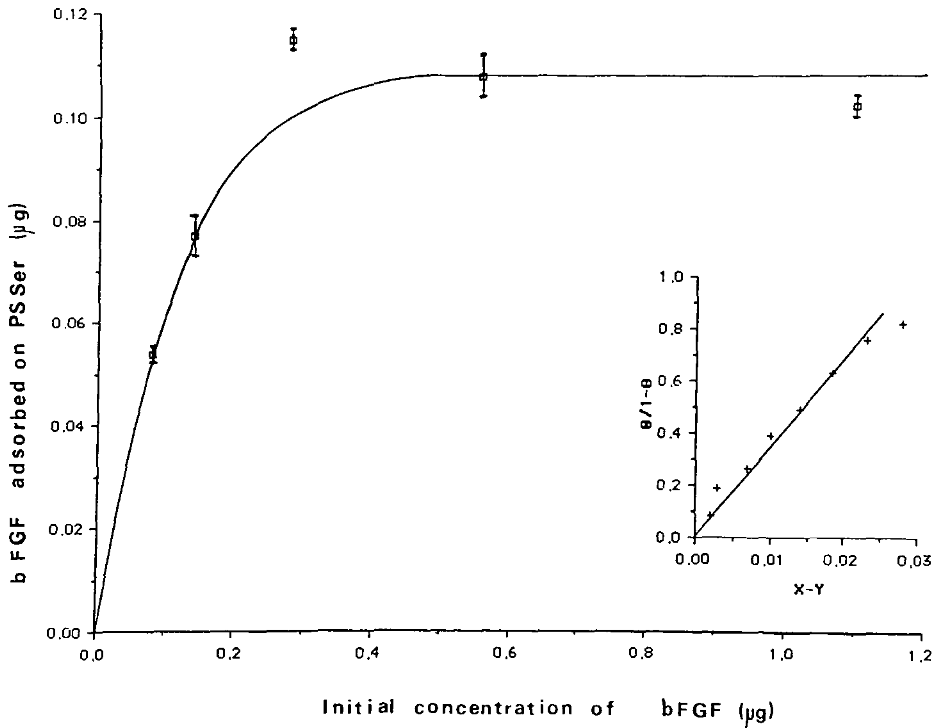

Fig. 2. Adsorption isotherm of bFGF on 4.85 mg of PSSer in 0.01 *M* phosphate buffer–0.4 *M* NaCl (pH 7.4).

RESULTS AND DISCUSSION

The composition of the resin, expressed as the percentage of monomeric units bearing the different substituted groups, is presented in Fig. 1. It was found that the polymer can develop different types of interactions with the proteins in solution[10]. It is obvious that the affinity constant relating to the adsorption isotherms is the sum of all these interactions. In fact, the hydrophobic character of the starting polymer is strongly reduced by the chlorosulphonation. However, the substantial percentage of sulphonate groups in the polymer structure confers a cation-exchange capacity to the resin. The adsorption capacity of the resin for the bFGF determined from the saturation level of the isotherm (Fig. 2) is 0.02 μg per mg of dry support. This adsorption corresponds to 2.5 ng/cm^2; the specific surface area determined by adsorption of radiolabelled serum albumin is 8.1 cm^2/mg[11]. The affinity constant of the growth factor for the functional polymer, determined from the adsorption isotherm, is 1.2×10^9 l/mol[11]. This thermodynamic function indicates the strong affinity of this substituted polystyrene for bFGF. Moreover, this affinity constant was determined under experimental conditions where bFGF competes with other proteins in solution.

The polystyrene resins functionalized by substitution with sulphonate and serine sulphamide residues (PSSer) are able to adsorb basic fibroblast growth factor at low ionic strength (0.4 *M* NaCl) under the HPAC elution conditions. Typical HPAC of bFGF on PSSer is shown in Fig. 3. The adsorbed growth factor can be desorbed using a linear salt gradient from 0.4 to 2 *M* with a maximum at 1.4 *M* NaCl. We can assume that the salt concentration required for the desorption of the bound growth factor reflects the strength of the interactions between bFGF and the solid surface. When the concentration of NaCl in the initial buffer is 0.5 *M*, the recovery of the bFGF in the separation (Table I) is similar to those obtained previously in the low-pressure chromatographic experiments with the same resin with a elution flow-rate of 0.5 ml/min[3]. Under these elution conditions, the pressure is *ca.* 80 bar.

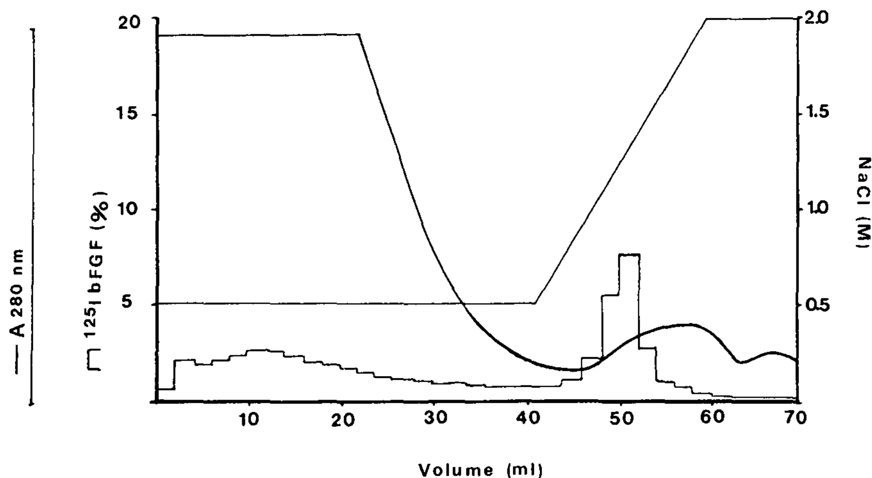

Fig. 3. Elution of radiolabelled bFGF mixed with 1 ml of bovine brain crude extract on 2 ml of PSSer support. Column, 5 × 0.7 cm I.D.; flow-rate, 0.5 ml/min; initial buffer, 0.01 *M* PBS–0.5 *M* NaCl (pH 7.4).

TABLE I

PERCENTAGE OF RADIOLABELLED bFGF RETAINED AND NON-RETAINED ON PSSer SUPPORT WITH DIFFERENT DESORPTION CONDITIONS.

Flow-rate (cm/min)	*Initial ionic strength (M NaCl)*[a]	*bFGF eluted at 0.4 M (%)*	*bFGF eluted by gradient (%)*	*Ionic strength of eluted peak (M)*
0.50	0.5	25	30	1.4
1.25	0.5	30	19	1.4
2.50	0.5	40	08	1.4
1.25	0.4	02	44	1.4

[a] Ionic strength of eluting buffer (0.01 M, PBS pH 7.4) in NaCl molarity.

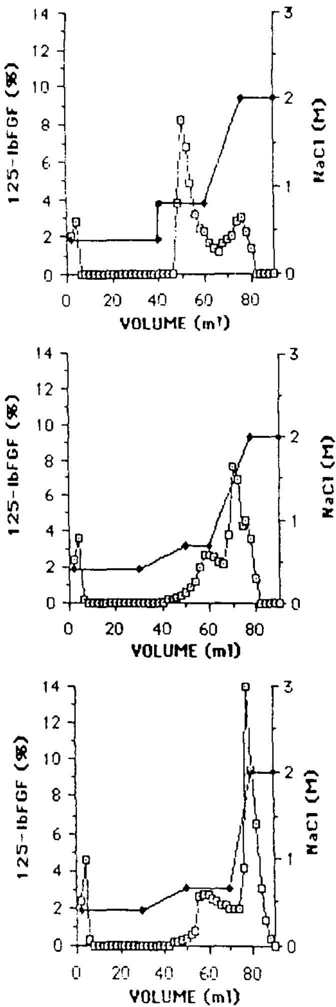

Fig. 4. Elution of radiolabelled bFGF mixed with 1 ml of bovine brain crude extract on 2 ml of PSSer support with different desorption conditions. Column, 5 × 0.7 cm I.D.; flow-rate, 0.5 ml/min; initial buffer, 0.05 M PBS–0.5 M NaCl (pH 7.4).

TABLE II

EFFECT OF INJECTED HEPARIN ON THE ADSORPTION OF RADIOLABELLED bFGF ON PSSer RESINS

Flow-rate, 0.5 ml/min (1.25 cm/min); initial buffer, PBS (pH 7.4)–0.4 *M* NaCl.

Amount of heparin injected (mg)	*bFGF eluted at 0.4 M NaCl (%)*	*bFGF desorbed at 2 M NaCl (%)*
0	6	56
0.05	8	50
4.4	71	25

The importance of the effect of elution rate on the chromatographic performance of the support is illustrated in Table I. With a low elution rate (0.2 ml/min), the amount of growth factor retained on the resin increases. This a clear indication that the heterogeneous reaction of complex formation is kinetically limited.

The ionic strength of the initial buffer is also an important parameter (Table I). When the salt concentration decreases, the percentage of bFGF retained on the solid phase increases considerably. This effect relects the importance of ionic interactions in the mechanism of adsorption. However, the complex formation can also be mediated by other components present in the injected sample.

The importance of desorption conditions on the recovery of the growth factor is demonstrated in Fig. 4. When bFGF is adsorbed on the stationary phase, it requires a considerable change in the ionic strength of the eluent for its desorption. If this change is limited, an substantial amount of growth factor remains absorbed. The salt concentration has to reach 2 *M* NaCl for the desorption and even under these conditions a percentage of growth factor remains adsorbed on the solid phase and can only be desorbed by a competitive elution.

Finally, if the sample is incubated with a small amount of heparin (50 μg), bFGF is normally adsorbed and desorbed from the active polymer (Table II). This seems to indicate that the binding site of the growth factor is not involved in the interaction with the functional polymer. However, a competive effect is observed when a large amount of heparin is injected. In fact, under these conditions, the adsorbed bFGF is completely washed out from the stationary phase. This effect probably reflects a dramatic change in the ionic interactions due to the anionic character of heparin and demonstrates the role of electrostatic interactions in the affinity of bFGF for the functional polymer.

CONCLUSION

Polystyrene resins substituted with sulphonate and serine sulphamide groups exhibit a specific and strong affinity for basic FGF in solution. The growth factor can be adsorbed on the solid phase at low ionic strength and desorbed by increasing the salt concentration as observed by HPAC. The influence of the elution rate demonstrates that the complex formation reaction between bFGF and the resin is kinetically limited. When the complex is formed, desorption can occur on increasing the ionic strength of

the eluent but a fraction of the growth factor remains adsorbed on the solid phase and can only be desorbed by a competitive elution. This adsorption is the result of different types of interactions and the importance of ionic interactions is revealed by the effect of a large amount of heparin on the chromatographic separation.

This functional polymer leads to a rapid and efficient separation of basic fibroblast growth factor and, because of the mechanical properties of the support, this separation process can easily be scaled up.

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CHROM. 22 408

Rapid protein purification using phenylbutylamine–Eupergit: a novel method for large-scale procedures

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ABSTRACT

Electrophoretic desorption was used to compare the protein binding capacities of some hydrophobic adsorbents [the phenylbutylamine (PBA) derivatives of Eupergit C and agarose and Phenyl-Sepharose] for low-pressure chromatography. The highest capacity was observed for the bifunctional adsorbent PBA–Eupergit. The hydrophobically adsorbed proteins can be selectively desorbed by decreasing the pH of the eluent due to electrostatic repulsion between positive charges on the adsorbed proteins and positively charged secondary amines on the adsorbent. This was used to purify 1500 U penicillin amidase from *E.coli* homogenates per gram wet weight of PBA–Eupergit in 50 adsorption–desorption cycles without organic solvents (>90% yield, purification factor = 5.3).

INTRODUCTION

Hydrophobic adsorbents can be used for the selective adsorption of extracellular proteins from fermentation media or intracelular proteins from cell homogenates[1–4]. The adsorbed proteins can then be selectively desorbed by either decreasing the ionic strength or increasing the content of hydrophobic organic solvents in the buffer. In the former instance complete desorption of very hydrophobic proteins is difficult, and in the latter the rate of protein denaturation is generally increased. The hydrophobic solvents must therefore be removed from the protein in subsequent purification steps and recycled.

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Bifunctional adsorbents where the adsorption is governed by hydrophobic interactions and the desorption is caused by electrostatic repulsion between the support and the protein offer an alternative[1,5]. Such adsorbents can be formed when a secondary amine is produced during the covalent immobilization of the hydrophobic ligate to the support. With decreasing pH the density of positively charged groups on the support increases. The same applies for proteins. Hence hydrophobically adsorbed proteins could be desorbed by increasing the electrostatic repulsion simply by changing the pH of the aqueous eluent below the isoelectric point. When this repulsion is stronger than the hydrophobic adsorption, the use of organic solvents or denaturing agents[5] can be avoided in the desorption step.

The aim of this study was to investigate whether such bifunctional adsorbents can be formed by covalent immobilization of phenylbutylamine to Eupergit C and other supports. Their application to the preparative isolation of penicillin amidase from *E. coli* homogenates, without organic solvents, was also investigated.

EXPERIMENTAL

Materials

α-Chymotrypsin (CT, EC 3.4.21.1; Worthington CDI, Freehold, NJ, U.S.A.), trypsin (TRY, EC 3.4.21.4; Merck 8350, Darmstadt, F.R.G.), N-acetyl-L-tyrosine ethyl ester (ATEE; Sigma, St. Louis, MO, U.S.A.), phenylbutylamine (PBA; Aldrich, Milwaukee, WI, U.S.A.), N-benzoyl-L-arginine ethyl ester (BAEE; Merck), 2-nitro-5-(phenylacetamido)benzoic acid (NIPAB; Sigma), penicillin G (Welding, Hamburg, F.R.G.), Phenyl-Sepharose (Pharmacia), phenylbutylamine–agarose (Sigma) and Eupergit C (Röhm, Darmstadt, F.R.G.) were used as purchased. Alkaline protease was provided by Röhm. Penicillin amidase (PA) from *E. coli* (EC 3.5.1.11) was purified from crude enzyme preparations as described[6]. All other chemicals were of analytical-reagent grade.

Preparation of phenylbutylamine–Eupergit C

Eupergit C was added to a 1 *M* solution of PBA in ethanol and boiled for 4 h. The support was washed with ethanol and water until the UV spectrum of the eluate was negligible, then boiled for 30 min in 0.5 *M* sulphuric acid and washed with water. The amount of PBA immobilized to the support was calculated from the mass balance determined from the UV spectra and volumes of the stock and washing solutions used. PBA–Eupergit was stored at 4°C in water.

Enzyme activities

The CT, TRY and PA activities were determined using ATEE, BAEE and NIPAB as substrates as described[6,7]. The PA activity was also determined using 2% (w/v) penicillin G as a substrate [phosphate buffer (pH 7.8), 37°C]; 1 U is the amount of enzyme that hydrolyses 1 μmol/min of substrate. The reaction was monitored by high-performance liquid chromatography or a pH-stat[6].

Charge density of PBA–Eupergit as a function of pH

A known amount of support was suspended in 0.1 *M* potassium chloride solution and the pH adjusted to 10.5. The suspension was titrated with hydrochloric acid to pH 2.0. A blank without support was also titrated.

Binding curves

Solutions with different enzyme contents were added to the same amount of support equilibrated with the same buffer as used to prepare the enzyme solutions. The suspensions were incubated using a rotary mixer at 25°C for 60 min. Then the free enzyme concentration, C, was determined in the filtrate. From the mass balance, the concentration of adsorbed enzyme, X, was determined.

Comparison of the protein binding capacities of different supports with isoelectric focusing

To 50 μl (wet volume) of different supports equilibrated with pH 7.5 buffer (phosphate, $I = 0.2\ M$), 1 ml of a crude enzyme solution was added. The suspension was incubated for 60 min using a rotary mixer and filtered. The support and 20 μl of the supernatant were then placed near the anode of an isoelectric focusing plate (LKB, pH 3.5–9.5) and analysed as described[7].

Preparative purification of penicillin amidase from E. coli homogenates

A sample of an *E. coli* homogenate (17 ml with 19 U/ml penicillin amidase) was applied to a column (volume 8 ml, diameter 1 cm, flow-rate 1 ml/min) packed with PBA–Eupergit, equilibrated with buffer A (0.05 *M* potassium phosphate–1 *M* sodium chloride, pH 7.5). After elution with 30 ml of buffer A, desorption with 35 ml of buffer B (0.1 *M* sodium formate, pH 3.8) was performed. After washing with 16 ml of buffer A, 40 ml of regeneration buffer (1% bacterial alkaline protease in 0.02 *M* glycine–sodium hydroxide, pH 10) and finally 60 ml of buffer A were pumped through the column before a new sample was applied. The protein contents in the sample and the eluates were determined as described[8].

RESULTS AND DISCUSSION

Protein binding capacities of different hydrophobic support with immobilized phenyl groups

The results are shown in Fig. 1. From the ratio of the intensity of the protein bands desorbed from the gel and the corresponding supernatant, the differences in the protein binding capacities of the adsorbents can be determined. The protein binding capacity was found to increase in the order Phenyl-Sepharose < PBA–agarose < PBA–Eupergit. The last adsorbent was therefore used in the subsequent experiments.

Charge density of PBA–Eupergit as function of pH

This was determined from the titration curve for a known amount of PBA–Eupergit corrected for the amount of titrant used to titrate the blank. The results are given in Fig. 2. It follows that the density of positive charges on the support increases with decreasing pH. The apparent pK values of the secondary amine groups that are protonated lie in the range 6–7. This value is lower than expected for free secondary amines owing to charge–charge interactions on the support[9].

Binding curves

Scatchard plots for the binding of CT, TRY and PA to PBA–Eupergit are given in Fig. 3. The non-linear binding curves show that the adsorbent sites differ with

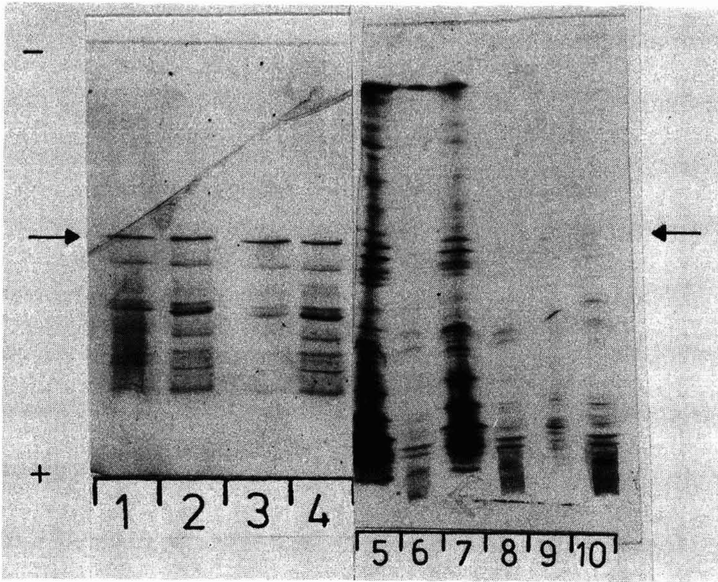

Fig. 1. Protein binding capacities of different hydrophobic adsorbents from a crude PA solution (lanes 1–4) and an *E. coli* homogenate (lanes 5–10). To 50 μl of the adsorbent (wet volume), 1 ml of crude enzyme or homogenate in phosphate buffer (pH 7.0, $I = 0.2$ *M*) was added. After equilibration for 60 min at 25°C, the adsorbent and 20 μl of the supernatant were placed near the anode (bottom) of an isoelectric focusing plate and separated. PBA–agarose (lanes 1 and 9, adsorbent; lanes 2 and 10, supernatant); Phenyl-Sepharose (lane 3, adsorbent; lane 4, supernatant); PBA–Eupergit (lanes 5 and 7, adsorbent; lanes 6 and 8, supernatant). Different preparations of this adsorbent were used in the two experiments. Arrows indicate the location of the main band with active PA (IP = isoelectric point = 7.0[6]).

respect to the dissociation constants, as is usually observed for affinity supports[10]. The average dissociation constants in buffer without added salt are 1, 4 and 5 μM for PA, TRY and CT, respectively. The binding capacity was found to increase in the presence of 1 *M* sodium chloride. For proteins of similar size, CT and TRY, the capacity decreases with increasing isoelectric point. This indicates that hydrophobic interac-

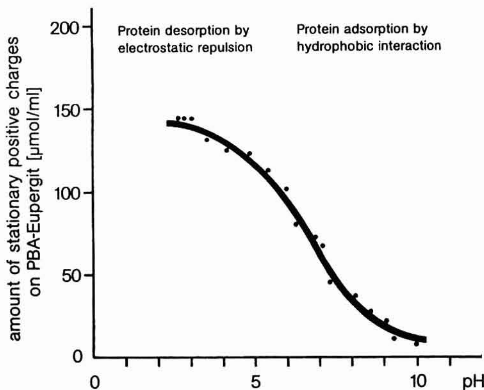

Fig. 2. pH dependence of the concentration of positive charges in PBA–Eupergit.

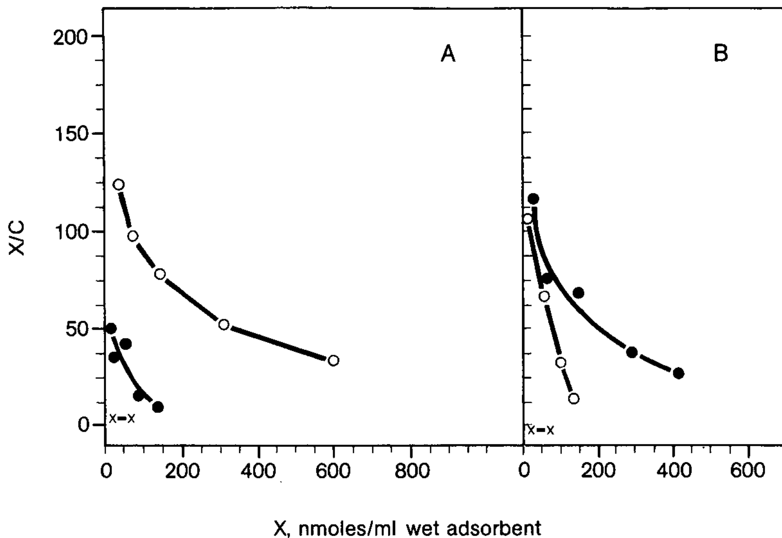

Fig. 3. Scatchard plots for the binding of different proteins to PBA–Eupergit at pH 7.5 (potassium phosphate–sodium phosphate buffer, I = 0.2 M) and 25°C. (A) CT, (×) with and (○) without 50% ethylene glycol; (●) TRY. (B) (○) PA from *E. coli*, (●) with 1 M NaCl and (×) with 50% ethylene glycol.

tions cause the adsorption, and that charge–charge repulsions on the support limit the adsorption capacity. From Fig. 2 it follows that these repulsive interactions should increase with decreasing pH. Hence it should be possible to desorb the adsorbed proteins simply by reducing the pH in the elution buffer. The negligible adsorption in the presence of ethylene glycol also demonstrates that hydrophobic interactions are responsible for the binding. Proteins adsorbed in the absence of this organic solvent could not be desorbed by the buffer with 50% ethylene glycol, with which no adsorption was observed. This indicates that an adsorbed protein is bound to the support by multiple-point interactions[3,11].

Preparative isolation of penicillin amidase from E. coli homogenates

The results for one adsorption–desorption–regeneration cycle are given in Fig. 4. The high salt content in buffer A was used to minimize the adsorption of negatively charged nuleic acids to the positively charged support. Their adsorption reduces the protein binding capacity of the column. The first peak in Fig. 4 has an absorption maximum at 260 nm, as expected for nucleic acids. The second peak with PA activity has an absorption maximum at 280 nm, indicating the selective adsorption of protein to the bifunctional support. The overall recovery of PA was found to be *ca.* 90% over 50 consecutive cycles. Hence at least 1500 U of PA per gram of PBA–Eupergit (wet weight) could be prepared by this method. The specific activity of the enzyme was increased from 2.0 U/mg protein in the sample to 10.6 U/mg protein in the eluate. The enzyme thus prepared can be used for immobilization without further purification in order to obtain a catalyst suitable for the hydrolysis of penicillin G or cephalosporin-G to prepare 6-aminopenicillanic acid and 7-aminodeacetoxycephalosporinic acid, respectively.

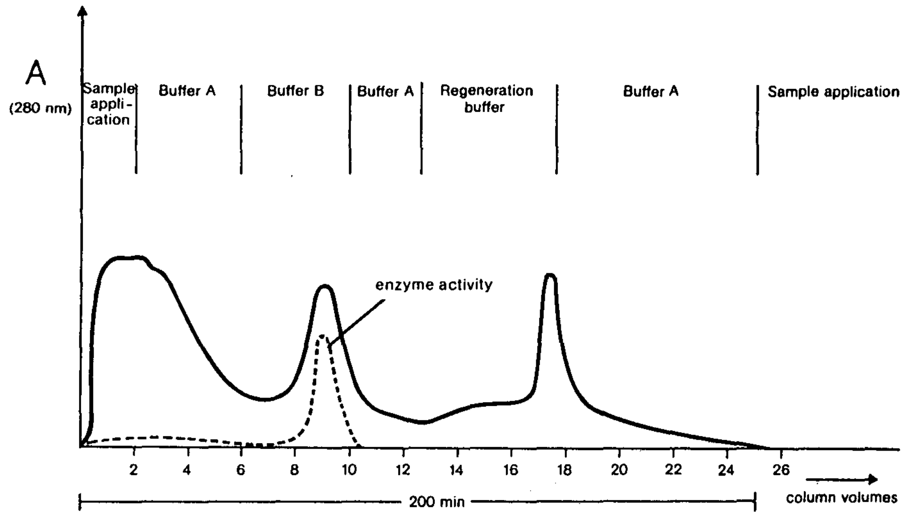

Fig. 4. One adsorption–desorption–regeneration cycle for the purification of penicillin amidase from an *E. coli* homogenate with a PBA–Eupergit column (8 ml, diameter = 1 cm) at a flow-rate of 1 ml/min at room temperature.

The results in Figs. 2–4 show that PBA–Eupergit is a bifunctional adsorbent that can be used for the isolation of enzymes on a preparative scale where the adsorbed proteins can be desorbed without the use of organic solvents.

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Screening method for large numbers of dye-adsorbents for enzyme purification

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ABSTRACT

A method is described by means of which 96 different dye-adsorbents can be tested simultaneously for their ability to bind enzymes and to test their biospecific elution. Small amounts of cell-free extract are applied to dye-adsorbents which are packed in a 96-well transplate cartridge. After biospecific elution, the amount of the eluted enzyme is tested in a microtitre plate assay. The method is illustrated by the purification of glycerol dehydrogenase (E.C. 1.1.1.72), 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44) and glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) from the hyphal fungus *Aspergillus nidulans*.

INTRODUCTION

Dye-ligand chromatography has been proved to be a very useful technique in small- and large-scale purification procedures for enzymes and proteins[1]. The reactive dye Cibacron Blue F3G-A has been used especially in the purification of dehydrogenases and kinases[2]. It was shown to bind to the nucleotide-binding domain of liver alcohol dehydrogenase, partly similar to the binding of NAD^{+}[3,4]. It is now accepted, however, that many other dyes can interact with proteins and other biomolecules, and are therefore of potential use in affinity chromatography[5]. Good purification factors and high yields can be achieved if a dye-adsorbent can be found that binds the desired protein selectively and in such a way that it can be eluted (bio)specifically.

Several strategies for the screening of dye-ligands have been proposed. Quadri and Dean[6] selected two dyes that inhibited free in solution the activity of the enzyme to be purified. These dyes were shown to be useful in the purification of 6-phosphogluconate dehydrogenase from *Bacillus stearothermophilus*. Hey and Dean[7] examined large numbers of dye-adsorbents to select useful dyes for the purification of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*. They proposed a two-step purification using first a "negative" column that binds much protein but not the protein of interest, followed by a "positive" column that binds the wanted protein but not many other proteins. Scopes[8] divided reactive dyes in into five groups accord-

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ing to their protein-binding capacities. He also studied factors that can affect protein binding. Guidelines were derived by which systematic screening of dye-adsorbents became possible. Kroviarski *et al.*[9] constructed an automated set-up by means of which eight mini-columns, containing 1.6 ml of dye-adsorbent, can be screened for their ability to retain certain enzymes.

We present here a screening method by means of which 96 different dye-adsorbents can be tested simultaneously within several hours for their ability to bind enzymes in such a way that they can be eluted biospecifically. The method is illustrated by screening which dyes are suitable in the purfication of three enzymes from the hyphal fungus *Aspergillus nidulans:* glycerol dehydrogenase (GLYDH), 6-phosphogluconate dehydrogenase (6PGD) and glucose-6-phosphate dehydrogenase (G6PD).

EXPERIMENTAL

Materials

Remazol dyes were gifts from Hoechst (Frankfurt, F.R.G.), Cibacron dyes from Ciba-Geigy (Basle, Switzerland), Levafix dyes from Bayer (Leverkusen, F.R.G.), Basilin dyes from BASF (Ludwigshafen, F.R.G.) and Procion dyes from ICI (Manchester, U.K.). Sepharose 4B-Cl, fast protein liquid chromatographic (FPLC) equipment and a MonoQ column were supplied by Pharmacia (Uppsala, Sweden). Phenazine methosulphate (PMS) and nitro blue tetrazolium chloride (NBT) were obtained from Serva (Heidelberg, F.R.G.). The transplate cartridge was produced by Costar (Cambridge, MA, U.S.A.). Bovine serum albumin (BSA), glucose-6-phosphate, 6-phosphogluconate, $NADP^+$ and Tris were from Boehringer (Mannheim, F.R.G.). All other reagents were of analytical-reagent grade from Merck (Darmstadt, F.R.G.).

Immobilization of dyes

Sepharose 4B-Cl agarose beads were washed extensively with deionized water on a sintered-glass funnel under suction. For every dye 2 g of moist agarose beads were resuspended in 6.5 ml of deionized water, to which were added 10–40 mg of a reactive dye and 1.3 ml of 3 *M* sodium chloride solution. These suspensions were stirred at room temperature for 1 h, then 1 *M* sodium carbonate solution was added to raise the pH to 10.5. After incubation for a further 1 h at 60°C, the gels were washed on a small glass funnel with deionized water, 4 *M* urea, deionized water, 2 *M* sodium chloride solution, deionized water and 0.05 *M* sodium phosphate solution (pH 7.0). The affinity adsorbents were stored in 0.05 *M* sodium phosphate solution (pH 7.0) containing 0.02% (w/v) of sodium azide.

Chromatographic procedure using a transplate cartridge

From a transplate cartridge, which is normally used in hybridoma technologies for filling 96-well plates, the plastic membrane was removed. In each of the 96 wells glass-wool was placed, on which 150 μl (bed volume) of affinity adsorbent were loaded. A different dye-adsorbent was placed in each well. The position of each dye was well documented. In this way a holder containing 96 "mini-columns" was created.

Before use, the mini-colums were equilibrated with a 10-fold volume of extrac-

tion buffer, after first having been rinsed with deionized water, 4 *M* urea, deionized water, 2 *M* sodium chloride solution and deionized water. Using a multi-channel pipette, 100 μl of cell-free extract were deposited on each of the mini-columns. After 10 min, non-adsorbed protein was washed away with a 10-fold volume of extraction buffer. The transplate cartridge was then placed above a 96-well microtitre plate. Proteins were eluted by pipetting into each well 250 μl of elution buffer, *viz.*, extraction buffer containing 1 m*M* $NADP^+$, in portions of 50 μl at 2-min intervals. The eluted fractions (one per well) were collected in a microtitre plate, which had previously been incubated for several hours with 1% (w/v) BSA in extraction buffer to block protein-binding sites. After washing it extensively with deionized water and cooling it to 4°C, the plate was ready for use. The microtitre plate containing the fractions was kept on ice. All operations were performed at 4°C.

After use, the transplate cartridge was washed with deionized water, 4 *M* urea, deionized water, 2 *M* sodium chloride solution and deionized water and then stored in 0.05 *M* sodium phosphate solution (pH 7.0) containing 0.02% (w/v) sodium azide at 4°C.

Colorimetric microtitre plate assays

G6PD and 6PGD were assayed in a microtitre plate assay essentially as described by Cairns[10]. In the case of G6PD, samples of 25 μl of the eluted fractions were added to 225 μl of 50 m*M* Tris–HCl (pH 8.0), 5 m*M* magnesium chloride, 5 m*M* glucose-6-phosphate, 0.4 m*M* $NADP^+$, 0.25 m*M* PMS and 0.2 m*M* NBT. 6PGD was assayed by adding 25 μl of the eluted fractions to 225 μl of 50 m*M* glycylglycine (pH 8.0), 2 m*M* 6-phosphogluconate, 0.2 m*M* $NADP^+$, 0.25 m*M* PMS and 0.2 m*M* NBT. Assays were performed at 25°C.

Spectrophotometric microtitre plate assay

GLYDH was assayed by adding 25 μl of the eluted fractions to 225 μl of 50 m*M* glycine–sodium hydroxide (pH 9.6), 50 m*M* glycerol and 0.2 m*M* $NADP^+$. The change in the absorbance at 340 nm was measured in a UVmax microtitre plate reader (Molecular Devices, Palo Alto, CA, U.S.A.). The results of the assay computations were printed using a Hewlett-Packard printer. Assays were performed at 25°C.

Enzyme assays

The activities of enzymes were measured spectrophotometrically with an Aminco (Silver Spring, MD, U.S.A.) DW-2 UV–VIS spectrophometer using the double-beam mode at 340 nm *versus* 380 nm.

G6PD was assayed in a reaction mixture containing 50 m*M* Tris–HCl (pH 8.0), 5 m*M* glucose-6-phosphate, 5 m*M* magnesium chloride and 0.4 m*M* $NADP^+$. 6PGD was assayed in 50 m*M* glycylglycine (pH 8.0), 2 m*M* 6-phosphogluconate and 0.2 m*M* $NADP^+$. GLYDH was assayed in 50 m*M* glycine–sodium hydroxide (pH 9.6), 50 m*M* glycerol and 0.2 m*M* $NADP^+$. Reactions were performed at 25°C.

Protein determination

Protein in cell-free extract and in the eluted fractions after affinity chromatography was determined by the micro-biuret method[11]. Protein in the fractions after MonoQ chromatography was determined by the bicinchoninic acid method accord-

ing to the instructions of the supplier (Pierce, Rockford, IL, U.S.A.). For both methods BSA was used as a standard.

Strains and growth conditions

A. nidulans wild type strain WG 096 (*yA2, pabaA1*) was used. For the preparation of conidiospores. *A. nidulans* was grown on complete medium[12] solidified with 1.2% agar using 25 m*M* sucrose as carbon source. Mycelium was grown by inoculating 10^6 ml^{-1} in minimal medium[12], supplemented with 2 $\mu g\ ml^{-1}$ *p*-aminobenzoate. For the purification of G6PD and 6PGD 100 m*M* glucose was used. The mycelium was grown for 18 h at 37°C in a New Brunswick Scientific (Edison, NJ, U.S.A.) orbital shaker (200 rpm) using 1-l flasks containing 300 ml of minimal medium. The mycelium was harvested by filtration, washed with cold saline and squeezed to remove excess of fluid. The mycelium was then frozen with liquid nitrogen and stored at −60°C.

Preparation of cell-free extract

Small volumes of cell-free extract were prepared by disrupting 1.0 g frozen mycelium using a Braun micro-dismembrator. The mycelial powder obtained was extracted with 2.0 ml of extraction buffer [20 m*M* Bis–Tris (pH 7.0)–1 m*M* $MgCl_2$–0.5 m*M* EDTA). Larger amounts of cell-free extract were prepared by grinding 20 g of frozen mycelium in a Waring blender with liquid nitrogen for 8 min. After evaporation of the liquid nitrogen, 50 ml of extraction buffer were added. Extraction was performed for 1 h at 4°C with gentle stirring of the suspension. This suspension was then centrifuged for 15 min at 20 000 *g*. The supernatant was used as the cell-free extract.

Electrophoresis

Electrophoresis in 10% polyacrylamide gel containing 0.1% SDS (SDS-PAGE) was performed according to Laemmli[13] in a minigel system (LKB, Bromma, Sweden). Carbonic anhydrase (mol.wt. 29 000), ovalbumin (45 000), BSA (68 000) and phosphorylase B (92 500) were used as protein standards.

Chromatographic procedures using 1- or 10-ml columns

Columns of 1 ml (5 cm × 0.50 cm I.D.) or 10 ml (6.5 cm × 1.4 cm I.D.) were packed with dye-absorbent and equilibrated with extraction buffer. One bed volume of cell-free extract was applied to each of the columns. After washing with three volumes of extraction buffer, extraction buffer containing 1 m*M* $NADP^+$ was used to elute. Fractions of 0.5 or 1 ml were collected.

Ion-exchange chromatography

Pooled fractions eluted from one of the dye-adsorbents were loaded onto a MonoQ ion-exchange column. After washing with extraction buffer until the absorbance at 280 nm had decreased to less than 0.01, a 20-ml linear sodium chloride gradient (0–0.4 *M*) was applied. The fraction collector was programmed to collect only peak fractions (1.0 ml).

RESULTS

Screening of dyes using a spectrophotometric microtitre plate assay

The screening procedure involves applying cell-free extract to the 96 mini-columns in the transplate cartridge, washing away unbound protein, followed by the elution of proteins, which are then collected in a microtitre plate. These fractions are used as samples in a spectrophotometric microtitre plate assay.

Cell-free extract (100 μl) of *A. nidulans* WG 096 was applied to each of the 96 mini-columns. After washing away unbound proteins, 250 μl of extraction buffer containing 1 m*M* $NADP^+$ were used for elution. In the eluted fractions GLYDH activity was measured, using a kinetic microtitre plate reader. Protein content was determined in the six most active fractions. Table I lists the data for these fractions, which show that there are several dye-adsorbents that are capable of binding GLYDH in such a way that it can be eluted biospecifically with $NADP^+$. Despite the fact that the yields and purification factors are low, the data suggest that Cibacron Brilliant Red 3B-A will give the best results in the purification of GLYDH.

TABLE I

BINDING AND ELUTION BEHAVIOUR OF GLYCEROL DEHYDROGENASE OF *A. nidulans* WITH RESPECT TO SIX DYE-ADSORBENTS

In the 96 eluted fractions GLYDH activity was measured using a kinetic microtitre plate reader (see Experimental for details). The yield is related to the amount of GLYDH applied to each of the mini-columns.

Well	*Dye*	*Specific activity (units/mg)*	*Yield (%)*	*Purification factor*
A5	Cibacron Brilliant Red 3B-A	0.408	44	8.5
D2	Basilin Red E-B	0.186	20	3.9
D12	Basilin Orange E-2R	0.209	23	4.4
E3	Procion Black 2-RPC	0.091	25	1.9
F3	Procion Red P-8B	0.096	28	2.0
H3	Levafix Blue E-3GLA	0.150	33	3.1

A 1-ml Cibacron Brilliant Red 3B-A column was used to test this dye-adsorbent. Cell-free extract (1 ml) of *A. nidulans* WG 096 was loaded on this column. After washing with 5 ml of extraction buffer, 1 m*M* $NADP^+$ was added. Fractions of 0.5 ml were collected. GLYDH appeared at the $NADP^+$ front as a sharp peak of activity. A 97-fold purification with 91% recovery was achieved (Table II). The active fractions were pooled and loaded on a MonoQ ion exchanger. A linear sodium chloride gradient (0–0.4 *M*) was used to elute GLYDH and the enzyme eluted at 80 m*M* sodium chloride. $NADP^+$ also binds to this column, but it is eluted at a higher sodium chloride concentration, so the GLYDH fraction will be free of $NADP^+$.

In this two-step purification GLYDH is purified to homogeneity as judged by SDS-PAGE (Fig. 1). The specific activity of 25.1 U mg^{-1} is in good agreement with another purification method for *A. nidulans* GLYDH[14].

TABLE II

PURIFICATION OF GLYCEROL DEHYDROGENASE, 6-PHOSPHOGLUCONATE DEHYDROGENASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE OF *A. nidulans* BY USING THREE TWO-STEP PURIFICATION METHODS

1 ml of cell-free extract (GLYDH) or 10 ml of cell-free extract (6PGD and G6PD) was loaded onto columns of 1 ml (GLYDH) or 10 ml (6PGD and G6PD) packed with immobilized Cibacron Red 3B-A (GLYDH), Procion Black 2 RPC (6PGD) or Levafix Navy P-RRL (G6PD). After washing, the columns were eluted with 1 m*M* $NADP^+$ in the extraction buffer. Pooled active fractions were applied to a MonoQ column and eluted with a linear sodium chloride gradient (see Experimental for details).

Step	*Volume (ml)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification factor*
GLYDH extract	1.0	0.23	0.048	100	1
Red 3B-A	1.5	0.21	4.67	91	97
Mono Q	1.4	0.20	25.1	87	623
6PGD extract	10.0	18.4	0.74	100	1
Black 2 RPC	4.0	15.6	48.9	85	66
Mono Q	1.0	14.4	115.0	78	156
G6PD extract	10.0	75.0	3.0	100	1
Navy P-RRL	5.0	40.5	270.0	54	90
Mono Q	2.8	37.5	357.8	50	119

Selection of dyes using a colorimetric microtitre plate assay

As kinetic microtitre plate readers are not widely available, we investigated the possibility of using less expensive means, *e.g.*, colorimetric assays. 6PGD and G6PD activity can be coupled to the reduction of the chomophore NBT[15]. As an intermediate electron donor PMS is used. Reduced NBT changes colour from yellow to blue, wich can be assessed by eye if the dehydrogenase activities are not too low.

A. nidulans cell-free extract (100 μl per well) was applied to the mini-columns. After washing with extraction buffer, 250 μl of extraction buffer containing 1 m*M* $NADP^+$ were used as the elution buffer. G6PD and 6PGD activity were determined in a microtitre plate assay by coupling the formation of NADPH to the reduction of NBT. In Fig. 2 a picture of the microtitre plate is shown in which the G6PD activity was determined. The G6PD microtitre plate assay gave a similar result (data not shown). We also measured the 6PGD activity and G6PD activity in a "normal" spectrophotometric assay of the ten most active fractions, ten non-active fractions and ten fractions with intermediate activities. The measured activities showed a good correlation with the colour development in the corresponding wells. The only clear exception was well D2, which showed as prominent a colour development in the 6PGD assay as in the G6PD assay, whereas the measured enzyme activities were about half as high as the activities in the most active fractions. The protein content in fraction D2 was 5–10 times higher than that content in other fractions. Cairns[10] has shown that colour development in a coupled NADP reduction to produce a formazan dye is enhanced by increased protein concentrations. This might explain why fraction

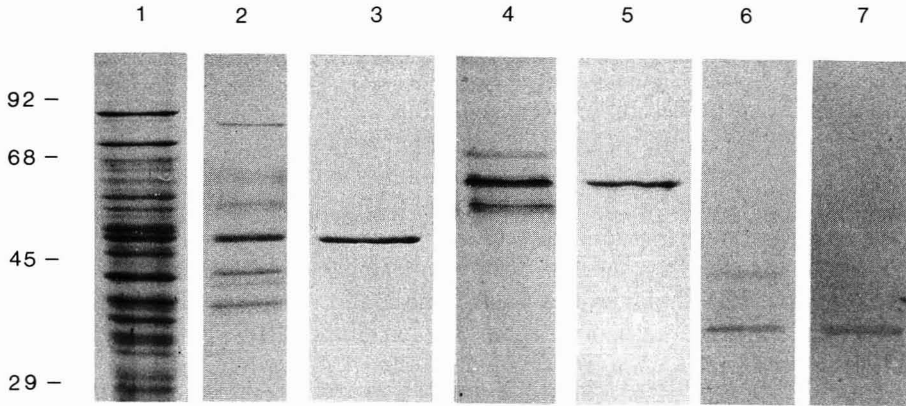

Fig. 1. SDS-PAGE of 6PGD, G6PD and GLYDH at different stages of purification. 1 = Cell free extract of *A. nidulans* 2 = 6PGD after chromatography on Procion Black 2 RPC; 3 = 6PGD after MonoQ chromatography using sample of lane 2; 4 = G6PD after chromatography on Levafix Navy p-RRL; 5 = G6PD after MonoQ chromatography using sample of lane 4; 6 = GLYDH after chromatography on Cibacron Red 3B-A; 7 = GLYDH after MonoQ chromatography using sample of lane 6. Positions and mass (in kilodalton) of molecular weight markers are indicated on the left.

D2 produced the strongest signal although it was not the fraction with the highest enzyme activity

Procion Black 2 RPC (well E3), Procion Rubine MX-B (well E9) and Procion Yellow P5-GN (well G6) were selected as potential dye-adsorbents for the purifica-

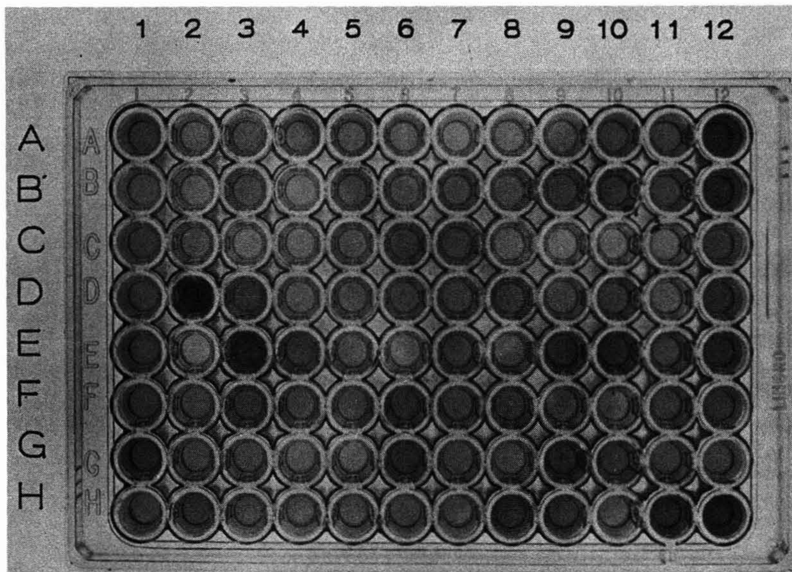

Fig. 2. Colorimetric microtitre plate assay of 6PGD. Collected fractions, eluted from the mini-columns in a transplate cartridge, were screened for 6PGD activity by coupling the reaction to the reduction of NBT, leading to darkening of the wells. The positions of the wells correspond to the positions of the dye-adsorbents in the transplate cartridge.

tion of 6PGD. These dyes were selected because in the corresponding wells the 6PGD activity was high (E3) or intermediate (E9 and G6), whereas the G6PD activity was low. The dye-adsorbents corresponding to wells A12, B12, E10, G9, H11 and H12 were rejected because they showed a clear colour development in the G6PD assay. These findings were confirmed by the measured enzyme activities.

The elution profiles of 6PGD from 10-ml columns of the three selected dye-adsorbents differ. From Procion Black 2 RPC 6PGD is eluted with 1 m*M* $NADP^+$ in a sharp peak (4 ml) with a yield of 85% (Table II). The use of Procion Rubine MX-B or Procion Yellow P5-GN resulted in peak broadening (6 and 10 ml) and lower yields (58% and 69%). These findings on a 10-ml scale confirm the indications obtained in the "mini-column scale" screening where Procion Black 2 RPC gave the strongest signal (apart from well D2) in the 6PGD microtitre plate assay.

The pooled fractions eluted from Procion Black 2 RPC were applied to a MonoQ column. Using a linear sodium chloride gradient, 6PGD was eluted at 230 m*M* sodium chloride. This fraction contained homogeneous 6PGD as judged by SDS-PAGE (Fig. 1). An overall purification factor of 156 and a yield of 78% were achieved in this two-step purification (Table II).

Levafix Navy P-RRL (well H8) was selected for study on a 10-ml scale. It was selected because an intense colour development was observed in well H8 (and also in wells H12 and D2) in the G6PD microtitre plate assay. In the 6PGD assay only a faint colour development was observed in well H8, whereas wells H12 (Cibacron Blue F3G-A) and D2 (Basilin Red E-B) showed an intense colour development. This indicated that Levafix Navy P-RRL would be more selective than Cibacron Blue F3G-A or Basilin Red E-B in the binding of G6PD.

A 10-ml Levafix Navy P-RRL column was loaded with 10 ml of cell-free extract. Almost all the 6PGD activity (83%) was found in the flow-through. The elution profile when using 1 m*M* $NADP^+$ in the extraction buffer showed a peak of G6PD activity at the $NADP^+$ front. This peak contained about half of the G6PD activity applied. Also, a long tailing effect was observed. Even after 10 ml a low G6PD activity was still found. This might be explained by the fact that two (or more) isoenzymes exist, which could behave differently on this dye-adsorbent or could differ in their affinity for $NADP^+$. Isoenzymes of G6PD differing only at the N-terminus are found in several organisms[16].

The fractions containing more than 3 U ml^{-1} were pooled and applied to a MonoQ column. G6PD activity was eluted at 200 m*M* sodium chloride using a linear 0–400 m*M* sodium chloride gradient. As judged by SDS-PAGE, this sample contained almost pure G6PD (Fig. 1). A purification factor of 119 and a yield of 54% were obtained in this two-step purification.

DISCUSSION

The development of a rapid and easy to set up purification procedure for a certain protein can be an important step in recombinant DNA work. Apart from being able to study the purified protein, it can be used as a means of obtaining a probe for the corresponding gene. Gas-phase microsequencing of small amounts of pure protein can be used to obtain data for the generation of oligonucleotide probes[17]. Purified protein can also be used to raise antibodies, which can be used to screen a

cDNA expression library[18]. For the characterization of mutant proteins or proteins modified by protein engineering, the availability of a rapid small-scale purification procedure is also of great importance, as these proteins may be less stable[19].

A particular enzyme or protein is generally found to have affinity for different textile dyes. Screening of a large number of different dyes to find the most suitable one is usually time consuming and therefore kept to a minimum in most instances. Here a method is presented by means of which 96 dye affinity adsorbents can be screened in several hours. This method uses components that have the same structure and therefore make it possible to handle 96 mini-columns at the same time. A transplate cartridge is modified to create 96 mini-columns. Fractions eluted from these columns can easily be collected in a microtitre plate. The amount of enzyme present in each of the fractions can be determined in a microtitre plate assay.

An important feature of the method presented is its versatility. Adsorption and (biospecific) elution conditions can be varied and tested in an integrated fashion in one cycle. In the eluted fractions the activities of more than one enzyme can be determined, in addition to the protein content. From these data a purification strategy may already be derived. A great diversity of enzyme detection methods can be used. In this paper a spectrophotometric assay (for GLYDH) and two assays based on colour development (for 6PGD and G6PD) are used. We have also succesfully used various discontinuous assays and some coupled enzyme assays in the screening of some other enzymes (data not presented).

As an illustration of the method we screened for dye-adsorbents that can be used in the purification of GLYDH, 6PGD and G6PD from *A. nidulans*. In the screening for GLYDH a spectrophotometric microtitre plate assay was used. Information about yields and purification factors on a microlitre purification scale were used to select Cibacron Red 3B-A as the most suitable dye. A yield of 91% and a purification factor of 97 were achieved using this dye-adsorbent in a millilitre scale purification. Colorimetric microtitre plate assays were used in the screening for dye-adsorbents for the purification of 6PGD and G6PD. Dye-adsorbents were selected that showed a strong (or intermediate) signal in one assay and a weak signal in the other assay. Using Procion Black 2 RPC a purification factor of 66 and a yield of 85% were achieved in one purification step. For G6PD a purification factor of 90 and a yield of 54% were obtained using Levafix Navy P-RRL. These three examples show that data obtained on a microlitre purification scale can be used to select from 96 dye-adsorbents the most suitable ones to be used on a millitre scale.

We currently use this screening method as a first routine step each time a new enzyme has to be purified. The transplate cartridge has so far been used for the purification of about ten different enzymes in several months. It always resulted in the identification of a suitable dye that proved was useful.

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Immobilization of reactive dyes on several matrices that allow high and very high flow-rates to be used

Application to the purification of a proteinase inhibitor from corn

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ABSTRACT

Several matrices were used for immobilization of the reactive dye Procion Red H3B: TSK HW 40, 65 and 55, Trisacryl GF 2000, Sephacryl S-200, Superose 6, Sepharose CL-4B and laboratory-prepared dextran-coated Spherosil XO15. The amounts of incorporated dye and the flow properties of the various supports were determined, in addition to their capacity for a trypsin inhibitor present in a corn-derived food product. It was shown that dextran-coated silica, TSK HW 55 F, Superose 6 and Sephadex G-50 F allowed immobilized dye chromatographic supports suitable for purification of this protein under high flow-rate conditions to be prepared. Actual results of purification obtained using dyed dextran-coated silica and dyed Sephadex G-50 F (settled in a squat column) are described and shown to compare favourably with those of a more traditional purification procedure.

INTRODUCTION

Immobilized dye chromatography has been shown to be a powerful technique for the purification of a host of different proteins[1]. It has been demonstrated by several workers that it could be beneficial when faced with the problem of purifying a given protein to search systematically among several tens of different dyes for that one which is most efficient for the purification[2–5]. Such approaches have been made using agarose as a matrix for dye immobilization, but it is well known that agarose is not

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a perfect support because it cannot withstand high pressures and flow-rates. It does not seem sound to perform screening experiments with agarose-based supports and subsequently to switch to better performing supports in terms of allowed flow-rates; indeed, it has been demonstrated that the nature of the matrix could be a significant factor in determining the capacity of immobilized dye chromatographic supports[6].

The optimum support for dye immobilization should conform to the following properties and conditions: it must be of good mechanical stability so as to withstand to high flow-rates; obviously full benefit of the eventual use of high flow-rates can be taken only if the capacity of the dyed support does not level off too much with increasing flow-rate; the support must allow the use of the very straightforward chemistry commonly used to graft directly textile reactive dyes onto agarose matrices (hence this implies in particular that the support must be able to withstand to the aggressive alkaline conditions that are commonly used for immobilization of reactive textile dyes); it must be commercially available or at least easily prepared from commercially available materials; and it must be inexpensive.

This paper presents the results of a search for the optimum support and its application to the purification of a trypsin inhibitor present in a corn-derived food product. This inhibitor, named popcorn inhibitor (PCI), is known to inhibit activated Hageman factor but not kallikrein, another serine proteinase implicated in the contact activation of plasma coagulation[7]. An effective purification procedure for PCI has already been described using immobilized dye in the first step[8].

EXPERIMENTAL

Materials

Dextran T-500, Sephadex G-50, Sephacryl S-200, Superose 6 Prep Grade and Sepharose CL-4B were obtained from Pharmacia (Uppsala, Sweden) and Trisacryl GF 2000 M and Spherosil XO15 M from IBF Biotechnics (Villeneuve la Garenne, France). TSK HW 55 and HW 65 gels were Tosoh products and were either purchased or obtained as a much appreciated gift from Merck Clevenot (Nogent sur Marne, France). Chemicals were bought from Aldrich Chimie (Strassbourg, France) or from Merck. Procion Red H3B was a kind gift from ICI France (Clamart, France).

Preparation of dextran-coated silica

The preparation of dextran-coated silica has been described in detail elsewhere[9]. Briefly, Spherosil XO15 M was impregnated with specially prepared DEAE-dextran (dextran T-500 derivatized with diethylaminoethyl chloride so that 4% of glucose units carried a DEAE substituent). After impregnation, the polymer layer was stabilized using butanediol diglycidyl ether as a reticulating agent.

Dye immobilization onto the various supports

The same method was used for grafting of dyes onto the various chromatographic supports and is similar to those in common use[1]. The chromatographic support was rinsed on a Buchner funnel with water then with 0.2 *M* sodium hydroxide solution containing 2% (w/v) sodium chloride. The support was then transferred to a vessel containing a suitable amount of reactive dye (20 mg per millilitre of support) and the same solution as above was added to the vessel up to a final volume twice that

of the settled support. The vessel was then tumbled at 60°C for 1 h. The support was then quickly rinsed with 2% sodium chloride in 0.2 *M* sodium hydroxide solution and transferred to a new vessel containing fresh reactive dye and treated as above. The experiments described below were done with various supports treated three times. At the end of the last treatment the supports were rinsed exhaustively with 10 m*M* sodium hydroxide solution, water, 6 *M* urea and water again.

This immobilization method will be referred later in the text as the direct immobilization method, as opposed to those other methods from the literature in which dye is first modified by grafting of a spacer arm before immobilization onto the chromatographic support.

Evaluation of amounts of dye grafted onto the supports

Amounts of incorporated dye in most supports were evaluated by hydrolysis methods; basically, one volume of dyed support was hydrolysed in ten volumes of hydrolysis medium for durations sufficient to induce either complete solubilization or at least complete discoloration of the supports (in this case the coloured supernatant was recovered by filtration).

The hydrolysis conditions were 50% acetic acid in water at 110°C for most supports, but for dyed Trisacryl hydrolysis was effected in methanol–sulphuric acid mixture (5:20, v/v) at room temperature for 30 min. The amounts of dye liberated through these hydrolysis methods were determined by spectroscopic measurements.

No satisfactory hydrolysis method could be worked out to evaluate the amount of dye incorporated into the deep red Sephacryl S-200. For determination of the amounts of dye incorporated into the dyed dextran-coated silica samples, an alkaline hydrolysis method taken from the literature[10] was found to be not satisfactory (a large residue of undissolved, uncompletely decolorized silica was constantly obtained even after long incubation times), so another method was adapted from data published elsewhere[11]: the dyed dextran layer was stripped from the coated silica by incubation in fuming nitric acid (2 ml for 50 mg of dyed support) for 15 h at 20°C. and the amount of solubilized dye derivative was then evaluated through spectroscopic measurements.

Evaluation of flow properties of supports

To evaluate the flow properties of the supports they were sedimented in Pharmacia FPLC HR 5/5 columns up to a height of 5 cm. The columns were equilibrated at 0.05 ml/min in 30 m*M* sodium phosphate buffer (pH 6.5) containing 2 *M* sodium chloride (buffer B), the pressure was recorded and thereafter the flow-rate was increased in increments of 0.05 ml/min, the pressure was allowed to come to a stable value and recorded. TSK gels were settled according to recommendations given by the supplier of the gel, *i.e.*, they were sedimented to constant height at a back-pressure of 4 bars.

Evaluation of capacities of dyed supports for popcorn inhibitor

The capacities were evaluated by frontal chromatography. The dyed supports were sedimented to a height of 2.8 cm in Pharmacia FPLC HR 5/2 columns, and equilibrated in 30 m*M* sodium phosphate buffer (pH 6.5) containing 20 m*M* sodium chloride (buffer A). Thereafter polenta extract prepared as indicated below was pumped to the column. The effluent was collected in 1-ml fractions and the amounts of

PCI present in the fractions were evaluated by measurement of its inhibitory activity towards trypsin.

Preparation of polenta extract and buffers used in the purification procedures

The advantage of using polenta, a traditional Italian corn-derived food product, as a source for purification of PCI instead of the more commonly used corn kernels has been described elsewhere[8]. Polenta obtained from a local food store was suspended in buffer A (400 ml of buffer for 100 g of polenta), toluene added (80 ml per 100 g of polenta) and the suspension shaken overnight in a cold room. The aqueous phase was then filtered through a Whatman 1 MM filter-paper and the filtrate used for purification. Buffers used for chromatography of polenta extracts on the immobilized dyes were buffer A, buffer B with the same composition as buffer A but containing 2 *M* sodium chloride) and buffer C (with the same composition as buffer B but containing 6 *M* urea).

RESULTS AND DISCUSSION

Amounts of dye immobilized on the different matrices

A list of the dyed supports and relevant characteristics taken from the manufacturers' data are given in Table I. Also are given the amounts of dye that were grafted on each individual matrix.

TABLE I

CHARACTERISTICS OF THE VARIOUS DYED SUPPORTS PREPARED FOR THIS STUDY

Matrix	*Bead diameter (μm)*	*Chemical nature of matrix*	*Amount of dye incorporated (μmol/ml of settled support)*	*Letter code used for identification of relevant chromatograms in Fig. 1*
TSK HW 40 F	32–63	Poly(vinyl alcohol)	22.6	A
TSK HW 65 F	32–63	Poly(vinyl alcohol)	11.6	B
TSK HW 65 S	25–40	Poly(vinyl alcohol)	11.3	C
TSK HW 55 F	32–63	Poly(vinyl alcohol)	31.0	D
TSK HW 55 S	25–40	Poly(vinyl alcohol)	16.3	E
Superose 6 prep grade	20–40	Cross-linked agarose	3.7	F
Sepharose CL-4B	40–165	Cross-linked agarose	11.2	G
Sephadex G-50 SF	20–50	Cross-linked dextran	18.8	
Sephadex G-50 F	20–80	Cross-linked dextran	5.6	H
Sephadex G-50 C	100–300	Cross-linked dextran	3.8	I
Sephacryl S-200 SF	40–105	Allyldextran cross-linked with N,N'-methylenebisacrylamide	n.d.	
Trisacryl GF 2000 M	40–80	Hydrophilic acrylic polymer	0.4	
Dextran-coated Spherosil XO15 M	40–100	Composite support	24.6	J

The smallest amount of incorporated Procion Red H3B was obtained with Trisacryl GF 2000, and indeed it was very low. The manufacturer of Trisacryl GF 2000 markets Red Trisacryl on which is grafted the related dye Procion Red HE 3B. However, Procion Red HE 3B is incorporated into Trisacryl by the manufacturer in a very different way: the dye is first derivatized with diaminohexane and thereafter aminohexyl dye is grafted onto the gel activated by suitable means. Another procedure was described recently[12] in which Red Trisacryl is prepared by copolymerization of the usual monomers of Trisacryl together with an acrylic derivative of the dye.

The disappointing results obtained with Procion Red H3B and Trisacryl GF 2000 preclude the use of this latter matrix as a first choice for the preparation of a panel of several different immobilized dyes for dye screening procedures, even though with other dyes and the same technique we could incorporate dyes into Trisacryl at a comparatively satisfactory level (data not shown).

It is worth noting that a fairly large amount of dye could be grafted onto dextran-coated silica, even though the grafting technique was very simple; this is an obvious advantage of the dextran coating. Another advantage is that the coating makes the silica resistant to alkaline conditions; this is demonstrated by the fact that the alkaline hydrolysis method allowing dyed silica (prepared by grafting of an aminohexyl derivative of the dye onto epoxy silica[10]) to be dissolved was found to be ineffective with dextran-coated silica.

Flow properties of the dyed supports

Plots of flow-rate *versus* pressure were established for most of the dyed supports listed in Table I. Three different dyed supports followed Darcy's law up to the highest linear flow-rate tested (25 cm/min), *viz.*, dextran-coated silica, Sephadex G-50 coarse and TSK HW 40 F (with the latter gel, the flow-rate *versus* pressure plot showed a slight upward curvature), the back-pressures generated at 25 cm/min being 0.8, 1.9 and 6 bar, respectively.

Several other gels followed Darcy's law only up to limiting values of flow-rate and pressure, higher flow-rates being followed by a very large increase in back-pressure. The limiting flow-rates found for dyed Sephadex G-50 F and SF were 7.5 and 1.25 cm/min, respectively (limiting pressure values 0.75 and 1 bar, respectively). The limiting values of flow-rate and pressure for dyed Sephacryl S-200 were found at 5 cm/min and *ca.* 0.5 bar, respectively, and for both Sepharose CL 4 B Superose 6 prep grade at ca. 12 cm/min and 1.3 bar, respectively. We assume that limiting values of pressure are reached when irreversible gel collapse occurs; in this regard, it should be emphasized that once the limiting pressure had been reached, a second recording of the curve of flow-rate *versus* pressure showed a greatly lowered permeability of the gel columns.

TSK gels HW 65 and 55 showed a different behaviour. With TSK HW 65 F and S the curves of flow-rate *versus* pressure deviated progressively from linearity with an upward curvature, but buffer could be pumped at velocities in excess of 20 and 18 cm/min, respectively, at a back pressure of 8 bar. The permeability of TSK HW 55 seemed to be lower and when the flow-rates were increased above 7.5 and 2 cm/min with F and S grade, respectively, large increases in the generated back-pressure were observed (it must emphasized, however, that TSK gels did not show signs of irreversible collapse of the gel structure; we recorded variations of pressure with

increasing flow-rate twice and the results were identical in both instances. This is in contrast with the results obtained with the polysaccharidic gels the performance of which has been described earlier and which showed a greatly diminished permeability after having been exposed once to elevated pressure.

Capacities of the dyed supports

Results of the measurements of capacity by frontal chromatography of crude polenta extract are shown in Fig. 1. The capacity for PCI was low with dyed TSK HW 40F (curve A). This poor result was not unexpected as this support has an exclusion limit for proteins of 5 kDa according to its manufacturer; hence the interaction of PCI (migrating as a 14.4 kDa protein on SDS gels) is likely to occur only with those dye molecules which are grafted on the outside surface of the gel beads.

TSK HW 55 gels were seen to perform better than TSK HW 65 gels (but less dye was incorporated in the TSK HW 65 gels). Curves B, B′ and C show the results obtained with dyed TSK HW 65 gels. With TSK HW 55 F it was possible at 0.5 cm/min to load more than 55 ml of crude extract before any inhibitory acitivity could be found in the eluate from the 0.56-ml volume column (see curve D). The capacity decreased with an increase in flow-rate but was still *ca.* 40 ml (see curve D′, which was obtained with this gel at 5 cm/min). Dyed TSK HW 55 S gel was less satisfactory than dyed TSK HW 55 F (compare curves E and D, respectively), but this lower performance can be explained by the lower level of substitution which was obtained with this matrix.

Dyed Superose demonstrated an adequate capacity for PCI, as *ca.* 50 ml could be pumped onto the gel at high flow-rate before the inhibitory activity did begin to elute from the column (see curves F and F′).

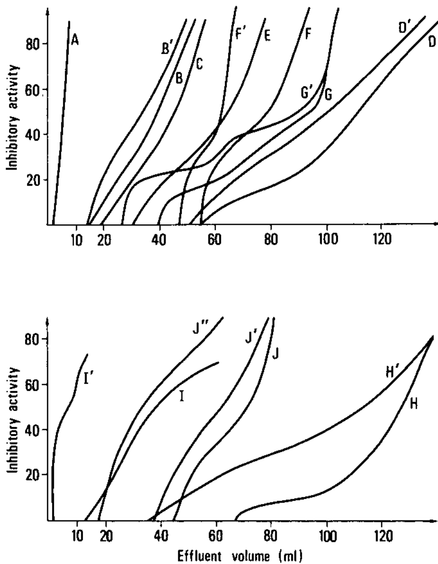

Fig. 1. Frontal chromatography of polenta extract on several different dyed chromatographic supports at high flow-rates. Inhibitory activities (ordinate) are expressed as percentages of the activity present in the starting material. Letter codes used to identify the chromatograms are given in Table I. The letter is followed with a prime when the chromatogram was obtained at a linear flow-rate of 5 cm/min. Chromatogram J″ was obtained at 25 cm/min and all other chromatograms at 0.5 cm/min.

Curves G and G′ show the performance of dyed Sepharose CL-4B at 0.5 and 5 cm/min, respectively. These curves and also data given earlier on the flow properties of this dyed gel demonstrate that Sepharose CL-4B can be used at much higher flow-rates than are commonly used.

Among all the gels tested, dyed Sephadex G-50 fine showed the highest capacity for PCI; *ca.* 70 ml of crude extract could be pumped at 0.5 cm/min onto the column before any inhibitory activity was eluted (see curve H). Indeed, when flow-rate was increased 10-fold the capacity decreased but to the still satisfactory value of *ca.* 40 ml of crude extract for the 0.56-ml column used (see curve H′).

The results obtained with dyed Sephadex G-50 coarse were much less satisfactory; the capacity for PCI at a linear flow-rate of 0.5 cm/min (see curve I) is less than 15 ml and when the linear flow-rate was increased to 5 cm/min the capacity decreased almost to zero (curve I′).

The results obtained with dyed dextran-coated silica demonstrate that the capacity was satisfactory [more than 40 ml of crude extract for a 0.56-ml volume column operated at a linear flow-rate of 0.5 cm/min (see curve J)]. The capacity did decrease slightly when the flow-rate was increased 10-fold (see curve J′), but the column could be operated at flow-rates as high as 25 cm/min and nevertheless retained a significant capacity (see curve J″).

Unexpectedly, PCI was not retained at all by dyed Sephacryl S-200 (frontal chromatogram not shown). Indeed, PCI permeates freely in the inside porous volume of the beads, hence it can only be said that dye is not available for interaction with protein, possibly because of some interaction with the matrix. We should emphasize here that our results do not demonstrate that the latter gel cannot be used for dye immobilization; it is possible that, by using a different chemistry for grafting, *e.g.*, by using spacer arms, efficient dyed supports could be obtained. Nevertheles, we stress that Sephacryl S-200 cannot be a good choice as a matrix for preparing by the direct immobilization method a panel of immobilized dyes for screening.

Taken together, these results show that several supports could be used to purify PCI from polenta extract, *i.e.*, they have a satisfactory capacity even at high flow-rates; those which could be considered as good choices are Superose, Sephadex G-50 fine, TSK HW 55 F and dextran-coated silica. Only the last support is sufficiently rigid to allow in practice the pressure strain induced by high flow-rates to be ignored. TSK HW 55 F seems to be fairly resistant (even if TSK HW 65 did show a better flow-rate *versus* pressure curve); with TSK HW 55 F no clue of gel collapse was observed when this support was submitted to a flow-rate and pressure of 22 cm/min and 8 bar, respectively, hence it seems probable that this gel would be easy to use for protein purification under high flow-rate conditions. The data presented show that the other gels (Sephadex G-50 F and Superose) can be used at fairly high flow-rates but the applied pressure must be kept below *ca.* 0.75 bar for dyed Sephadex G-50 F and 1.3 bar for dyed Superoseses in order not to induce irreversible collapse of the gel structure; this obviously sets limits to the column geometry as high flow-rates will be possible only in short columns.

Purification of PCI using dyed dextran-coated silica

Dextran-coated silica was used for the large-scale purification of PCI from polenta extract. A column of small volume (10 cm × 1 cm I.D.) has been used but

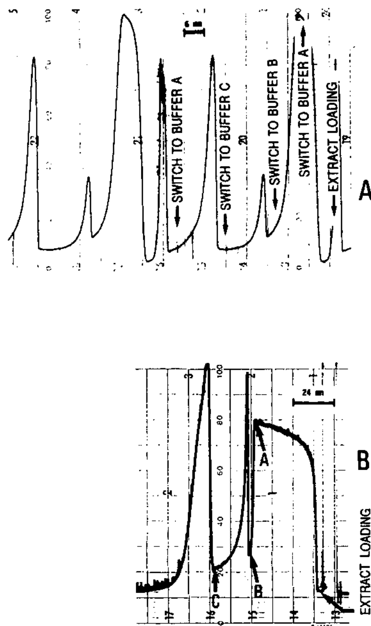

Fig. 2. (A) Recorder trace of automated cyclic chromatography of polenta extract on a Procion Red H3B–dextran-coated silica column. Flow-rate, 5 cm/min. PCI, eluted by buffer C, was adequately automatically collected. (B) Recorder trace of chromatography of polenta extract on a Procion Red H3B–Sephadex G-50 fine column under high flow-rate conditions: the linear flow-rate was 2.5 cm/min during extract loading and column rinsing with buffer A and B and was lowered to 1.25 cm/min during elution of partially purified PCI by buffer C. Arrows A, B and C indicate when buffers A, B and C, respectively, were pumped to the column.

significant amounts were nevertheless treated by repeating purification cyclically under full automatic control. The column was operated at a linear flow-rate of 5 cm/min. To date the column has been used for more than 600 cycles. Fig. 2 shows the recorder trace for some cycles. The yields, and purification factors are given on Table II.

Even if dyed dextran-coated silica seems to fulfil most of the criteria which have given in the Introduction, it is not yet commercially available and even though the technique for coating is relatively straightforward, to date we have not prepared dextran-coated silica on a large scale. It must be said also that less favourable results

TABLE II

COMPARISON OF THREE DIFFERENT PROCEDURES FOR PURIFICATION OF PCI FROM POLENTA BY IMMOBILIZED DYE CHROMATOGRAPHY

	Yield (%)	*Purification factor*
Procion Red H3B–dextran-coated silica	86	6.4
Procion Red H3B–Sephadex G-50 F	89	4.1
Procion Red HE3B–agarose[8]	84	6.7

have been obtained with another dye, another protein and another batch of dextran-coated silica[13].

From the data in Fig. 1 and current prices, it could be calculated that to treat the same amount of polenta extract in a single pass at high flow-rate, the cost of the purchase of Superose 6 would be 40 times that of Sephadex G-50 F (and those of TSK HW 55 F and Spherosil XO15 would be five times those of Sephadex G-50 F). Clearly dyed Sephadex G-50 F, which has already been shown to be the gel with the highest capacity, also has the highest capacity/cost ratio. This provided the impetus to attempt to use this latter support for the large-scale preparation of PCI from polenta extract.

Purification of PCI using dyed Sephadex G-50 F

Obviously, because of pressure limitations with this matrix, we had to use a squat column (1.8 cm × 5 cm I.D.). The flow-rate was 2.5 cm/min (50 ml/min), except during elution with buffer C (it was observed that during elution of proteins retained on the column by this urea-containing buffer the back-pressure increased, so, for safety, we halved the flow-rate). A recorder trace is shown on Fig. 2. Partial purification of PCI from 1800 ml of polenta extract could be completed in 100 min. The yield was satisfactory (see Table II), but the purification factor was lower than that one obtained using immobilized dye chromatography in a more conventional way, and with a lower througput (1300 ml of extract were treated per day in an immobilized dye column five times larger than that one described here and filled with Procion Red HE 3B–agarose[8]). PCI obtained after a second and anyway necessary purification step (reversed-phase chromatography) was in fact of the same purity with both methods.

General comments and conclusions

We are well aware that our statement that Sephadex G-50 can be chosen as a matrix for dye immobilization for the chromatography of proteins at high flow-rates could seem in some ways provocative. Many efforts are being devoted to the development and marketing of efficient supports for protein purification based on mineral matrices or new resistant polymers but nevertheless our results demonstrate that Sephadex G-50, the development of which began *ca.* 30 years ago[14], can be used advantageously under high flow-rate conditions provided that care is taken to use it in short columns.

Indeed Sephadex G-50 F, which is cheap and commercially available, could be used for preparing a panel of different immobilized dyes in order to establish those most suitable for protein purification. We obviously bear in mind that this gel is of limited porosity and therefore it cannot be envisioned that one would use it to purify proteins of high molecular weight. However, proteinase inhibitors from plants in which we are interested are usually low-molecular-weight proteins and therefore in this instance Sephadex G-50 F could be proposed as an advantageous substitute to agarose, commonly used for making panels of immobilized dyes. Indeed, Sephadex G-50 F, because of its pressure limitations, can be used under high flow-rate conditions only in short columns, hence it sets practical limits to column volume, and therefore probably use of Sephadex G 50 F under large flow-rate conditions will be limited to laboratory use.

In contrast, dextran-coated silica, because of the mechanical strength of the silica matrix, can be settled in columns of almost any volume[15]. Our results show that

dextran coating imparts great chemical resistance to the matrix and also allows very convenient grafting of dyes onto the support, this enabled us to prepare easily an efficient support for protein purification.

Several papers have already demonstrated that dyes could be immobilized onto silica and used advantageously for protein purification under high flow-rate conditions[10,16–18] and even on a process scale[19]. However, from reported data it appears that in order to obtain dyed supports with sufficiently high levels of incorporated dye it was necessary either to prepare aminohexyl derivatives of the dyes prior to grafting them onto epoxy-activated silica[10,16,17] or to use only the more reactive dichlorotriazinyl reactive dyes for direct grafting onto diol silica[17,19]. Direct grafting of monochlorotriazinyl dyes onto aminoalkylated silica was also shown to be feasible, but a large proportion of amino alkyl chains remained unmodified at the end of the derivatization procedure, which might be troublesome for protein purification even though satisfactory results have been obtained with one model protein[18].

In contrast, from our data it is clear that dye immobilization on dextran-coated silica is as straightforward as it is on agarose, moreover dextran-coated silica prepared as described above has been used for size-exclusion chromatography of proteins, making it clear that such coated silica is devoid of unwanted interactions with proteins[9].

Finally, we are aware that the satisfactory results which we have presented in this paper must be backed up by further studies with other dyes and other proteins, but nevertheless it seems plausible that dextran-coated silica could be a matrix of choice for immobilization of dyes and the industrial use of immobilized dye chromatography. Probably such developments will have to await the commercial availability of coated silica.

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Affinity chromatography on novel perfluorocarbon supports

Immobilisation of C.I. Reactive Blue 2 on a polyvinyl alcohol-coated perfluoropolymer support and its application in affinity chromatography

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ABSTRACT

The triazine dye C.I. Reactive Blue 2 has been immobilised on a particulate perfluorocarbon support by preparation of a hydrophilic polymeric coating comprising polyvinyl alcohol (average molecular weight, M_r 14 000) esterified with perfluorooctanoyl chloride and securely adsorbed on the perfluorocarbon support by multiple Van der Waals interactions. This polyvinyl alcohol-based coating wets the perfluorocarbon support and provides a neutral barrier to non-specific adsorption of proteins. Reaction with the triazine dye C.I. Reactive Blue 2 allows secure immobilisation of this versatile pseudo-affinity ligand and yields a remarkably stable adsorbent. The performance and capacity of the perfluorocarbon-based adsorbent was assessed: an 8-fold purification of lactate dehydrogenase was achieved from a crude rabbit muscle extract in 100% yield. The albumin-binding capacity of a dyed perfluorocarbon support containing 2.2 ± 0.2 μmol dye per g wet support was determined to be 15.7 mg/ml by frontal analysis. These novel supports compare favourably with conventional polysaccharidic affinity supports.

INTRODUCTION

The reactive textile dyes are known to participate in complex hydrogen bond, electrostatic, hydrophobic and steric interactions at the active sites of biological macromolecules and are becoming popular as versatile group-specific ligands for application in affinity chromatography[1]. The reactive chlorotriazine ring enables facile coupling to hydroxyl-containing chromatographic matrices, thereby allowing the preparation of affinity adsorbents. However, the stability of the triazine dyes to a wide range of stringent chemical conditions highlights the inherent limitations of the base matrix to which they are invariably attached. In particular, there is still a requirement for the development of novel chromatographic matrices that are able to

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withstand not only high pressures for application in high-performance affinity chromatography, but also extremes of acid and alkali to allow *in situ* column clean-up, chemical sanitisation and depyrogenation[2].

Perfluorocarbons are chemically and biologically inert, high-density (1.8–2.1 g/ml), thermally stable synthetic materials that are totally insoluble in aqueous and organic solvents. Aggregates of powdered polytetrafluoroethylene have been used as adsorbents for gas chromatography[3] and as hydrophobic adsorbents for the fractionation of small organo-fluorine-containing compounds[4] and the separation of proteins and nucleic acids[5]. However, perfluorocarbon surfaces display extremely low surface tensions and are wetted by water only in the presence of fluorosurfactants that adsorb to the surface[6]. Consequently, enzmes may be immobilised to fluorocarbon surfaces by cross-linking with the hydrophilic moieties of the adsorbed fluorosurfactant layer[7]. In an alternative approach, urease may be immobilised on perfluorocarbon surfaces by prior perfluoroalkylation of the protein with perfluorooctyl isothiocyanate[8]; the macromolecule is adsorbed on the perfluorocarbon surface by strong fluorocarbon–fluorocarbon Van der Waals interactions between the perfluoroalkyl chains of the covalently modified protein and the exposed perfluoromethyl groups of the polymer. Similarly, we have recently described the immobilisation of three dichlorotriazinyl dyes on solid and liquid perfluorocarbon surfaces by prior bis-perfluoroalkylation of the triazine rings of the dyes[9]. Lactate dehydrogenase was successfully purified from a crude rabbit muscle extract on dyes immobilised on a modified perfluoropolymer matrix. The adsorbents exhibited similar capacities and degrees of purification as previously reported for dyed agarose[10] and silica[11]. However, further work clearly showed that these bis-perfluoroalkylated triazine dyes were insufficiently anchored to the perfluorocarbon matrix and leached in the presence of albumin and organic solvents. Secure anchorage could be achieved by the introduction of additional perfluorinated groups to the ligand. It has recently been reported[12] that the strength of interaction between highly fluorinated compounds and perfluorocarbon stationary phases increases exponentially as the number of perfluoroalkyl chains per molecule increases. Furthermore, double-stranded molecules are more strongly retained than the single-stranded compound containing the equivalent overall number of fluorinated carbon atoms, indicating the strong cooperative effect of multiple anchorage sites.

Adsorbed-coating technology has been developed by Alpert and Regnier[13] to prepare alternative ion-exchange and reversed-phase silica and organic packings for use in high-performance liquid chromatography (HPLC). The technology involves the polysulphonation of the matrix followed by electrostatic adsorption of polyethyleneimine. In an analogous fashion, perfluorocarbon matrices may be coated with polymers by hydrophobic interactions, provided that the polymers contain highly fluorinated regions that are able to interact with the perfluorocarbon surface. However, in order to be effective in affinity chromatography, the polymeric coating should be neutral, stable, easily derivatised and exhibit low non-specific adsorption of proteins. Polyvinyl alcohol is a common neutral polymer derived from the hydrolysis of polyvinyl acetate and used extensively in the paper, food and printing industries. This paper describes the application of a perfluorooctanoyl-polyvinyl alcohol coating to a particulate perfluorocarbon matrix, its subsequent derivatisation with the triazine dye C.I. Reactive Blue 2 and its stability and performance as a chromatographic

adsorbent in the purification of lactate dehydrogenase from a crude rabbit muscle extract and albumin from human plasma.

EXPERIMENTAL

Materials

The triazine dye C.I. Reactive Blue 2 was obtained from BDH (Poole, U.K.). Polyvinyl alcohol [average molecular weight (M_r) 14 000, 100% hydrolyzed] was purchased from Sigma (Poole, U.K.), together with the biochemicals tris(hydroxymethyl)methylamine, NADH (grade 2), sodium pyruvate and the diagnostic reagent kit for albumin determination. Lactate dehydrogenase [L-lactate:NAD^+ oxidoreductase, EC1.1.1.27; rabbit muscle (500 U/mg)] and pure human serum albumin were also purchased from Sigma, while human plasma was obtained from a known donor at the National Blood Transfusion Centre (Nottingham, U.K.) and tested negative for HIV III, HBS antigen and syphilis. Perfluorooctanoyl chloride was obtained from Fluorochem (Old Glossop, U.K.) while pre-packed Sephadex G-25 (PD-10 columns) was purchased from Pharmacia Biotechnology (Milton Keynes, U.K.). With the exception of acetone (SLR), all solvents were of analytical grade. The particulate perfluoropolymer matrix (6–8 m2/g, mean particle size 35 μm) was kindly provided by E.I. Du Pont de Nemours (Wilmington, DE, U.S.A.).

Modification of the perfluorocarbon matrix

Polyvinyl alcohol (1 g, 72 μmol) was dissolved in 100 ml water to which was added perfluorooctanoyl chloride (400 μl, 15 mmol). The mixture was shaken for 10 min, after which perfluorocarbon matrix (100 g) was added, and the suspension stirred overnight at room temperature. The modified support was sequentially washed on a sintered glas funnel with distilled water (1 l), acetone–water (50:50, v/v) (1 l), acetone (500 ml), acetone–water (50:50, v/v) (500 ml), acetone–water (30:70, v/v) (500 ml) and finally distilled water (1 l). Care was taken to avoid the exposure of the perfluorocarbon support to air between washes.

Immobilisation of C.I. Reactive Blue 2

Wet polyvinyl alcohol-coated–perfluorocarbon matrix aliquots (4 g) were stirred with distilled water (20 ml) to which were added various weights of commercial C.I. Reactive Blue 2 (0, 50, 100, 150, 200 and 250 mg). The suspension was stirred at 50°C for 30 min, after which sodium hydroxide (5*M*, 6 ml) was added and stirring continued at 70°C for a further 30 min. The suspension was allowed to cool and washed on a sintered-glass funnel with water (200 ml), acetone–water (50:50, v/v) (100 ml), acetone (100 ml), acetone–water (50:50, v/v) (100 ml), acetone–water (30:70, v/v) (100 ml) and finally distilled water (200 ml), once again avoiding exposure of the support to air. Higher degrees of dye loading on the support were achieved by repeating both dye application and washing stages.

Determination of immobilised dye concentration

The immobilised dye concentration of a dyed support was estimated by sucking the adsorbent dry on sintered glass and suspending a weighed aliquot (50 mg) in methanol–water (50:50, v/v) (0.5 ml) to which was added boiling agarose (2%, w/v),

(0.5 ml). After continuous inversion of the cuvette until the gel had set, the difference spectrum of the support between 700 and 500 nm could be prepared against an equivalent sample of undyed support. The system was calibrated as follows: various dilutions of free dyed perfluorooctanoyl polyvinyl alcohol conjugate were prepared in a methanol–water mixture (50:50, v/v) (5 ml) and then incubated with aliquots of wetted perfluorocarbon support (50 mg wet weight) by rotary tumbling for 12 h. The decrease in absorbance of the methanol–water solutions at 620 nm after incubation was directly attributed to the adsorption of the dyed polymer on the support. After extensive washing, this yielded a series of dyed supports with known immobilised dye concentrations. These supports were centrifuged at 4500 *g* for 5 min and resuspended in plastic cuvettes in the methanol–water mixture (0.5 ml), set in agarose as previously described. Comparison of the measured difference spectra of the dyed versus undyed supports with the known dye concentrations showed a difference by a factor of 1.25 which was thereafter incorporated into the estimation of immobilised dye concentrations on all supports.

Determination of adsorbent stability

A wet perfluorocarbon adsorbent containing 2.2 μmol/ml C.I. Reactive Blue 2 was sucked dry, divided into 100-mg aliquots and suspended in 2 ml volumes of various solutions and solvent by rotary tumbling at room temperature for 24 h. The adsorbent was allowed to settle, the solution/solvent removed and, where necessary, adjusted to neutral pH with a known volume of acid or base. The absorption at 620 nm (λ_{max}) of the solution was then measured against appropriate solution/solvent blanks, tand the dye concentration estimated assuming a molar extinction coefficient of 12 750 $M\ 1^1\ cm^{-1}$. The percentage dye lost from the support was estimated assuming that the leached dye remained intact.

Protein preparation, determination and assay

Protein preparations were desalted where necessary by passage on disposable Sephadex G-25 pre-packed PD-10 columns pre-equilibrated in the appropriate buffer. Protein determination was routinely carried out using the Bradford assay[14], the standard reagent (Coomassie brilliant blue G-250, 0.01%, w/v; ethanol, 4.7%,w/v; phosphoric acid, 8.7%, w/v) being diluted one fifth in water prior to use. Protein concentrations in stock solutions were initially determined by absorbance at 280 nm assuming extinction coefficients for crude albumin and crude lactate dehydrogenase as 0.53 (ref. 15) and 1.14 (ref. 16) ml $mg^{-1}\ cm^{-1}$, respectively. Serial dilutions (20 μl) of stock protein solutions were incubated with fresh assay mixture (980 μl) for 10 min before measuring the absorbance at 595 nm against a buffer blank, to prepare standard curves from which the protein concentration in unknown samples could be estimated.

The bromocresol green assay[17] for serum albumin was used to determine the albumin content in plasma samples. Diluted plasma samples were compared to a known standard by incubating aliquots (200 μl) with the standard assay mixture (Sigma) (800 μl) for 10 min, followed by measuring the absorbance at 628 nm against the appropriate buffer blank. The activity of lactate dehydrogenase was monitored spectrophotometrically at 340 nm and 25°C by measuring the oxidation of NADH in disposable plastic cuvettes arranged in a Perkin Elmer Lambda 5UV–VIS spectrophotometer. Small volumes of enzyme (10 μl), diluted if necessary, were added to

a prepared solution (1 ml) containing Tris–HCl (200 mM), sodium pyruvate (1 mM) and NADH (200 μM) at pH 7.3. One unit is defined as that amount of enzyme needed to convert 1 μmol of substrate to product in 1 min at 25°C, assuming a molar extinction coefficient of 6230 l mol^{-1} cm^{-1} for NADH at 340 nm.

Chromatographic procedure

Adsorbents were washed in the appropriate running buffer, then packed in Pharmacia HR 5/10 columns and used in conjunction with the Pharmacia FPLC system (P500 pumps, LCC 500+ controller and UV1 single-path monitor). The flow-rate used throughout was 2 ml/min. Typically, about 10 mg crude protein were loaded (1 ml) and purified protein was eluted as follows: albumin was recovered using potassium chloride (1 M), while lactate dehydrogenase was eluted with NADH (5 mM). Recovered protein fractions were analysed for activity and protein content and were stored at 4°C for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Dynamic load capacity

Dynamic load capacities of the adsorbent were determined by frontal analysis[18]. For example, human serum albumin-binding capacity was measured by pumping 10 mg/ml pure albumin until the absorbance at 280 nm of the output and input streams were identical. Albumin was subsequently eluted with potassium chloride (2 M), and the recovery of bound protein determined. Bound lactate dehydrogenase was eluted with the specific eluent, NADH (5 mM).

SDS-PAGE

SDS-PAGE was carried out essentially as described by Laemmli[19] using an LKB Multiphore II vertical electrophoresis unit, comprising gel lanes (1.5 mm in width) of 10% (w/v) acrylamide resolving gel and 3% (w/v) stacking gel. Protein samples (10–20 μl) were loaded and the gels stained with Electran PAGE Blue G-90 (BDH, Poole, U.K.). Gels were stored in aqueous acetic acid (3.5%, v/v).

RESULTS AND DISCUSSION

Fig. 1 shows two possible reaction routes to achieve the immobilisation of the triazine dye C.I. Reactive Blue 2 on a perfluorocarbon matrix via a highly fluorinated polymeric coating. The preferred strategy involves the preparation of the poly(perfluorooctanoyl)-substituted polyvinyl alcohol in water, coating the perfluorocarbon matrix and, finally, coupling the triazine dye to the adsorbed hydroxyl-containing coating. Alternatively, the dye may first be coupled to the polymer, thereby allowing the spectrophotometric estimation of the dye polymer ratio; the perfluorooctanoyl chloride can then be added separately or in conjunction with the perfluorocarbon matrix. Typical estimates for the extent of coupled dye, determined after reaction in free solution, lie in the range 1–4 mol dye per mol polymer (M_r 14 000). With regard to the perfluorooctanoyl-anchoring groups, a least a 200-fold molar excess of perfluorooctanoyl chloride was used to ensure secure anchorage. Alternative highly fluorinated reactive compounds may also be used, of which pentafluorobenzoyl chloride proved the most viable alternative. This compound has been used by Keese

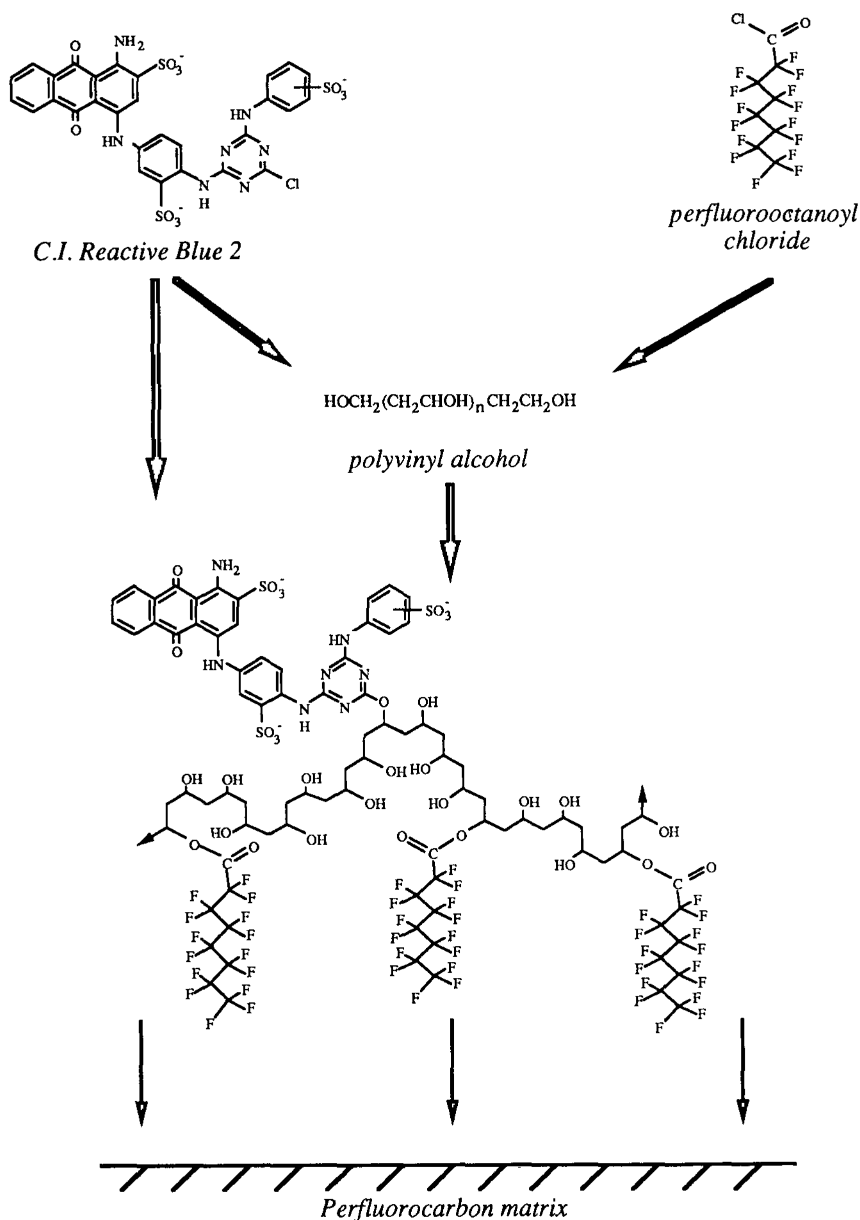

Fig. 1. Strategies for the preparation of C.I. Reactive Blue 2 securely adsorbed on a perfluorocarbon surface (mean n = 324).

and Giaever[20] to anchor polylysine on liquid perfluorocarbon droplets as a substrate for cell growth in culture media.

Estimation of the concentration of dye immobilised on the perfluorocarbon matrix is complicated by the inert nature of the base material, which precludes its

solubilisation. Solid perfluorocarbons are extremely unreactive at room temperature and cannot be dissolved, hydrolysed, oxidised or otherwise degraded. Measurement of the visible spectrum of the support suspended in water is complicated by rapid sedimentation of the particles; the use of a viscous medium such as glycerol only marginally improves this situation. By preparing mixed suspensionsin hot agarose, the perfluorocarbon are held in place upon gelling such that the difference spectra between dyed and undyed adsorbents can be determined. However, because of light loss froms scattering, a correction factor was estimated using lightly dyed supports prepared with known concentrations of immobilised dye (<0.5 μmol/g) (only lightly dyed supports can be prepared by this method). By assuming that this correction factor applies across the entire range of dye concentrations it is possible to estimate the concentration of immobilised dye on more heavily dyed supports.

The difficulties experienced in estimating the immobilised ligand concentration reflect the durability of the perfluorocarbon support. Table I assesses the stability of the polyvinyl alcohol-coated perfluorocarbon matrix derivatised with the triazine dye C.I. Reative Blue 2, by measuring the extent of dye leakage after exposure to a variety of conditions for 24 h. In strong acid and basic conditions (5 *M* hydrochloric acid, 6 *M* sodium hydroxide) the perfluorocarbon-based support showed some leakage of dye, while in long-term studies lasting more than nine months, no leakage of the triazine dye from the perfluorocarbon matrix was apparent, within the spectrophotometric limits of detection, in water, sodium hydroxide (1 *M*), hydrochloric acid

TABLE I

STABILITY OF PERFLUOROCARBON-IMMOBILISED C.I. REACTIVE BLUE 2 UNDER VARIOUS CONDITIONS

Suction-dried wet dyed perfluorocarbon support containing 2.2 μmol/ml C.I. Reactive Blue 2 was divided into 100 mg aliquots, suspended in 2 ml of solution/solvent and incubated at 18°C by rotary tumbling for 24 h. The absorption at 620 nm of the supernatants were measured after appropriate adjustment to neutrality with a known volume of acid or base. Dye concentrations were estimated assuming a molar extinction coefficient of 12 750 $M\ l^{-1}\ cm^{-1}$. Dye concentrations below the spectrophotometric limits of detection (<0.005 A.U. at 620 nm; <0.4 μ*M*) are indicated by a dash.

Solution/solvent	*Concentration of C.I. Reactive Blue 2 released (μM)*
Distilled water	–
Sodium hydroxide (1 *M*)	–
Sodium hydroxide (6 *M*)	7.7
Hydrochloric acid (1 *M*)	–
Hydrochloric acid (5 *M*)	7.1
Urea (8 *M*)	–
Aqueous glutaraldehyde (1%, w/v)	–
Aqueous triethylene glycol (25%, v/v)	–
Aqueous glycerol (25%, v/v)	–
Acetone	–
Acetonitrile	–
Dimethyl formamide	–
Dimethyl sulphoxide	–
Methanol	–

(1 *M*), urea (1 *M*) or acetone. Thus, the perfluorocarbon-based adsorbent appears to offer superior stability to conventional polysaccharide-based absorbents, particularly under the harsh conditions necessary for column cleaning and depyrogenation[2]. We are at present comparing dye leakage from a number of supports using both radioisotopic and immunological (competitive enzyme-linked immunosorbent assay) methods.

The chromatographic behaviour of the polyvinyl alcohol-coated perfluorocarbon support in the absence of immobilised dye was examined by repeated applications at room temperature of small aliquots (100 μg, 0.1 ml) of pure rabbit muscle lactate dehydrogenase (in 50 m*M* Tris–HCl, pH 7.3) or human serum albumin (in 20 m*M* phosphate, pH 5.0). No non-specific adsorption of either protein was observed. The polyvinyl alcohol coating effectively wets the strongly hydrophobic perfluorocarbon surface and provides an effective barrier to the non-specific adsorption of proteins on the underlying perfluorocarbon matrix.

Fig. 2a shows the purification of lactate dehydrogenase from a crude rabbit muscle extract achieved by chromatography on C.I. Reactive Blue 2 immobilised on the polyvinyl alcohol-coated perfluorocarbon support. Elution with the coenzyme NADH (5 m*M*) promoted an overall 8-fold increase in specific activity in 100% yield, the main eluted fraction showing a 12.7-fold purification (Fig. 2b, lane B); the purity of the eluted protein compares favourably with commercial pure rabbit muscle lactate dehydrogenase (lane A). Table II presents the data for the overall purification.

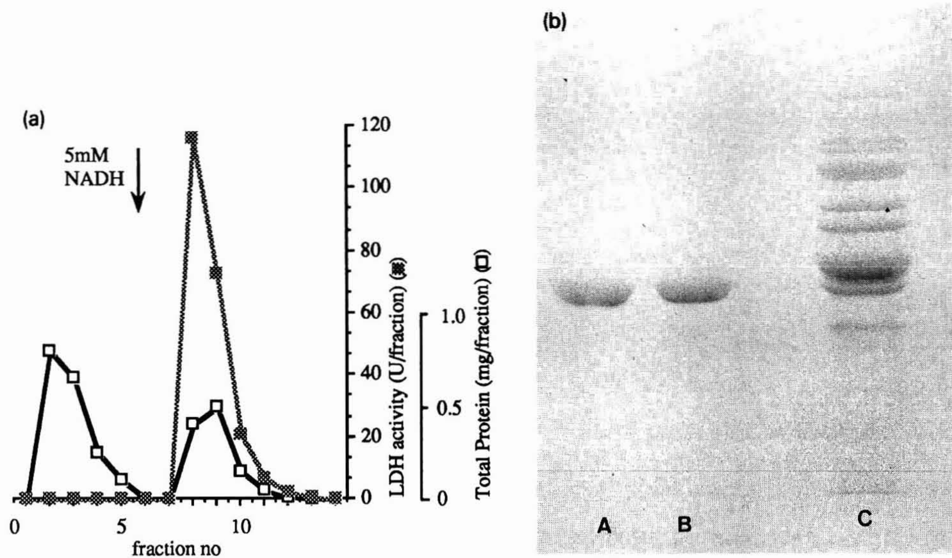

Fig. 2. (a) Purification of lactate dehydrogenase (LDH) from a crude rabbit muscle extract on C.I. Reactive Blue 2 immobilised on polyvinyl alcohol-coated perfluorocarbond Column volume, 1 ml; immobilised dye concentration, 2.2 μmol/ml; running buffer, 50 m*M* Tris–HCl, pH 7.3; eluting buffer, 5 m*M* NADH in running buffer; fraction volume, 1 ml; flow-rate, 2 ml/min. Protein measured by Bradford assay, enzyme activity detected as described in the text. (b) SDS-PAGE of lactate dehydrogenase from a crude rabbit muscle extract. (A) Pure lactate dehydrogenase, Sigma type II; (B) pooled NADH-eluted fractions from C.I. Reactive Blue 2 polyvinyl alcohol-coated perfluorocarbon adsorbent; (C) crude rabbit muscle lactate dehydrogenase extract, Sigma.

TABLE II

PURIFICATION OF LACTATE DEHYDROGENASE FROM CRUDE RABBIT MUSCLE EXTRACT USING PERFLUOROCARBON-IMMOBILISED C.I. REACTIVE BLUE 2

Experimental details as for Fig. 2. Fraction, 8, 9, 10 and 11 pooled as "eluted fraction".

Stage	*Total protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*	*Yield (%)*	*Purification (fold)*
Crude sample	8.8	213.0	24.2	100	1
Eluted fraction	1.1	214.0	194.5	100	8.04

TABLE III

PURIFICATION OF SERUM ALBUMIN FROM HUMAN PLASMA USING PERFLUOROCARBON-IMMOBILISED C.I. REACTIVE BLUE 2

Albumin was purified from diluted plasma (1:4) on a column containing perfluorocarbon-immobilised C.I. Reactive Blue 2; applied sample, 1 ml; column volume, 1 ml; immobilised dye concentration, 2.2 μmol/ml; running buffer, 20 m*M* phosphate, pH 5.0; eluting buffer, 2 *M* KCl, 20 m*M* phosphate, pH 8.0; fraction volume, 1 ml; flow-rate, 2 ml/min. Protein measured by the Bradford assay; specific albumin detected by the bromocresol green assay.

Stage	*Total protein (mg)*	*Albumin (mg)*	*Albumin content (%)*	*Yield (%)*	*Purification (fold)*
Crude sample	18.5	11.6	62.5	100	1
Eluted fraction	6.6	6.3	93.9	98.5	1.5

Determination of the dynamic load capacity for lactate dehydrogenase by frontal analysis for a matrix containing 2.2 μmol dye per g wet adsorbent yielded 5.3 mg/ml bound enzym per ml perfluorocarbon support.

Table III presents the data for the purification of serum albumin by chromatography of human plasma on the blue perfluorocarbon adsorbent (2.2 μmol dye per g); albumin was recovered in 93.9% purity and 98.5% yield. On repeated passage of human plasma, a 1.5-fold purification of albumin was typically achieved in greater than 90% yield. Fig. 3a shows the SDS-PAGE analysis of the purification and clearly demonstrates that the eluted fraction (lane C) is of comparable purity to commercial pure human serum albumin (Sigma, fraction V) (lane B). Fig. 3b illustrates the frontal analysis chromatogram for the measurement of the dynamic load capacity for human serum albumin of the perfluorocarbon adsorbent. The adsorbent, with an immobilised dye concentration of 2.2 μmol/g, bound 15.7 mg albumin per ml support, corresponding to approximately 11% usage of dye on a molar basis. This capacity compares favourably with the quoted albumin capacities for most commercial adsorbents containing C.I. Reactive Blue 2 (5–18 mg/ml). Fig. 4 examines the capacity as a function of the immobilised dye concentration and suggests that the capacity for both albumin and lactate dehydrogenase might be greater if the immobilisd dye concentration were increased. Furthermore, double reciprocal plots of the capacity data given in Fig. 4 indicate that maximum capacities for albumin and lactate dehydrogenase of 32.3 and 8.5 mg/g could be realised at higher dye concentrations.

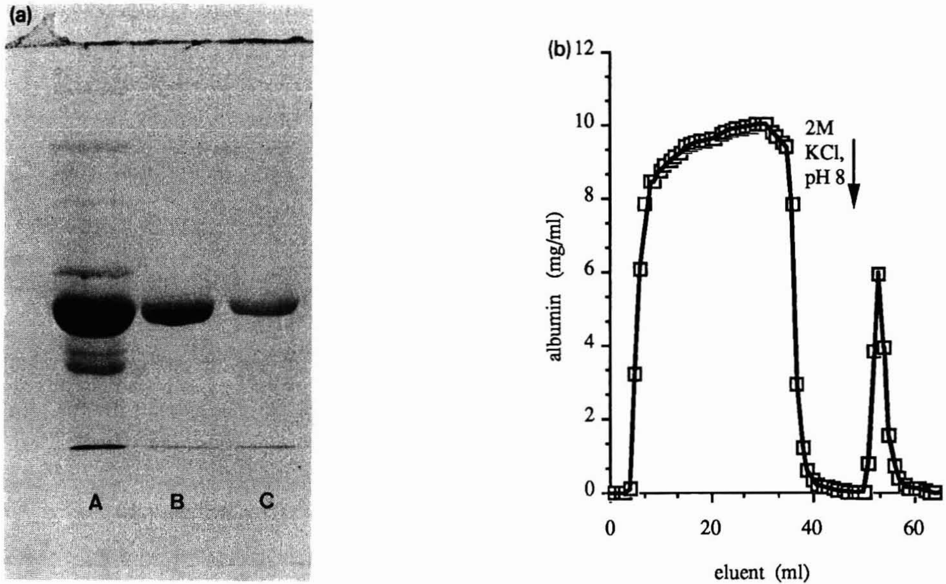

Fig. 3. (a) SDS-PAGE analysis of purification of albumin from human plasma. (A) Crude human plasma; (B) pure human serum albumin, fraction V. Sigma; (C) purified fraction, C.I. Reactive Blue 2 polyvinyl alcohol-coated perfluorocarbon adsorbent. (b) Determination of dynamic loading capacity for albumin by frontal analysis. C.I. Reactive Blue 2 polyvinyl alcohol perfluorocarbon support. Column volume, 1 ml; immobilised dye concentration, 2.2 μmol/ml; running buffer, 20 m*M* phosphate, pH 5.0; eluting buffer, 2 *M* KCl in 20 m*M* phosphate buffer, pH 8.0; flow-rate, 2 ml/min; loading, 10.0 mg/ml pure human serum albumin, fraction V, Sigma; 16.1 mg bound, 15.7 mg eluted with salt. Protein determined by absorbance of fractions at 280 nm (diluted if required).

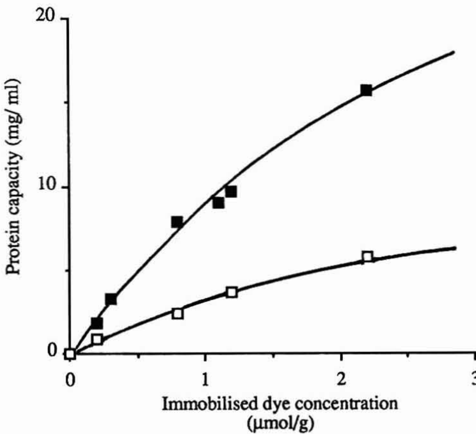

Fig. 4. Comparison of the dynamic loading capacities for human serum albumin (■) and rabbit muscle lactate dehydrogenase (□) of perfluorocarbon supports prepared with varying immobilisd dye concentrations. Immobilised dye concentrations were determined using the difference spectra between dyed perfluorocarbon supports and an equivalent weight of undyed support suspended in a 1% (w/v) agarose gel; observed absorbances at 620 nm were corrected by a factor of 1.25 (see text) to account for losses through light scattering. Dynamic loading capacities were determined by frontal analysis. All column volumes, 1 ml. Chromatographic details for pure albumin —loading concentration, 10 mg/ml; running buffer, 20 m*M* phosphate, pH 5.0; eluting buffer, 2 *M* KCl in 20 m*M* phosphate buffer, pH 8.0. Chromatographic details for lactate dehydrogenase loading concentration, 4.5 mg/ml; running buffer, 50 m*M* Tris–HCl, pH 7.3; eluting buffer, 5 m*M* NADH in running buffer. Flow-rate, 2 ml/min throughout. Protein was determined by absorbance of fractions at 280 nm (diluted if required).

The use of perfluorocarbon supports with smaller particle sizes, and therefore increased total surface areas, may offer still higher capacities while maintaining their overall performance as affinity adsorbents.

CONCLUSIONS

Perfluorocarbon polymers have hitherto found little application in the affinity chromatography of biological macromolecules because of the inherent limitations associated with ligand immobilisation and non-wettability. The preparation of a hydrophilic polymeric coating of polyvinyl alcohol, multiply derivatised with perfluoroalkyl tails with strong affinity for the perfluorocarbon surface, provides a securely adsorbed polyhydroxylic layer on the perfluorocarbon surface to which may be attached a variety of affinity ligands such as the triazine dyes. The chromatographic results suggest that perfluorocarbon supports may offer a viable alternative to many of the current commercially available matrices, while offering superior stability under the harsh conditions required for cleaning *in situ* and depyrogenation.

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New aspects of dye-ligand affinity chromatography of lactate dehydrogenase applying spacer-mediated beaded cellulose

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ABSTRACT

The interaction of lactate dehydrogenase (LDH, EC 1.1.1.28) with triazine dyes spacer-linked to beaded cellulose was studied. The length of the spacer influences neither the binding capacity of the dye–affinity adsorbent nor the elution of the enzyme by NAD–sulphite. However, the length of the extension arm strongly affects the elution of LDH by salt. The results with beaded cellulose carrying butyl, hexyl and decyl residues point to pure hydrophobic interaction with LDH. The introduction of a terminal amino or carboxylic group in the aliphatic chain changes significantly the binding and elution behaviour of the enzyme. It is assumed that in addition to specific interactions realized by the dye chromophore and specific domains on the surface of the enzyme the spacer arm generates a second type of binding force which points to hydrophobic interactions. The latter might be the result of specific orientation governed by the dye-ligand–protein interaction.

INTRODUCTION

The interaction of lactate dehydrogenase (LDH, EC 1.1.1.28) from bovine heart muscle with Cibacron Blue F3G-A and related reactive dyes has been thoroughly investigated[1]. In addition to Cibacron Blue F3G-A, which is known to act as a pseudo-ligand to the NAD-binding domain of numerous enzymes[2,3], a number of other dyes have been found to bind more or less specifically, often forming stochiometric dye–enzyme complexes[4]. Among diverse methods for studying dye–enzyme interactions, dye-ligand affinity chromatography is one of the most commonly applied technique, for both analytical and preparative purposes.

Owing to the wide variety of commercially available reactive dyes and to the facility to form a covalent bond with the support, a number of polymers such as agarose, cross-linked dextran, polyacrylamide and porous silica beads have proved useful as matrices[5,6]. In addition to the chemical requirements to act with reactive dyes, the matrices should show minor non-specific adsorption of proteins, high porosity and sufficient rigidity to achieve adequate flow-rates in packed columns, and showed to be inexpensive. Recently, beaded cellulose has become more attractive as

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a general support in chromatographic techniques[7]. As shown by Mislovičová *et al.*[8] and in our laboratory[9], this material seems to be well suited for affinity chromatography applying dyes as ligands.

Affinity chromatography of enzymes on dye-liganded matrices is generally carried out by (i) adsorption of the enzyme from the crude material, (ii) washing the gel to remove unbound material and (iii) desorption of the target enzyme either non-specifically by increasing the ionic strength or more specifically by adding competitive effectors. In the case of LDH, the substrate NAD in the micromolar range in the presence of millimolar concentrations of sodium sulphite is able to displace the bound enzyme by forming a high stable ternary NAD–sulphite–enzyme complex[10].

The success of affinity chromatography not only depends on the specificity of the ligand–enzyme interaction but is also governed by the mode of coupling of the ligand to the matrix. As shown by Lowe[6], a spacer of certain chain length was used advantageously in order to increase the mode of specific docking if, for example, the ligand-binding site is hidden in a hydrophobic pocket or the surface of the matrix causes in some manner a steric hindrance of the ligand–enzyme interaction.

We found recently[9] that adsorption and desorption of heart muscle LDH depends not only on the intrinsic properties of the dye-ligand but also on the length of the spacer between the dye and the cellulose matrix. For example, the Procion dyes Scarlet MX-G, Orange MX-G, Yellow HE-3G and Green H-4G exhibited strong interactions with LDH, as demonstrated by affinity partitioning[9], but their respective dye–cellulose matrices revealed no adsorption when the dyes were coupled directly to the resin. However, if the immobilization of the dye was realized via a spacer of sufficient chain length, both the specific adsorption of LDH increased markedly and the conditions for the elution of the enzyme were changed. On the other hand, Cibacron Blue F3G-A and Procion dyes such as Red HE-3B, Red HE-7B, Yellow HE-4R and Navy H-ER showed strong binding even if they were coupled directly to the beaded cellulose.

In order to obtain a deeper insight into the function of the spacer in performing dye-ligand affinity chromatography of LDH, Procion Scarlet MX-G was selected as a model ligand to elucidate the chemical requirements of the dye–enzyme interaction and the influence of the spacer length for optimum fitting of the ligand to the binding centre.

EXPERIMENTAL

LDH was partially purified from bovine heart muscle with 12-fold enrichment as described elsewhere[9]. The enzyme had a specific activity of 40–70 units/mg protein.

The dye derivatives of beaded cellulose (Divicell, VEB Arzneimittelwerk Leipzig, G.D.R.) were prepared according to the method of Lowe and Pearson[11]. The synthesis of dye–(spacer)–beaded cellulose (Fig. 1) was described in a previous paper[9] using N-chlorocarbonyloxy-5-norbornene-2,3-dicarboximide (Cl-CO-ONB) activated beaded cellulose[12] and the respective aminoalkyl dye.

The determination of spacer-mediated dye substituents by acid hydrolysis or by the use of Cadoxen[9] was unsuccessful because of the insolubility of the cross-linked dye–affinity adsorbents. The degree of dye substitution may be assumed to be roughly equal for this series because the same batch of activated cellulose was used and the dye substitutions were performed under identical conditions.

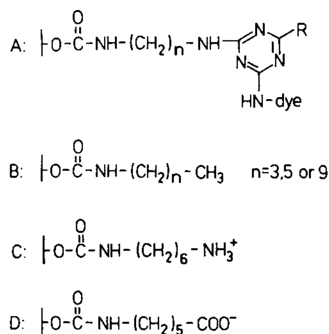

Fig. 1. Structures of dye–spacer–beaded cellulose (A) and of beaded cellulose with different extension arms (B–D).

For the preparation of liganded cellulose as presented in Fig. 1 0.05 mol each of 1-aminobutane, 1-aminohexane (Fig. 1B) and 1,6-diaminohexane (Fig. 1C) were dissolved in 10 ml of distilled water; 1-aminodecane (Fig. 1B) was dissolved in 10 ml of dimethyl sulphoxide–water (1:1, v/v), and ε-aminocaproic acid (Fig. 1D) was dissolved in 20 ml of 0.1 *M* sodium hydrogencarbonate. The solution containing the ligand was adjusted to pH 9 with 5 *M* hydrochloric acid. For coupling, 10 ml of Cl-CO-ONB-activated beaded cellulose were added stepwise to the ligand solution. The suspension was gently shaken at room temperature for 20 h, then the liganded cellulose was filtered off and washed thoroughly with distilled water.

For affinity chromatographic runs, prepurified LDH dialysed against 20 m*M* potassium phosphate buffer (pH 7.0 or 5.5) containing 5 m*M* 2-mercaptoethanol and 1 m*M* EDTA was loaded onto columns (5 × 1 cm I.D.) packed with the respective affinity adsorbent. To remove unbound protein the resin was washed extensively with the same buffer. The adsorbed LDH was then eluted with buffer containing either 1 *M* potassium chloride or 0.05 m*M* NAD plus 1 m*M* sodium sulphite. The main fractions of enzyme activity were pooled and concentrated by ultrafiltration.

The activities of LDH and malate dehydrogenase (MDH, E.C. 1.1.1.37) were measured spectrophotometrically at 340 nm using the following tests: 0.1 *M* potassium phosphate buffer (pH 7.0), 0.8 m*M* pyruvate for LDH and 0.8 m*M* oxaloacetate for MNH and 0.2 m*M* $NADH_2$. Protein was assayed according to Bradford[13].

RESULTS

Immobilized Procion Scarlet MX-G and some structurally related dyes (Fig. 2) were studied for their binding to LDH and their elution. None of these dyes was able to interact with the enzyme if they had been coupled directly to beaded cellulose. Immobilizing the ligands via 1,6-diaminohexane attached to the triazine ring (Fig. 1A) resulted in sufficient binding capacities for all dye matrices (Table I). The desorption of LDH was only achieved with NAD–sulphite and not with potassium chloride. No significant differences with respect to yield and purification factor were observed (Table I).

Procion Scarlet MX-G was used as a model ligand in order to study the influence of the spacer on the interaction of LDH. As demonstrated in Table II, no signif-

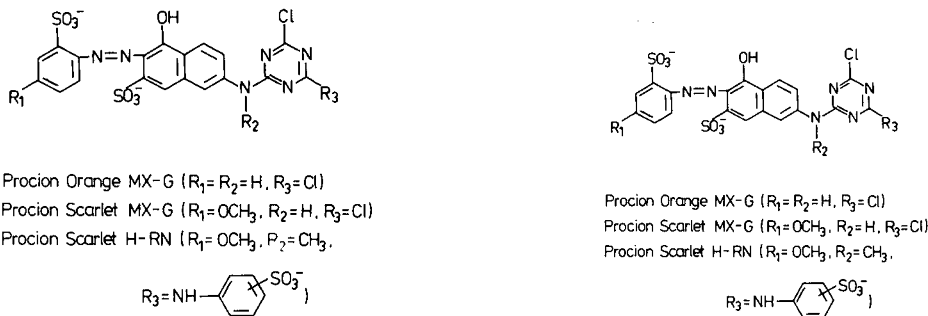

Fig. 2. Structures of Procion dyes used.

icant differences with respect to the dependence of the binding capacity of the prepurified LDH on the length of the spacer was observed. However, although 80–100% of bound LDH could be eluted specifically by NAD–sulphite independent of the number of methylene groups in the spacer, the length of the extension arm strongly affects the elution of LDH with potassium chloride. The non-specific elution with salt decreased with increasing spacer length of the matrix.

However, the specific activity of the enzyme with 180–200 units/mg and the SDS pattern of the main fractions of LDH eluted with NAD–sulphite were similar with all kinds of spacer length probes. Regarding the binding specificity it is worth noting that about 30% of bound LDH can be eluted from the diaminohexyl- and

TABLE I

BINDING CAPACITY AND AMOUNT OF ELUTED ACTIVITY OF PREPURIFIED LDH FROM DYE–(DIAMINOHEXYL)–BEADED CELLULOSE

The binding capacity of dye–beaded cellulose was determined using prepurified LDH by DEAE-Sephadex chromatography[9]. Columns (5 × 1 cm I.D.) containing 2 g of the respective dye–cellulose were loaded with an excess of LDH at 10°C. The amount of activity adsorbed (defined as 100%) was calculated from the difference in the total activity of LDH loaded onto the column and the unbound LDH determined in the breakthrough fraction and in the wash pool. The purification factor was calculated from the increase in the specific activity of the enzyme. For the elution of LDH, 20 m*M* potassium phosphate buffer (pH 7.0) containing 5 m*M* 2-mercaptoethanol, 1 m*M* EDTA, 0.05 m*M* NAD and 1 m*M* sodium sulphite was used. The specific activity of eluted LDH varied between 130 and 230 units/mg protein, depending on the purity of the starting material.

Procion dye	*Binding capacity (units/g moist gel)*	*Eluted LDH activity (%)*	*Purification factor*
Orange MX-G	400–690	60–70	2.5
Scarlet MX-G	500–770	60–80	3
Scarlet HR-N	400–600	60–80	4
Red MX-8B	500–650	50–70	3
Red H-3B	500–650	70–85	3
Red S6	350–500	70–80	4.5

TABLE II

BINDING AND ELUTION BEHAVIOUR OF LDH ON PROCION SCARLET MX-G-(SPACER)-BEADED CELLULOSE

Experimental details as in Table I. The LDH was desorbed by 1 *M* KCl followed by NAD–sulphite. Before use of NAD–sulphite containing buffer the column was washed free of KCl. Procion Scarlet MX-G-(NH-C_n-NH)COO-cellulose.

n	*Binding capacity (units/g moist adsorbent)*	*Activity of bound LDH (%)*		
		Elution with 1 M KCl	*Followed subsequently with NAD–sulphite*	*Elution with NAD–sulphite alone*
2	310–350	20–60	42–16	80–100
6	350–400	5–20	85–65	80–95
10	350–450	1– 3	95–85	90–100

diaminodecyl-beaded cellulose using ethylene glycol (50%, v/v), but the elution did not occur if affinitity adsorbents with a shorter spacer length were applied.

In Table III the desorption of LDH from Cibacron Blue F3G-A and Procion Red HE-3B coupled either directly to cellulose or via a spacer is shown. The elution of LDH by 1 *M* potassium chloride was significantly hindered if the diaminohexyl spacer between the dye and the cellulose was introduced, whereas the specific elution of LDH with NAD–sulphite was not changed. In addition, no difference in the specific activity were found. The purification of the enzyme was 4–5-fold in both types of dye–affinity matrices.

The different behaviour of directly coupled and spacer-mediated Cibacron Blue F3G-A with respect to the elution of LDH was used to separate LDH and MDH in a crude heart muscle extract. Both affinity adsorbents bound MDH with 2–4-fold higher capacity compared with LDH; 90–95% of the bound activities of MDH and LDH were recovered by desorption with salt from the directly bound dye–absorbent with

TABLE III

DESORPTION OF LDH FROM CIBACRON BLUE F3G-A AND PROCION RED HE-3B DIRECTLY BOUND TO BEADED CELLULOSE (BC) OR ATTACHED VIA A DIAMINOHEXYL SPACER

Experimental details as in Tables I and II.

Adsorbent	*Activity of bound LDH (%)*	
	Elution with 1 M KCl	*Elution with NAD–sulphite*
Cibacron Blue F3G-A–BC	95	95
Cibacron Blue F3G-A–(C_6)–BC	10–30	90
Subsequent elution with by NAD–sulphite	50–80	
Procion Red HE-3B–BC	95	95
Procion Red HE-3B–(C_6)–BC	5–30	70–90
Subsequent elution with NAD–sulphite	30–60	

slow retardation of LDH. Applying spacer-linked Cibacron Blue F3G-A, 85% of bound MDH and only 20% of adsorbed LDH were eluted by 1 *M* potassium chloride, resulting in a 3-fold enrichment of MDH.

To study the function of the spacer for the binding of LDH, three adsorbents with extension arms differing in length and in the terminal group were prepared (Fig. 1). No binding of LDH was observed with adsorbents carrying a hydrophobic butyl residue (Fig. 1B), as studied at pH 7.0 and 5.5. However, LDH was bound by beaded cellulose liganded with hexyl or decyl residues (binding capacity 200–400 units per gram of moist adsorbent). Partial desorption of the enzyme from hexyl- and decylcellulose (45% and 11% of the bound LDH, respectively) was achieved only if ethylene glycol-containing buffer (50%, v/v) was used. Potassium chloride and NAD–sulphite were unable to desorb the enzyme.

If a hexylamino residue was coupled to activated cellulose (Fig. 1C), this modified adsorbent bound LDH at both pH 5.5 and 7.0 without a difference in the binding capacity (300–500 units per gram of moist absorbent). Under both conditions 70–100% of the activity can be desorbed by 1 *M* potassium chloride but not by NAD–sulphite or ethylene glycol.

As an alternative ligand, caproic acid was attached to beaded cellulose (Fig. 1D). This adsorbent did not bind LDH at pH 7.0 but showed an interaction with LDH at pH 5.5. Elution of the enzyme occurred specifically with NAD–sulphite (50–60% of the bound enzyme) or with ethylene glycol (20% of the bound enzyme).

DISCUSSION

Triazine dyes of different classes are well established as biomimetic ligands in affinity separation techniques. Owing to the chemical reactivity, most of the dyes have been coupled to matrices by forming a covalent bond with the support via the triazinyl ring of the dye.

In a previous paper[9] we showed that several triazine dyes having the potential to interact with LDH do not bind the enzyme if they are coupled directly to beaded cellulose. The binding of the enzyme to the dyes, however, was achieved by the introduction of a spacer between the matrix and the affinity ligand. Among the group of analogues of Procion Scarlet MX-G no significant changes in the binding capacity, recovery or purity of the enzyme were observed. On changing the spacer length from two to ten methylene groups, the degree of non-specific desorption of LDH with potassium chloride decreased drastically (Table II). This means that in addition to specific interactions realized by the dye chromophore and specific domains on the surface of the enzyme, a second type of binding forces is generated which points to hydrophobic interactions.

This suggestion was confirmed by the results summarized in Table III, in which the elution behaviour of LDH from Cibacron Blue F3G-A– and Procion Red HE-3B–cellulose are directly compared with respect to the mode of dye coupling. Both types of affinity adsorbents were able to bind LDH. However, the introduction of a diaminohexyl spacer diminished the non-specific desorption of LDH by potassium chloride drastically and the main part of the enzyme was desorbed specifically by NAD–sulphite. This allows the assumption that after weakening the specific binding sites by the competitive inhibitor the enzyme might rearrange the conformation in such a manner that the hydrophobic interaction is also abolished.

In contrast, MDH can be displaced by salt from the dye–spacer–cellulose. The introduction of a spacer arm does not retard the enzyme to the same extent as LDH. The results support the findings of Lowe[6] that the increase in the strength of the binding is related to the apparent molecular weight of the protein. In order to establish the intrinsic effect of the spacer on binding and elution of LDH, the matrix was coupled with aminoalkanes of different chain length (n= 2–10). The results demonstrate that aliphatic spacers with more than four carbon atoms are able to bind LDH by pure hydrophobic interaction. However, the introduction of a terminal amino group obviously changed the mode of interaction. The enzyme was bound at pH 5.5 and 7.0 and was eluted by potassium chloride but was not desorbed by NAD–sulphite and ethylene glycol. Because of the positive charge of the terminal amino group ($pK \approx 10$), a poor ion-exchange mechanism is assumed to be responsible for the behaviour of LDH. On the other hand, the introduction of a terminal carboxylic group ($pK_{COOH} \approx 4.8$) in the aliphatic chain revealed no interaction of LDH with the liganded cellulose at pH 7.0, probably owing to repulsion of the negative charge of the LDH used, which is composed mainly of isoenzymes 1 and 2. The binding of LDH at pH 5.5 may be caused by electrostatic interaction between the negatively charged carboxylic group and positively charged groups of the enzyme which become dominant at pH 5.5 in addition to hydrophobic interaction. The ability of NAD–sulphite to elute LDH also from this type of adsorbent suggests that the formation of the ternary complexes becomes dominant and weakens both the electrostatic and hydrophobic interactions either directly or indirectly by a conformation change of the protein.

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Competitive elution of lactate dehydrogenase from Cibacron Blue–bead cellulose with Cibacron Blue–dextrans

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ABSTRACT

The efficiencies of elution of lactate dehydrogenase (LDH) from Cibacron Blue (CB)–bead cellulose with eluents ensuring competitive (Cibacron Blue–dextran), biomimetic (NADH) and displacing (KCl) mechanisms were compared. Competitive elution with CB–dextran T 10 was shown to be the most effective providing a 38 fold purified enzyme in 83% yield. As shown by fast protein liquid chromatography and polyacrylamide gel electrophoresis, this LDH preparation was free from protein contaminants but contained CB–dextran. CB–dextran was then removed by ion-exchange chromatography and the yield of LDH decreased to 62%. When using a longer colum, the enzyme was resolved partially in two fractions. The isoelectric point of the main fraction was 7.3.

INTRODUCTION

The use of dye-ligand chromatography has expanded in several directions, *e.g.*, with the rational development of new dyes[1,2] and their derivatives[3] and the search for a universal strategy for their testing[4,5]. In a search for the most effective elution strategy, attention was focused on the utilization of competition between immobilized and mobile dyes for the same binding site of the enzyme[6,7]. The efficiency of competitive elution was evaluated from parameters determined by analytical zonal chromatography[6,7] and batchwise adsorption[6].

This paper describes a study of competitive elution of the enzyme under the conditions of preparative dye-ligand chromatography. For this study, as in previous work[6,7], Cibacron Blue 3G-A (C. I. Reactive Blue 2) was chosen as the effective component of both the immobilized dye-ligand and the competitive eluent. Cibacron Blue (CB) was used in the conjugated form, namely with porous bead cellulose

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(immobilized dye-ligand) and with dextrans of the T series (competitive eluent). Lactate dehydrogenase (LDH) from beef muscle was the enzyme that provided biomimetic interaction with dye-ligands. All three basic components of the model system, *i.e.*, reactive dye (CB), dye-ligand matrix (bead cellulose), and enzyme (LDH) from various sources have been used previously in systematic studies of dye-ligand chromatography[6–9].

EXPERIMENTAL

Materials

Bead celluloses Ostsorb B [porosity 90%, dry weight 12.5% (w/w), particle diameter in the range 100–400 μm] and Ostsorb DEAE [ion-exchange capacity 1.1 mmol g^{-1}, dry weight 25% (w/w)] were obtained from Spolchemie (Ústí n/L, Czechoslovakia), dextran T 10 from Pharmacia (Uppsala, Sweden) and low-molecular-weight dextran substance (molecular mass 4000) and dextran 70 Spofa pulvis (molecular mass *ca.* 60 000) from Biotika (Slovenská Ľupča, Czechoslovakia). Cibacron Blue 3G-A (C.I. Reactive Blue 2) was kindly provided by Ciba Geigy (Basle, Switzerland). Coomassie Brilliant Blue G-250 was purchased from Serva (Heidelberg, F.R.G.).

Ostsorb B and the dextrans were derivatized (under base catalysis) with Cibacron Blue 3G-A at 80°C[6,9]. The degree of substitution (DS) was determined spectrophotometrically either in aqueous solution (CB–dextrans) or in cadoxene [cadimium tris-(ethylenediamine)-hydroxide] solution (CB–bead cellulose) at 610 nm[9] and/or 630 nm[6]. An extract from beef flank muscle[6], after filtration and lyophilization, contained *ca.* 4.4 units of LDH (L-lactate:NAD oxidoreductase, E.C. 1.1.1.27) per milligram of solid material. This material was used throughout this work.

Methods

The activity of LDH was established spectrophotometrically[6,10]. The protein concentration was established by the method of Bradford[11]. Zonal chromatography was performed[6] at 25 $\pm$ 0.5°C using an immobilized dye column (29 $\times$ 1.1 cm I.D.) which was equilibrated and developed with a fixed concentration of NADH or dye–dextran T 10 conjugate in 20 m*M* phosphate buffer (pH 8.5) at a flow-rate of 15 ml h^{-1}. Chromatographic analyses were started by application of 200 μl (42 mg of lyophilizate) of LDH (*ca.* 200 U). The results obtained in zonal chromatography were analysed using the equation for monovalent interactions[6,12].

Elution experiments

Dye-affinity chromatography of LDH on CB–bead cellulose (DS = 1.155 mmol l^{-1}) was performed at ambient temperature using a column (12 $\times$ 1.1 cm I.D.) equilibrated with 20 m*M* phosphate buffer (pH 8.5). Chromatography was started by application of 5 ml of a solution of crude LDH (500 mg of lyophilizate). The unbound proteins were washed out with equilibration buffer (*ca.* 300 ml). The elution of LDH was performed using three different eluent solutions of KCl (3 *M*), NADH (1 m*M*) or CB-dextran T 10 (50 μ*M*) in equilibration buffer. The flow-rate was 35 ml h^{-1} and 5-ml fractions were collected. The fractions with maximum activity of LDH were pooled and subjected to repeated ultrafiltration on Ultracell Amicon YM 10 immediately

after elution (KCl, NADH) or after removing CB–dextran on a DEAE-bead cellulose column (5 × 1.1 cm I.D.). The products were stored at −18°C in 50% glycerine.

Ion-exchange chromatography

Chromatography was performed on a DEAE-bead cellulose column (11.5 × 1.5 cm I.D.) at ambient temperature. The column was equilibrated with 20 m*M* phosphate buffer (pH 8.5) and loaded with 10 ml of a solution of LDH (purified on CB–bead cellulose by elution with CB–dextran T 10). LDH was then eluted with a linear gradient of 0–0.1 *M* ammonium sulphate in equilibration buffer. The flow-rate was 42 ml h^{-1} and 10-ml fractions were collected. For stabilization of the enzyme during ultrafiltration and chromatography on DEAE-cellulose it was advantagenous to add L-cysteine (5 m*M*) and EDTA (1 m*M*) to the LDH solution.

Fast protein liquid chromatography (FPLC)

The purity of LDH was checked by size-exclusion chromatography. Pharmacia FPLC equipment with a standard prepacked column (30.0 × 1.0 cm I.D.) of Superose 12 HR 10/30 was used. The column was preconditioned as follows: eluting buffer 50 m*M* phosphate (pH 7), flow-rate 30 ml h^{-1}, UV absorbance monitored at 280 nm, ambient temperature. Fractions of 0.5 ml were collected in order to measure the catalytical activity of LDH.

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed according to the method of Laemmli[13] using a gel which contained 12.5% of acrylamide. Proteins were stained with Coomassie Brilliant Blue G-250. Ultrathin-layer isoelectrofocusing in polyacrylamide gels on polyester films was performed according to Radola[14]. Proteins were stained with Coomassie Brilliant Blue G-250.

RESULTS AND DISCUSSION

The results of zonal analytical chromatography of LDH on CB–bead cellulose are summarized in Table I. From these data it follows that, in the system CB–cellulose–LDH-mobile ligand, there are significant differences in the $K_{M\text{-}L}$ values, representing the interaction of LDH with CB–dextran T 10 and of LDH with NADH. The values presented suggest that CB–dextran should be a more effective competitive eluent than NADH. However, the elution profiles of LDH are dependent on the concentration of the dye immobilized on bead cellulose. At a low concentration of CB (137.6 μmol l^{-1}) the elution with CB–dextran T 10 is characterized by a single LDH peak. When the used bead CB–cellulose was substituted to a greater extent (1155 μmol l^{-1}), double peaks occurred in the elution profiles of LDH in competitive elution with CB–dextran T 10 (not shown). They were ascribed[7] to partial separation of LDH isoenzymes.

LDH was eluted from CB–cellulose (1155 μmol l^{-1}) by CB–dextrans differing in average molecular mass (4000, 10 000 and 60 000; Fig. 1). The differences in DS values of these three CB–dextrans (see Experimental) were compensated by uniform concentration of the dye (50 μ*M*) in the eluent. The best elution profile and almost total

TABLE I

DISSOCIATION CONSTANTS K_{M-L} AND K_{I-L}

K_{M-L} represents the dissociation constant of the LDH–immobilized dye complex and K_{I-L} the dissociation constant of the LDH–mobile dye complex.

CB-cellulose: concentration of immobilized dye (μmol l^{-1})	*Mobile ligand*	K_{M-L} (μM)	K_{I-L} (μM)
137.6	CB–dextran T 10[a]	9.1	1.6
	NADH	200.0	1.8
1155.0	CB–dextran T 10[a]	20.0[b]	3.15[b]
		8.0	1.0

[a] The CB–dextran T 10 as the mobile dye was 130 μmol g^{-1}.
[b] Dissociation constants calculated from double peak in the elution profiles.

recovery (93%) of LDH was achieved after elution with CB–dextran T 10 (Fig. 1).

The desorption effect of CB–dextran T 10 was compared with that of KCl and NADH. The elution profiles of LDH were similar (Fig. 2) despite the fact that the eluents were applied in very different concentrations, *i.e.*, 50 μM CB–dextran T 10, 1 mM NADH and 3 M KCl. However, the yields of LDH were different (Table II). They decreased in the same order as the concentrations of the eluents increased. The separation effect achieved with all eluents was sufficiently strong to provide an almost equally purified (38-fold) LDH (Table II). This was also shown by chromatograms of the purified LDH obtained by size-exclusion FPLC. The chromatograms were almost identical in all three instances. They revealed only one peak at the same position corresponding to active LDH, as is demonstrated by the chromatogram obtained after elution with 50 μM CB–dextran T 10 (Fig. 3). Purification of LDH was confirmed also by the SDS-PAGE method (Fig. 4).

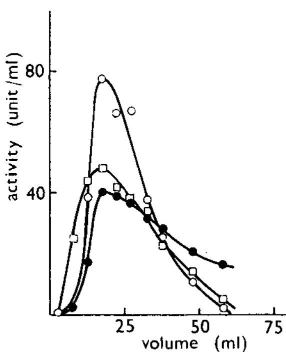

Fig. 1. Elution of LDH from CB–bead cellulose by using CB–dextrans as eluting agent. The elution of LDH was performed after washing out the unbound proteins from a 10-ml column of CB–bead cellulose with equilibration buffer; 500 mg of crude enzyme (2100–2200 U) was loaded onto this column. For elution of bound LDH solutions of CB–dextrans of various molecular mass were used: (○) CB–dextran T 10; (□) CB–dextran 60 000; (●) CB–dextran 4000.

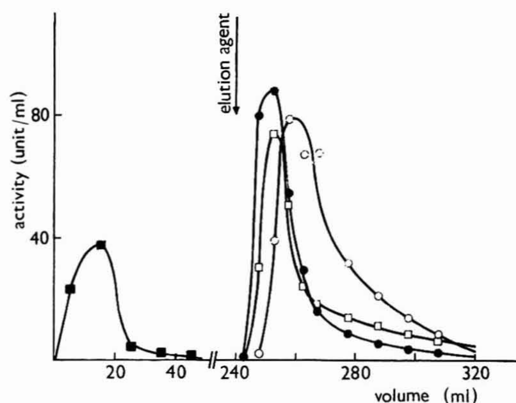

Fig. 2. Elution profiles of beef muscle extract from CB–bead cellulose. Eluting agents: (□) 3 *M* KCl; (●) 1 m*M* NADH; (○) 50 *μM* CB–dextran T 10; (■) equilibration buffer.

The most effective means of separating of LDH from CB–dextran was shown to be ion-exchange chromatography on DEAE-bead cellulose. Here CB–dextran is adsorbed on the ion-exchanging cellulose. It was found further that by using a sufficiently long column and gradient elution with ammonium sulphate, it is possible to remove CB–dextran and, at the same time, to resolve LDH into two fractions. Fig. 5 shows the results of the gradient elution of LDH by ammonium sulphate (0–0.1 *M*) from the DEAE-bead cellulose column (20 ml). Both LDH fractions are free from protein contaminants, as indicated by their FPLC traces (not shown). However, from the elution profile in Fig. 5 it follows that the separation of these two fractions was not

TABLE II

COMPARISON OF COMPETITIVE, BIOMIMETIC AND DISPLACING ELUTION OF LDH FROM CB–BEAD CELLULOSE

The solution of 500 mg of crude LDH in 5 ml of buffer (2100–2200 units, specific activity 4.4 units/mg solid) was loaded on an equilibrated CB–bead cellulose column (10 ml, concentration of immobilized dye = 1155 μmol l^{-1}) and the unbound proteins were washed out with equilibration buffer. The bound LDH was eluted with one of the solutions of KCl, NADH or CB–dextran T 10 each in equilibration buffer. Separation of CB–dextran T 10 from LDH was performed on a DEAE-bead cellulose column (4 ml) with equilibration buffer.

Eluent	*Volume (ml)*	*Activity (units ml^{-1})*	*Total activity (units)*	*Specific activity (units mg^{-1})*	*Yield (%)*	*Purification factor*
Equilibration buffer	250	1.2	300	1.2	13.6	0.14
3 *M* KCl	70	20.2	1417	329.5	66.2	38.8
1 m*M* NADH	70	23.5	1644	328.8	79.8	38.7
50 *μM* CB–dextran T 10	50	34.8	1740	–[b]	82.85	–[b]
$(NH_4)_2SO_4$ gradient[a]	70	18.5	1294	306.0	61.6	36.0

[a] Performed by linear gradient elution (0–0.1 *M*).

[b] Not determined.

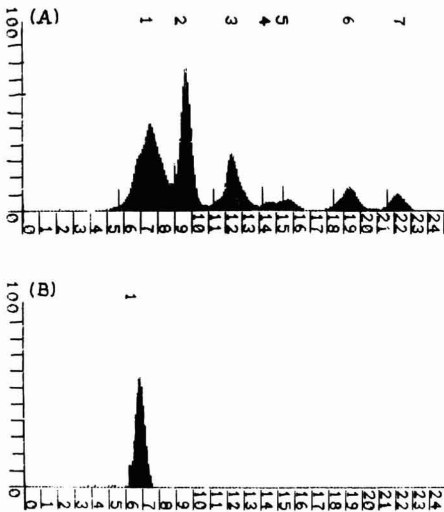

Fig. 3. Elution profiles of (A) crude and (B) purified LDH recorded with the aid of FPLC equipment. The absorbance of the effluent was monitored at 280 nm (ordinate); the numbers on the abscissa signify fraction numbers. The column (Superose 12 HR 10/30) was loaded with 20 U of LDH contained in 0.2 ml.

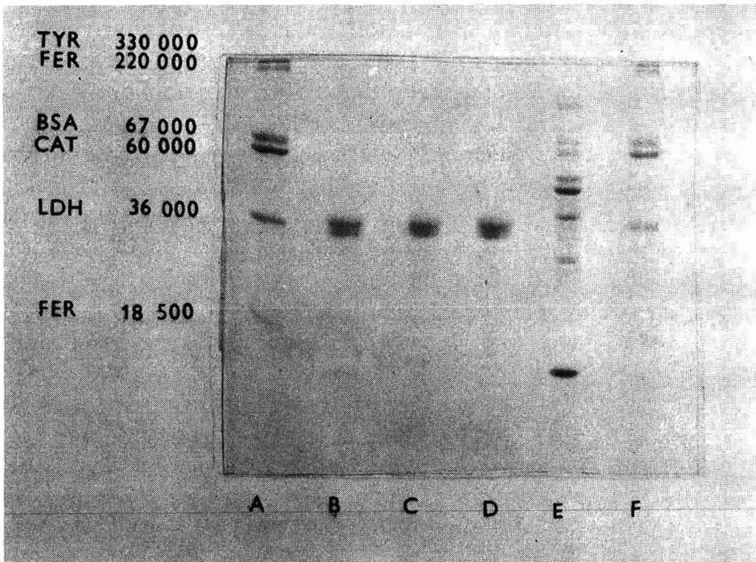

Fig. 4. SDS-PAGE of LDH. The gel consisted of 12.5% acrylamide in Tris–glycine buffer. Gels were stained with 0.2% (w/v) Coomasie Brilliant Blue G-250 in methanol–acetic acid–water (50:10:40) for 6 h, then destained in the same solvent for the same time. Samples A and F, Pharmacia electrophoresis calibration kit containing TYR = hog thyroid thyroglobulin, FER = horse spleen ferritin, CAT = beef liver catalase, LDH = beef heart lactate dehydrogenase and BSA = bovine serum albumin. Sample B, product from NADH elution. Sample C, product from KCl elution. Sample D, product from CB–dextran elution. Sample E, crude LDH from beef muscle.

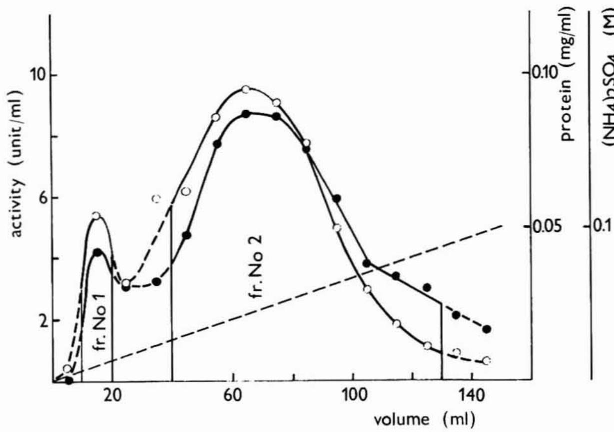

Fig. 5. Elution profile of LDH on DEAE-bead cellulose column. Ion-exchange chromatography was performed on a 20-ml DEAE-bead cellulose column by linear gradient elution with 0–0.1 M $(NH_4)_2SO_4$ in phosphate equilibration buffer; 10-ml fractions were collected and analysed for (○) LDH activity and (●) protein. The two main fractions (1, 10 ml; 2, 90 ml) were pooled.

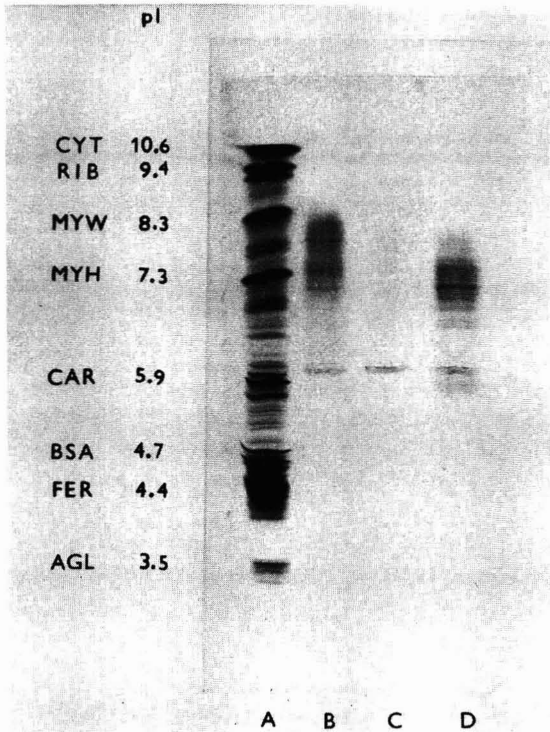

Fig. 6. Ultrathin-layer isoelectrofocusing of LDH in 50 μM polyacrylamide developed gel on silanized polyester film. Gel: 5% T, 3% C, Servalyt pH 3–10 carrier ampholyte 3%. Protein staining with Coomassie Brilliant Blue G-250. Sample A, mixture of marker proteins: CYT = cytochrome *c*; RIB = ribonuclease; MYW = sperm whale myoglobin; MYH = horse myoglobin; CAR = carbonic anhydrase; BSA = bovine serum albumin; FER = horse spleen ferritin; AGL = amyloglucosidase. Sample B, product from CB–dextran elution. Sample C, fraction No. 1 and sample D, fraction No. 2 from ion-exchange chromatography of LDH.

satisfactory. Therefore, only those pooled parts of both fractions which would least interfere with each other were subjected to isoelectric focusing. After this procedure, only fraction 2 (Fig. 6) could be analysed satisfactorily by isoelectric focusing. This fraction was enriched by an isoenzyme of LDH with a lower isoelectric point (p*I* 7.3).

Removal of the competitive eluent (CB–dextran T 10) and simultaneous resolution of LDH into two fractions by ion-exchange chromatography was accompanied by loss of material and a decrease in specific activity. These losses were minimized (20% and 7%, respectively) when CB–dextran was removed on a short column (4 ml) of DEAE-bead cellulose (Table II).

It can be concluded that competitive elution of LDH from CB–bead cellulose with CB–dextran T 10 is more effective than biomimetic (NADH) and displacing (KCl) elutions. Removal of CB–dextran after elution is accompanied by a loss of material and a decrease in specific activity. Despite these negative aspects, competitive elution may be utilized in dye-ligand chromatography mainly in those instances when difficulties are encountered in trying to elute a protein from too tight a binding to the immobilized dye.

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Centrifugal affinity chromatography

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ABSTRACT

A new technique termed centrifugal affinity chromatography (CAC) is presented in this paper. CAC combines a high flow-rate, created by centrifugal force, with the specificity of affinity chromatography. This technique has been used for the purification of human immunoglobulin G. Furthermore this technique has been used to remove human albumin from serum and the effect of centrifugal force, ionic strength and pH has been studied. A test for determining the percentage of glycosylated hemoglobin in hemolysates has also been developed. This test, employing centrifugal chromatography, is more than three times faster than commonly used gravity flow methods.

INTRODUCTION

We have designed a simple and rapid technique, centrifugal affinity chromatography (CAC), which is an extension of centrifugal chromatography. Several authors have described the use of centrifugal chromatography for ion exchange and gel permeation[1] but a centrifugal force has never been used before for performing affinity chromatography processes.

High-performance liquid affinity chromatography (HPLAC) has been widely used to analyse the protein content of various samples. HPLAC is best utilized for the routine analysis of multiples of the same, or similar, samples utilizing the same column and HPLAC requires considerable technological expertise. The cost of HPLAC is a serious draw-back for a laboratory where only occasional applications are needed. Conversely, affinity chromatography, based on gravity flow, can be very time consuming when many different columns must be screened. CAC can be a good alternative for these techniques. The rapid flow-rate created by centrifugal force saves time compared with gravity flow chromatography. CAC is extremely easy to perform and requires little training of the users.

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As an initial example, we employed CAC to purify human immunoglobulin G (IgG) on Protein A columns. The second system we studied was the specific removal of human albumin from human serum using Blue-Trisacryl® gel[2–5]. The system was optimized with regard to the centrifugal force, binding-time, ionic strength and pH.

Finally, CAC was adapted to a test for the determination of the percentage of glycosylated hemoglobin and was found to be superior to gravitiy flow methods[6,7].

EXPERIMENTAL

Materials

Centrex® columns, Affinica® gel and Affinica equilibration, elution and regeneration buffers were the kind gift of Schleicher & Schuell (Keene, NH, U.S.A.). the 5-ml columns contained a supporting cellulose acetate membrane (pore-size: 0.2 mm in experiments with human albumin experiments; pore-size: 0.45 mm in case of experiments utilizing human IgG and diabetic hemoglobin) and the columns themselves were made of polypropylene.

The centrifuge used was the IEC Model HN-SII (Intl. Equipment, Needham Heights, MA, U.S.A.). The centrifugal force was determined from the radius, at the membrane-level, of each of the centrifugal columns.

In the human IgG purification experiments the Centrex columns were filled with 1-cm settled bed-height of Affinica Protein A gel.

Blue-Trisacryl gel from IBF (Reactifs IBF Soc. Chim. Pointet Girard, Villeneuve la Gareneu, France) was used to remove human albumin from blood serum (1 cm settled bed-height was used in experiments with purified human albumin, 2 cm settled bed-height in experiments with whole serum). The maximum binding capacity of this gel, as described by the manufacturer, was 15 mg of human serum albumin/ml settled gel. Experiments were performed at saturating amounts unless otherwise specified. To compare CAC analysis of glycosylated hemoglobin with the gravity flow system, the Pierce Glyco Test, utilizing the instructions from the manufacturer, was used as the comparative standard.

For the determination of glycosylated hemoglobin, the wash buffer consisted of 0.25 *M* ammonium acetate and 0.05 *M* $MgCl_2$, adjusted to pH 8.5 with hydrogen chloride. The elution buffer contained 0.1 *M* Tris · HCl and 0.2 *M* sorbitol, adjusted to pH 8.5. Columns were regenerated by washing with 0.05 *M* acetic acid. All buffers contained 0.02% sodium azide.

Human IgC was purified from serum by ammonium sulfate precipitation and ion-exchange chromatography[8] and stored in 5 m*M* phosphate buffer pH 6.8 and 0.02% sodium azide. Purfied human albumin (25% solution) was the kind gift from the Waco Chapter of the American Red Cross. For hemolysis, human blood (0.1 ml) was mixed with 0.9 ml sodium phosphate KCN buffer (pH 6.7) and incubated for 10 min with gentle shaking.

As standards we used glycosylated hemoglobin controls from Pierce (Rockford, IL, U.S.A.).

Methods

In the purification of human IgG, Protein A affinity columns were centrifuged between each step for 1 min at 600 *g*. Initially the columns were equilibrated with

Affinica equilibration buffer (5 × 1 ml). Subsequently, 4 ml, containing about 4 mg of human IgG, was applied to the column, after which the columns were centrifugally washed with the same buffer. In the last step, human IgG was eluted with 2 × 3 ml elution buffer.

To prepare Blue-Trisacryl centrifugal columns for the removal of albumin from human serum, they were first treated with 6 *M* guanidine hydrochloride (3 × 1 ml). This treatment removed any excess dye which may have been physically absorbed to the column and permitted a contamination-free product to be obtained. Between each column application, the columns were centrifuged for 2 min at 600 *g*. After the first treatment, the columns were equilibrated in 0.025 *M* citrate (pH 4) or 0.025 *M* phosphate buffers adjusted to various ionic strengths with sodium chloride and to various pH values (pH 5.2, 7.2, 9). Subsequently, 0.5 ml of a 25% pure human albumin solution diluted eight fold with the same buffer was added to the columns (whole blood serum was 10 times diluted). After this, columns were washed twice with the same buffer used to equilibrate the columns (2 × 3.5 ml). Finally the human serum albumin was eluted with 0.5 *M* KSCN (2 × 2.5 ml).

Electrophoretic analysis was done in a Protean cell (BioRad Laboratories, Richardson, CA, U.S.A.) on a 10% polyacrylamide sodium dodecyl sulphate (SDS) denaturing gel by the method of Laemmli[9].

Before analyzing the elution fractions by electrophoresis they were concentrated eight fold utilizing the Centricon 10 micro concentrator (Amicon, MA, U.S.A.). A 10% acrylamide SDS gel was used to analyze the completeness of the removal of human serum albumin from the samples[8]. Staining was performed with 0.1% Coomassie Blue dissolved in 5% glacial acetic acid containing 10% methanol.

Determination of glycosylated hemoglobin was performed by a modification of the Pierce gravity-flow glycosylated hemoglobin procedure. Centrifugal columns containing Pierce Glycogel B were initially equilibrated with 2 ml of wash buffer and subsequently 50 ml of hemolysate was applied to each column. After this, the columns were washed twice with wash buffer and the non-bound fraction was collected. Between each wash the columns were spun at 160 *g* for 3 min. The receiver tubes were then changed and the bound glycosylated hemoglobin fraction was eluted with 3 ml of elution buffer for each column. The amount of glycosylated and non-glycosylated hemoglobin was spectroscopically measured at 414 nm.

RESULTS AND DISCUSSION

Human IgG was the first material to be purified in our laboratory using CAC. At least 85% of human IgG was bound to the Affinica gel columns when an amount of human IgG comparable with the maximum capacity (15 mg human IgG/ml settled gel as determined by the manufacturer) was applied. The residence time was altered by changing the incubation time for the human IgG sample in the column before centrifugation. No influence of the binding time was detected. The elution time was varied by changing the length of time for the incubation of the Affinica gel columns with elution buffer prior to centrifugation. Such alterations had no significant influence on the amount of human IgG recovered in the elution fractions. The minimum achievable residence time was 1 min and 20 s. This time consisted of 20 s for fluid application and 1 min for centrifugation and braking.

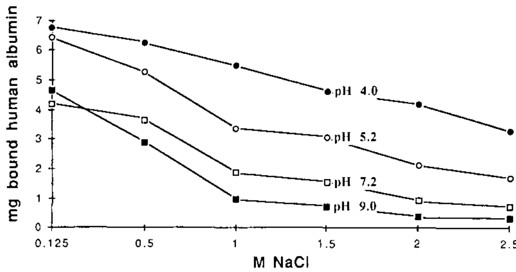

Fig. 1. The binding of purified human albumin to Blue Trisacryl during centrifugal affinity chromatography (600 *g*) at various pH values and ionic strengths.

The removal of human serum albumin from blood serum was the second example of CAC which we investigated. To study the influence of ionic strength and pH on the binding of human serum albumin to Blue-Trisacryl gel during CAC we applied purified human serum albumin to these columns. More human serum albumin was bound to the Blue-Trisacryl gels at lower ionic strength and pH (see Fig. 1). The same experiment was performed with whole serum. At pH 5.2 and 0.125 *M* sodium chloride, all serum proteins seemed to bind to the column. In order to get a more specific separation of human serum albumin from other blood proteins, a higher pH and salt concentration had to be employed. The best selective removal of human serum albumin from other blood proteins was achieved at pH 7.2 and 1 *M* sodium chloride (Fig. 2, lane 1). It is easily seen from Fig. 2 that it is possible to use CAC to completely remove human serum albumin from blood serum. The electrophoresis of concentrated whole serum is precluded by the presence of enormous quantities of human serum albumin which creates a long smear pattern on the surface of the electrophoresis gel and obliterates many other serum components. However, after CAC-based removal of human serum albumin from serum, the samples can be considerably concentrated prior to electrophoresis. This permits the detection of protein bands which would be otherwise obliterated.

Changing the flow-rate by increasing the applied centrifugal force from 350 to 600 *g* had no significant influence on the binding of purified human albumin to the columns. Alternatively, the flow-rate through the column can be changed by changing the membrane pore-size. Varying the membrane pore-size from a molecular-weight cut-off from 1 000 000 to 100 000 dalton did not significantly alter the binding of human serum albumin to Blue-Trisacryl columns. These data show that the flow-rates utilized in these experiments permit maximum binding of human serum albumin to the Blue-Trisacryl gel. It can also be noticed that some cracking of Blue-Trisacryl gel occurred occasionally.

To provide another example of the application of CAC we developed a test to determine the percentage of glycosylated hemoglobin in hemolysates utilizing a centrifugal column filled with boronate gel. Fig. 3 shows the profile of non-glycosylated and glycosylated hemoglobin in this CAC-based test. This suggests the intriguing possibility that CAC can be used for monitoring diabetes mellitus in clinical laboratories since the results obtained with the CAC-based test and the gravity based test were similar (Fig. 4), but the centrifugal method was three times faster. The resulting

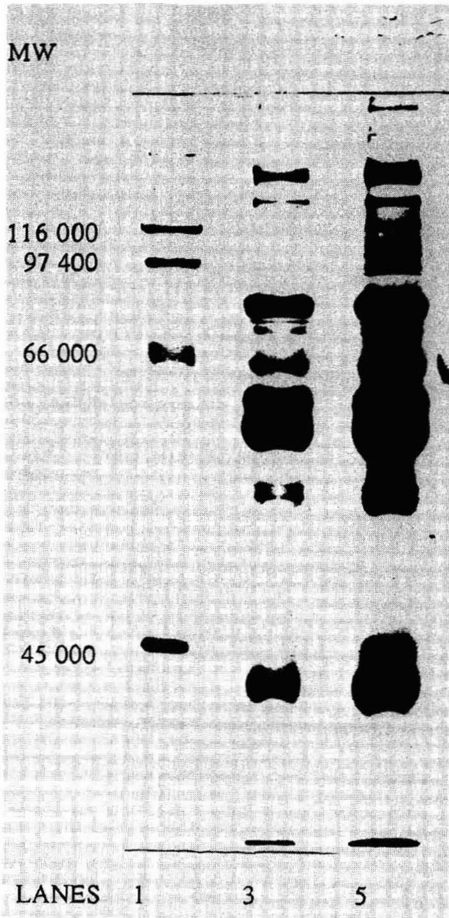

Fig. 2. The removal of human albumin from whole serum using centrifugal affinity chromatography (pH 7.2, 1*M* sodium chloride Blue-Trisacryl gel). Lane 1 contains markers, lane 3 human serum from which albumin has been removed prior to an eight-fold concentration and lane 5 contains whole serum. [Markers were: ovalbumin, molecular weight (MW) = 45 000; bovine serum albumin, MW = 66 000; phosphorylase B, MW = 97 400; and β-galactosidase (*E. coli*), MW = 116 000.]

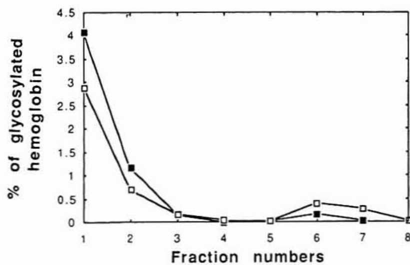

Fig. 3. The profiles of non-glycosylated (1–5) and glycosylated (fractions 6–8) hemoglobin of diabetic (□) and non-diabetic (■) hemolysates.

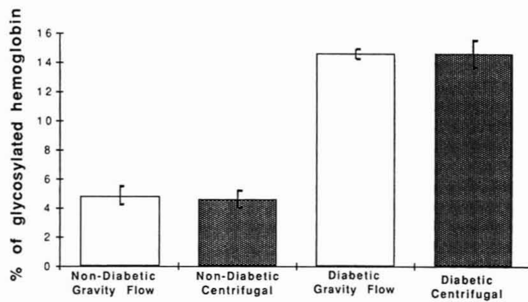

Fig. 4. Comparison of the centrifugal affinity chromatography based test for the detection of the percentage glycosylated hemoglobin in standard hemolysates with a gravity based method. The percent of glycosylated hemoglobin was determined using normal and diabetic standards (Pierce) as described in the text.

reduction of work time could reduce the labor costs significantly and, therefore, the centrifugal based method would be less expensive than the commonly used gravity based method.

In the above described experiments some cracking of the gel was noticed. Scouten and Elhardt[10] described cracking during gel permeation chromatography. They selected shrinking and cracking resistant matrices for gel permeation chromatography to prevent this problem. During CAC, however, cracking may even be an advantage. Cracking can explain the low binding and elution residence time needed in our experiments. When all of the interstitial water, and some of the intrastitial water, is removed from the beads, they shrink and finally the gel cracks. When a sample is subsequently applied to this column, an intimate absorption and contact between the sample and the column matrix occurs and, therefore, the effective local concentration is higher than in case of a normal chromatography column.

The advantage of CAC compared with gravity chromatography is not only the speed of analysis but also the fact that higher final product concentration can be obtained. During CAC, samples become less diluted since most of the interstitial, and even some of the intrastitial, water is removed. Furthermore, CAC is a very useful technique, unlike HPLAC, for the screening of different types of matrices, in that, using the HN-SII centrifuge, 18 different columns can be analysed simultaneously. Other centrifuges with other configurations may permit even larger number of column types, possibly hundreds, to be investigated simultaneously.

We have also noticed that the type of matrix and the applied centrifugal speed can be very important. The choice of matrix and the applied centrifugal speed are influenced by the hardness and the hydrophobicity of these beads. A matrix which is too rigid and/or too hydrophobic may have all of the fluid removed from the internal pores by the centrifugal process. Since it is difficult to rewet these types of matrices, some loss of capacity may occur. Conversely, poor yields may also be seen on elution due to the fact that some internal pore area will not be accessible to the elution buffer. Such matrices can be used but have to be employed at an appropriate, decreased centrifugal speed. Centrifuging such matrices until they lose all their interstitial fluid causes the development of air bubbles in the openings of such pores and subsequently they are inaccessible to exchange with the surrounding buffers. Conversely, a matrix which is excessively soft might collapse under the centrifugal force applied in the process and clog the membrane at the bottom of the column.

CONCLUSIONS

CAC has been demonstrated to be a very useful technique for many varied applications. The potential of this technique has been demonstrated in terms of the speed of analysis, ease of operation and costs of analysis. The purification of human IgG is an example of preparative application of CAC. The removal of human albumin from serum and the determination of the percentrage of glycosylated hemoglobin in hemolysates are both clinical applications. It is the authors' belief that CAC can be useful for many other applications, including the purification of nucleic acids, hydrophobic chromatography and dye chromatography. Unlike HPLAC, which is very useful for screening many samples on the same column, CAC is very useful for screening the same type of sample on many different columns, an obvious first step when one is trying to optimise an affinity chromatography system.

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Composite affinity sorbents and their cleaning in place

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ABSTRACT

Making large-scale affinity sorbents that are reusable under acceptable hygienic conditions implies specific treatments for cleaning in place with known aqueous solutions of chemical agents. However, common agents such as sodium hydroxide are frequently considered too drastic for the stability of macromolecular biologically active immobilized ligands. According to a large series of trials, it was found that only a mixture of sodium hydroxide and ethanol was actually effective in sterilizing a sorbent in a single step. When hydroxide or an ethanol–acetic acid mixture were used alone, they were not totally efficient in the inactivation of sporulated *Bacillus subtilis*. Conversely, they were efficient when used sequentially. All these solutions were able to remove pyrogens from chromatographic sorbents. As the sterilizing solutions contained a certain amount of ethanol, the most suitable chromatographic affinity sorbents had to be based on an incompressible matrix. When washing an affinity silica sorbent that had proteins as ligands with solutions such as sodium hydroxide, ethanol–acetic acid or ethanol–sodium hydroxide, it was found that certain sorbents were able to tolerate the treatments without a noticeable decrease in their biochemical activity.

INTRODUCTION

Scaling up affinity chromatographic systems implies repeated utilization of sorbents for the sake of economical exploitation of the technique. For the separation of therapeutic components, the sorbents must be washed periodically either to remove adsorbed undesirable substances (*e.g.*, pyrogens, pigments, lipids or protein aggregates) or for sterilization purposes. These treatments are well known in ion-exchange chromatography or in gel filtration but they still give problems in affinity chromatography, in particular when the ligand is not extremely stable. The proteins used as ligands (lectins, antibodies, etc.) may be denatured during the regeneration cycle and sterilization operations.

In this paper, we report preliminary results concerning the cleaning in place of composite silica–dextran supports bearing biologically active proteins. These supports, specially designed for preparative applications, are obtained by coating the

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available surface of porous silica with dextran molecules[1,2]. Once the complex has been stabilized by cross-linking, the affinity ligands are immobilized on the dextran part by classical methods[3–5]. We have found that these affinity sorbents are particularly resistant to acidic, alkaline or hydroorganic media. First, the silica is well proteced by the dextran layer against aggressive alkaline media. Second, the silica or the silica-based composite does not shrink in organic media, in contrast to clascical organic supports and particularly agarose-based supports.

During this study, we examined the depyrogenation and sterilization effect of three aqueous or hydroalcoholic solutions, and then studied the chromatographic behaviour of affinity supports bearing a proteic ligand in these solutions.

EXPERIMENTAL

Chemicals and biological materials

Silica porous microbeads were obtained from our production unit or purchased from Merck (Chelles, France) or Amicon (Epernon, France). Dextran cationic derivatives were obtained from Pfeiffer and Langen (Dormagen, F.R.G.), human IgG from Sigma (La Verpillère, France) and protein A from Fermentech (Edinburgh, U.K.). All other chemicals and biochemicals were purchased from Aldrich (Strasbourg, France) and Sigma.

Microorganisms such as *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans* and *Escherichia coli* were obtained from Institut Mérieux (Lyon, France) and limulus test from Hemachem (St. Louis, MO, U.S.A.).

Coating of silica with dextran

Dry silica beads with a porous volume of 1 cm^3/g and a mean pore size of 1200 Å were coated with dextran according to a previously described method[1,2]. Dextran had a molecular weight between 70 and 500 kDa and possessed a small amount of cationic groups (about 0.5–1%).

Briefly, the coating was done as follows: 25 g of dextran derivative were dissolved in 150 ml of 0.2 *M* carbonate buffer (pH 10.5), then 5 ml of butanediol diglycidyl ether were added with stirring, followed by 100 g of dry silica beads (40–100 μm). The suspension was then filtered under vacuum to remove the excess of the dextran solution. The gel was placed in a ventilated oven and kept at 80°C for 24 h, and the dry product obtained was repeatedly washed with water and acidic and alkaline solutions and finally stored in the presence of 1 *M* sodium chloride. Dextran-coated silica was ready for use as an affinity sorbent after activation of the dextran hydroxyl groups and ligand immobilization.

Immobilization of a protein as a ligand: IgG, concanavalin A and protein A

Dextran-coated silica was first activated with *p*-nitrophenyl chloroformate according to Wilchek's method[5,6] and then the proteins were immobilized in the presence of 0.2 *M* carbonate buffer (pH 8–9). The protein concentration before immobilization was 3 mg/ml for protein A and 10 mg/ml for human IgG and concanavalin A. The amount of immobilized protein was 2.5 mg/ml of sorbent for protein A, 7.5 mg/ml for immunoglobulins G and 8 mg/ml for concanavalin A. The sorbents obtained were stored in aqueous suspensions in the presence of 1 *M* sodium chloride and 0.02% sodium azide as a bacteriostatic agent.

Treatments for cleaning in place (sterilization and pyrogen removal)

Three separate treatments were performed to check their efficiency against four standard strains: 0.1–1 *M* sodium hydroxide, 60% ethanol–0.5 *M* acetic acid and ethanol (up to 60%)–0.2 *M* sodium hydroxide.

The strains used were *Bacillus subtilis* (sporulated form) ATCC 6633, *Candida albicans* 562, *Escherichia coli* ATCC 10536 and *Staphylococcus aureus* ATCC 14154.

The washing efficiency of the various treatments was determined by mixing a volume of chromatographic sorbent with $6 \cdot 10^6$–$7 \cdot 10^6$ microorganisms/ml and then mixed with three volumes of sterilizing solution. After incubation (up to 24 h at 25°C), the amount of remaining microorganisms was detected by standard culture. Briefly, after sterilization the supernatant was neutralized and diluted 5-fold with phosphate-buffered saline (PBS). Solutions (0.5 ml) to be tested were serially diluted into BTS (tryptase-soya sterile broth in a ratio of 1:10) in triplicate per dilution. The tubes were incubated at 33°C for 14 days. Observations were effected every 24 h. The determination of the number of microorganisms per millilitre was calculated according to Spearman-Karber[10].

Experiments were also done on-column; contamination was induced by injecting into the column inlet the strain suspension (see above) and then the decontaminating solution was perfused from the top to the bottom of the column for 4 h. The column was neutralized by washing with five volumes of PBS and then the gel was treated as indicated above.

The solutions used for microorganism sterilization were also studied to determine their efficiency in pyrogen removal. These operations were effected on-column. To remove the pyrogens from contaminated sorbent, the column filled with the sorbent was washed with the same solutions as used for the microorganism decontamination and the amount of endotoxin (pyrogens) was determined at the column outlet using the standard limulus lysate test.

Stability studies on affinity sorbents

The decontaminating solutions used for sterilization and pyrogen removal were specifically studied in association with immobilized protein A, immunoglubulins G and concanavalin A on dextran-coated silica. The sorption capacity of the affinity sorbents was determined after repeated cleaning cycles alternated with separation cycles.

In the case of protein A–dextran-coated silica, the column was repeatedly used for the separation of human IgG. The sorption was effected in physiological buffer (PBS) and the elution using 1 *M* acetic acid. Between runs, four volumes of sterilizing solution were passed through the column at room temperature. The linear flow-rate was 15 cm/h and the time of contact with the cleaning solution was about 45 min per run.

The column of immobilized IgG was used for the separation of rabbit anti-human IgG antibodies. An excess of total rabbit antiserum was injected into the column previously equilibrated with PBS. After washing, the antibodies were desorbed using 0.2 *M* glycine–HCl buffer (pH 2.8). Between runs, four column volumes of sterilizing solution were passed through the column at room temperature for about 15 min (linear flow-rate 15 cm/h).

Concanavalin A–dextran-coated silica was used for the separation of egg

albumin in 0.05 *M* Tris–HCl buffer (pH 7.4) containing 0.15 *M* sodium chloride and 1 m*M* calcium and manganese chloride. The elution was performed by adding 0.3 *M* α-methyl mannoside to the adsorption buffer. Between runs, four volumes of sterilizing solution were passed through the column at room temperature for about 45 min (linear flow-rate 15 cm/h).

RESULTS AND DISCUSSION

Sorbent synthesis

Coating porous silica beads with dextran was necessary to eliminate the well known non-specific protein adsorption. Indeed, silica has a very acidic character and most proteins are strongly adsorbed and frequently denatured.

Complete coating with a polysaccharide avoided direct contact between the protein and the silica acidic groups; this is clearly evidenced by the progressive decrease in the adsorption of cationic proteins such as cytochrome *c* (see Fig. 1). An increase in the dextran content lowered to zero the interaction between cytochrome *c* and the silica gel. The dextran layer also contributes to making the silica more resistant to strongly alkaline media and, further, makes easy the activation of polysaccharide-based supports involving the clasical reagents used in affinity chromatography. Although a large amount of dextran is beneficial to the elimination of non-specific adsorptions, it also diminishes the support porosity. The value of V_e/V_t (ratio of the elution volume for bovine serum albumin to the total column volume) decreased when the amount of dextran was increased. This decrease is linked with progressive reduction of the pore

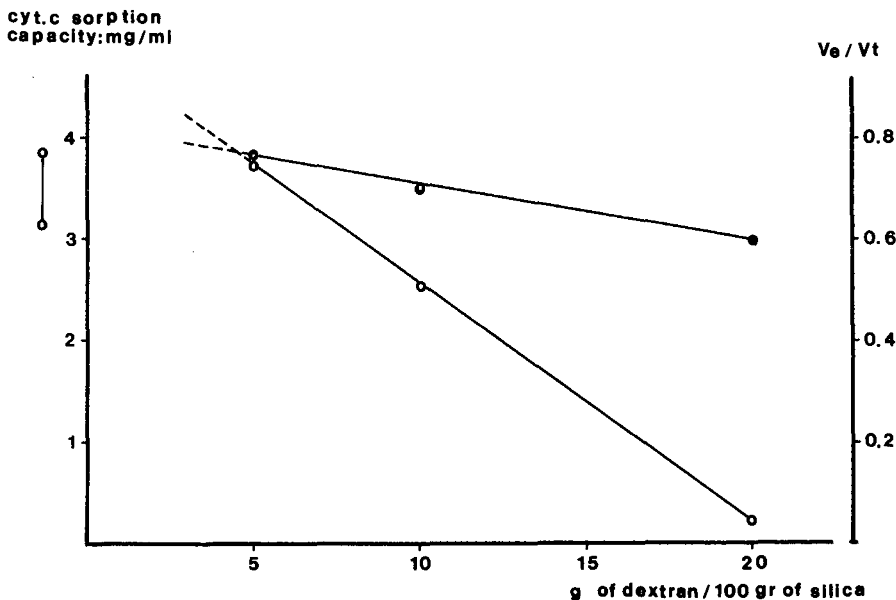

Fig. 1. Influence of the amount of dextran used for coating silica (○) on the adsorption of cytochrome *c* (non-specific adsorption) and (●) on the porosity (V_e/V_t) for bovine serum albumin. V_e = elution volume determined by gel chromatography in a physiological buffer; V_t = total volume of the column.

size inside the silica network. The degree of cross-linking (different amounts of cross-linking agent) did not significantly modify the behavior of the silica–dextran composite towards cytochrome *c* and bovine serum albumin.

The degree of activation of a silica–dextran composite (containing about 10 g of dextran for 100 g of silica) in the *p*-nitrophenyl chloroformate (*p*-NPC) method was directly proportional to the amount of the activating reagent added to the reaction mixture. The amount of *p*-NPC regularly used in our laboratory for activating these supports prior to protein immobilization is 25 mg/g of dry silica, which provides an active support containing about 20–30 μmol/ml of *p*-nitrophenol. Under these conditions, the amount of immobilized proteins (protein A, immunoglobulins G and concanavalin A) were 2.5, 7.5 and 8.0 mg per ml of support, respectively. Their sorption capacities towards human immunoglobulins G, anti-human IgG antibodies and egg albumin were 15–20, 2.5–3.5 and 3–4 mg/ml, respectively.

Sterilization and pyrogen removal

It was found that in the sodium hydroxide treatment of chromatographic supports previously contaminated by different microorganisms, the inactivation of the latter was dependent on the nature of the strains used. *Escherichia coli* was the most sensitive to alkaline media (Fig. 2), being totally inactivatad by sodium hydroxide at concentrations as low as 0.05 *M*. *Candida albicans* and *Staphylococcus aureus* were also sensitive to sodium hydroxide treatment but their total inactivation was observed only when the alkali concentration was 0.1 *M* or higher. However, sodium hydroxide at any concentration (and at temperatures of 20–25°C for 3 h) was not found to be very efficient in the inactivation of the sporulate form of *Bacillus subtilis* (Figs. 2 and 3), even when the sodium hydroxide treatment was extended to 24 h. In all instances, the

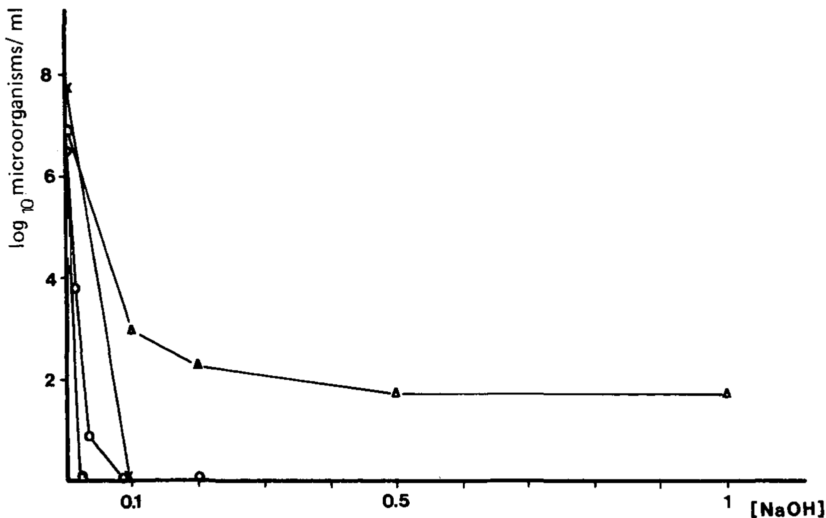

Fig. 2. Inactivation of different microorganisms using various concentrations (0–1 *M*) of sodium hydroxide. Experiments were effected at room temperature for a contact time of 3 h. ○ = *S. aureus*; **O** = *E. coli*; × = *C. albicans*; ▲ = *B. subtilis*.

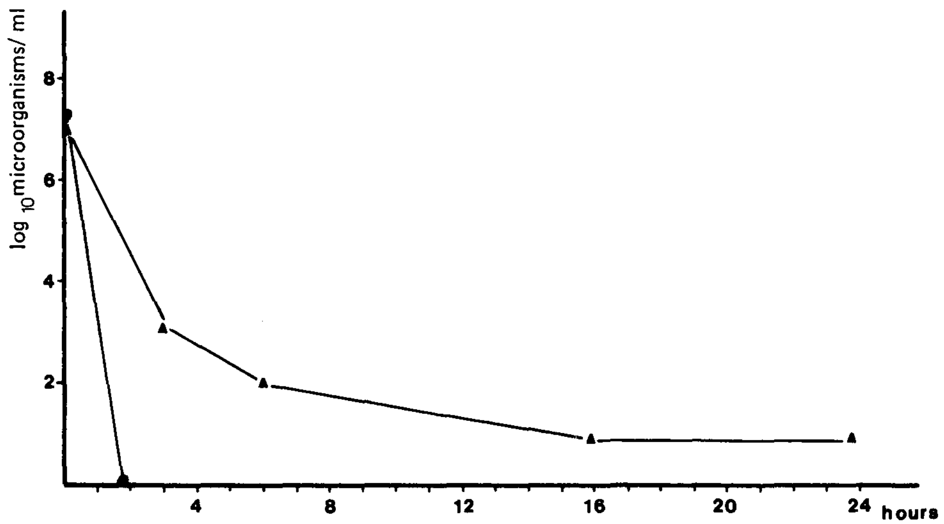

Fig. 3. Inactivation of different microorganisms by means of 0.2 *M* sodium hydroxide solution. Experiments were performed at room temperature. ▲ = *B. subtilis*; ● = *S. aureus*, *E. coli* and *C. albicans*.

diminution of the amount of *Bacillus subtilis* was about 5 logs, which means that in the studied case, from an initial concentration of about 10^7 microorganisms/ml, about 100 microorganisms/ml (on average) were still present at the end of the treatment.

The treatment of contaminated supports with an aqueous mixture of 60% ethanol and 0.5 *M* acetic acid elicited extreme sensitivity from all the strains studied, except *Bacillus subtilis*. Within 1 h of ethanol–acetic acid treatment, all *E. coli*, *C.*

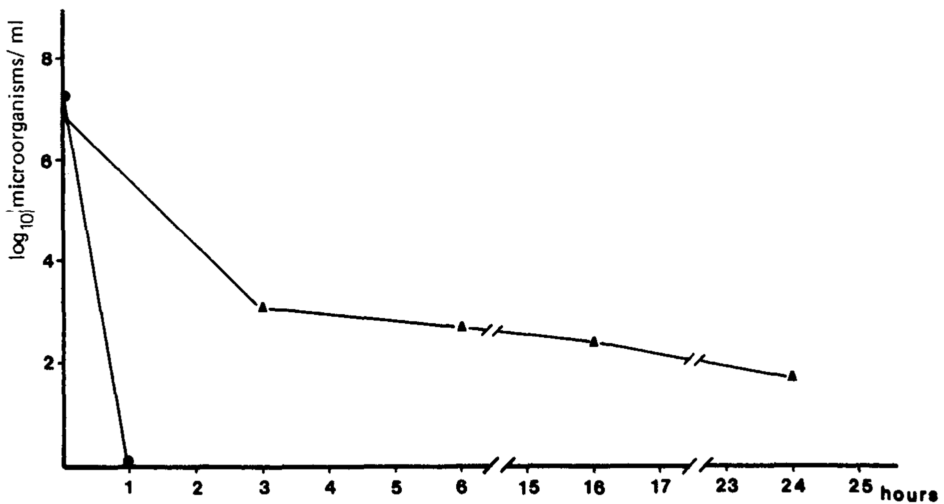

Fig. 4. Inactivation of different microorganisms by means of 60% ethanol–0.5 *M* acetic acid. Experiments were performed at room temperature. Symbols as in Fig. 3.

albicans and *S. aureus* microorganisms were inactivated (Fig. 4); the number of *B. subtilis* decreased greatly during the first hour of treatment (from 10 millions to 1000 germs) and then very slowly in the subsequent hours. After 24 h of treatment, a significant level of contamination (180 microorganisms/ml) still persisted. The strong stability of *B. subtilis* towards the treatment with sodium hydroxide and ethanol–acetic acid represents a fundamental problem in the sterilization of the chromatographic support.

These results are in accordance with those reported by Whitehouse and Clegg[7], who found a strong resistance of *B. subtilis* toward treatments with sodium hydroxide. It was demonstrated that at 22°C, 1 *M* sodium hydroxide decreased the initial amount of *B. subtilis* by about 4 logs in 12 h, whereas under the same conditions it took 25, 49 and about 70 h to obtain similar results with 0.6, 0.4 and 0.2 *M* sodium hydroxide, respectively.

On the basis of the above results, we tried to check the efficiency of two methods: (i) with 0.2 *M* sodium hydroxide treatment followed by treatment with ethanol–acetic acid and (ii) a unique treatment involving mixtures of ethanol (20, 40 and 60% concentration) and 0.2 *M* sodium hydroxide. Fig. 5 shows that alternate treatments for 1.5 h each with sodium hydroxide and acetic acid-containing ethanol totally eliminated *B. subtilis*. The results obtained with these experiments led us to consider a treatment with a solution composed of 0.2 *M* sodium hydroxide and ethanol at concentrations between 20 and 60% for 3 h. Under these conditions, the ethanol concentration played the most important role: when it was 50 and 60%, the inactivation of sporulated *B. subtilis* was complete. On decreasing this concentration to 20%, the efficiency of this mixture is sterilizing contaminated solutions was very good for *E. coli*, *C. albicans* and *S. aureus*, but *B. subtilis* was not totally destroyed (Fig. 6).

From the above-mentioned results, it can be ascertained that, if total elimination of microorganisms in a chromatographic support is to be achieved, it is necessary to

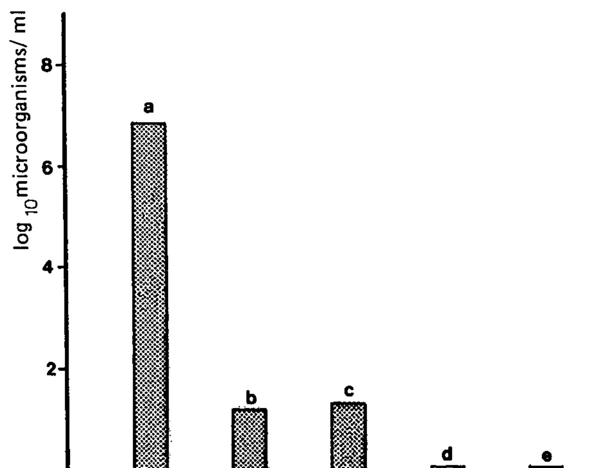

Fig. 5. Inactivation of sporulated *Bacillus subtilis*, (b) with 0.2 *M* sodium hydroxide, (c) with 60% ethanol–0.5 *M* acetic acid, and with successive inactivations (d) using solution b followed by the solution c or (e) using solution c followed by solution b. (a) Microorganism content before inactivation. All experiments were done at room temperature; the inactivation time was 3 h.

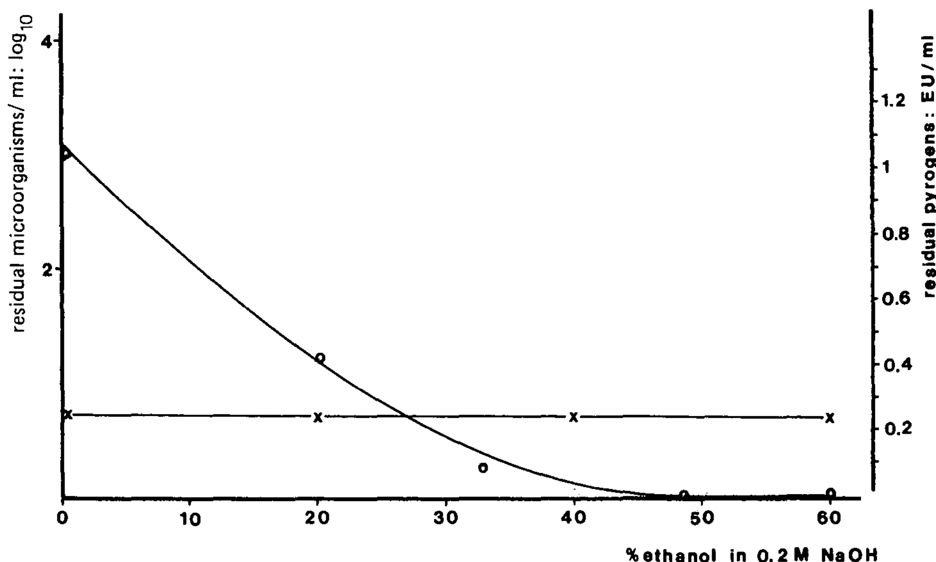

Fig. 6. Effect of solutions of 0.2 *M* sodium hydroxide containing different amounts of ethanol on sterilization (○) and pyrogen removal (×). The strain studied was *Bacillus subtilis* seeded at a concentration of $6.8 \cdot 10^6$ microorganisms/ml of sorbent. The time of contact with sterilizing solutions was 3 h for microorganism inactivation and about 30 min for pyrogen removal. The initial pyrogen content was about 100 EU/ml. All experiments were performed at room temperature.

resort either to alternate treatment with 0.2 *M* sodium hydroxide and ethanol–acetic acid or to a low-concentration alkaline treatment in the presence of ethanol. In this situation, two general problems could occur for affinity sorbents at the practical level: (i) with such treatments in the presence of ethanol, soft or semi-rigid gels may shrink to various degrees, thus making the operation on-column very difficult; and (ii) the stability of the biologically active ligand attached to the sorbents may be affected. To avoid at least the first problem, rigid sorbents (dextran-coated silica) were selected in order to support treatments with high concentrations of ethanol without shrinking and channelling.

Concerning the pyrogen removal problem, it is known that washing chromatographic supports with 0.1 or 0.2 *M* sodium hydroxide permits any pyrogens to be removed. The mechanism of pyrogen removal is based on good solubility of bacterial lipopolysaccharides in alkaline media and on the possible modification of their chemical structure[8], reducing the pyrogenicity level substantially. For completing our study on chromatographic support sterilization, the efficiency of pyrogen elimination by using these sterilizing solutions was checked.

As shown in Table I and Fig. 6, effectively all the washing solutions tested eliminated pyrogen completely. Whatever the initial degree of contamination was, the pyrogen content, after 2–3 column volumes of washing solution, fell below the level of acceptability or limit of the detection by the limulus test [0.12 Endotoxin unit (EU)/ml].

We conclude that the proposed treatments for sterilizing supports also permit pyrogens to be eliminated completely.

TABLE I

PYROGEN REMOVAL FROM A DEXTRAN–SILICA SORBENT WITH DIFFERENT SOLUTIONS

Solution	*Initial pyrogen content (EU/ml)*	*Final pyrogen content (EU/ml)*
0.2 *M* NaOH	100	<0.12
Ethanol–acetic acid[a]	600	<0.12
Ethanol–NaOH (a)[b]	100	<0.24
Ethanol–NaOH (b)[c]	100	<0.24
Ethanol–NaOH (c)[d]	100	<0.12

[a] 60% Ethanol–0.5 *M* acetic acid (final concentrations).
[b] 60% Ethanol–0.2 *M* sodium hydroxide (final concentrations).
[c] 40% Ethanol–0.2 *M* sodium hydroxide (final concentrations).
[d] 20% Ethanol–0.2 *M* sodium hydroxide (final concentrations).

Study of affinity support stability in sterilizing media

Protein A immobilized on the dextran-coated silica was resistant to repeated treatment with the 60% ethanol–0.5 *M* acetic acid mixture; its chromatographic behaviour remained almost unchanged and its sorption capacity for human IgG and the chromatographic pattern did not vary significantly (Table II). Alkali treatment without ethanol moderately affected its sorption capacity for human IgG. There was on average a 12% decrease in its sorption capacity in the two first treatments and then the capacity remained constant.

The treatment with ethanol–sodium hydroxide mixture was also well supported by immobilized protein A with an overall decrease in the sorption capacity of about 7%. Hence this biospecific sorbent showed a remarkable stability which is probably related with the intrinsic properties of protein A.

When immobilized concanavalin A was treated with ethanol–acetic acid, a 25% decrease in the sorption capacity was observed. Treatment with 0.2 *M* sodium hydroxide solution denatured concanavalin A very quickly: after four cycles, the sorption capacity for ovalbumin had decreased to zero. The treatment of immobilized concanavalin A with ethanol–sodium hydroxide resulted in total disruption of the sorbent binding capacity. This was probably due both to the higher sensitivity of this protein in comparison with protein A and to the impossibility of stabilizing it by addition of divalent ions. It should be noted in fact that concanavalin A is tetrameric whereas protein A is a single chain.

Immobilized immunoglobulins G did not seem to lose their capacity to interact with specific antibodies after repeated treatment with ethanol–acetic acid. This may be explain by the stability of these macromolecules in acidic medium (elution in affinity chromatography is often realized at pH between 2.5 and 3) and by their stability in ethanol. It should be recalled that ethanol is classically used for purifying IgG from human plasma according to the Cohn's *et al.* method[9].

In strongly alkaline medium, the denaturation of immobilized human IgG was also very limited; the support sorption capacity diminished by about 2% after the first treatment in 0.2 *M* sodium hydroxide solution and then remained constant. However,

TABLE II

EFFECT OF STERILIZING SOLUTIONS ON THE SORPTION CAPACITY OF AFFINITY SORBENTS

Solution	*Cycle No.*	*Sorbent capacity (mg affinant/ml sorbent)*		
		Immobilized protein A[a]	*Immobilized h-IgG*[b]	*Immobilized Con A*[c]
0.2 *M* Sodium hydroxide	0	21.15	3.47	3.30
	1	14.20	3.43	1.47
	2	18.77	3.68	0.71
	3	18.66	3.70	0.58
	4	18.98	3.80	0.5
	5	19.45	3.70	0.0
60% Ethanol–0.5 *M* acetic acid	0	18.36	3.48	3.34
	1	18.79	3.23	2.50
	2	17.87	3.34	2.69
	3	18.44	3.40	2.70
	4	16.90	3.60	2.23
	5	17.17	3.35	2.48
60% Ethanol–0.2 *M* sodium hydroxide	0	20.05	4.11	2.51
	1	19.85	3.90	0.48
	2	19.75	3.30	0.0
	3	20.05	3.20	0.0
	4	19.05	3.30	0.0
	5	19.06	3.50	0.0

[a] Specific sorption determined using human IgG.
[b] Specific sorption determined using rabbit anti-human IgG antibodies.
[c] Specific sorption determined using egg albumin.

after 24 h of contact in the same solution, the sorption capacity for these antibodies diminished by about 25%.

The properties of immobilized immunoglobulins after repeated cycles with 60% ethanol–0.2 *M* sodium hydroxide were almost unchanged. The diminution of the antigen–antibody association was only about 15% after five treatments but it was about 70% after a 24-h incubation.

The results obtained with immobilized immunoglobulins G do not necessarily mean that all the imombilized antibodies, independent of their species, class or specificity, remain stable towards these treatments. In our experimental model, immobilized IgGs were used as antigens. The solution to the problem of the stability of immobilized antibodies, and particularly monoclonal antibodies (mAbs), is still to be studied in detail. It seems likely, however, that the monoclonal antibody stability cannot be generalized for all classes and species or specificities.

In preliminary work, it was found in fact that an immobilized rat mAb specific for mouse IgG_1 was partially damaged by the 60% ethanol–0.5 *M* acetic acid mixture.

The behaviour of the affinity support towards the studied sterilizing solutions seemed dependent on the nature of the protein. The ethanol–acetic acid mixture seemed to be the least denaturing treatment. The sorption capacity of protein A for human IgG diminished slightly; the capacity of immobilized IgG to adsorb specific antibodies and the concanavalin A binding to ovalbumin decreased by less than 20%

after repeated treatments. The latter adsorbent is doubtless the most sensitive but its sensitivity may be decreased considerably when glucose is added to the sterilizing solution at a concentration of 0.3–0.5 *M*. Preliminary tests have actually shown that the sorption capacity of concanavalin A was partially protected when 0.3 *M* glucose or 0.3 *M* α-methyl mannoside was added to the sterilizing mixtures (data not shown).

CONCLUSION

The problem of support sterilization in affinity chromatography when ligands are biologically active is a vast topic which is just being explored. This type of sorbent is, as explained by its nature, particularly sensitive to contamination and easily inactivated. In this situation, a decontamination procedure must be efficient, rapid and non-denaturing for the ligand. Such decontamination treatments are necessary when these sorbents are used to purify products for diagnostic or therapeutic purposes and when, for economic reasons, these expensive supports must last for a long time.

The results obtained showed that all the sterilizing treatments devised for chromatographic supports are not totally efficient in any instance. Only alternate treatments with sodium hydroxide and ethanol–acetic acid mixture and alkali treatments in the presence of ethanol showed total efficiency. These two possibilities are applicable only on rigid supports, such as those based on a silica matrix, which are being increasingly used in preparative and industrial applications. However, sterilization methods should be set up according to the stability of the immobilized macromolecule while bearing in mind that *a priori* a single treatment with ethanol–sodium hydroxide is the simplest, the most practical and often the most efficient.

The study reported here represents a novel and almost unexplored approach to the sterilization of supports bearing biologically active macromolecules. The extension of these sterilization methods to other microorganisms is doubtless desirable; the search for the best compromise between the concentration of the sterilizing agent and the denaturation of macromolecular ligands should also be investigated in detail case by case.

Finally, another field to be investigated is the protection of macromolecular ligands against denaturation during sterilization. When we consider the encouraging results obtained on the protection of concanavalin A in the presence of glucose, this line of research may be very promising. Further studies are being carried out in our laboratories and will be reported elsewhere.

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Sensitive blotting assay for the detection of glycopeptides in peptide maps

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ABSTRACT

A dot blotting assay using digoxigenin hydrazide (Glycan detection kit, Boehringer Mannheim Biochemicals) was used to screen an endoproteinase Lys-C peptide map of ribonuclease B for the presence of glycopeptides. The carbohydrate content of the identified glycopeptide fraction was then further characterized by monosaccharide analysis using high-pH anion-exchange chromatography with pulsed amperometric detection (HPAE–PAD). The tandem use of a hydrazide dot blotting technique to screen peptide maps for glycopeptides and subsequent use of HPAE–PAD to identify the monosaccharide composition of glycopeptide hydrolyzates proved to be a quick, sensitive and reliable method for identifying glycopeptides and analyzing their glycan composition without derivatization of the carbohydrate.

INTRODUCTION

Electrophoretic separation of proteins and/or peptides resolved by sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS-PAGE) and followed by Western blotting[1] has become a widely used technique for analysis of complex protein and/or peptide mixtures[2–4]. The antibody probes used in Western blotting to identify particular epitope(s) can be substituted with reagents which recognize proteins containing specific functional groups such as amino and sulfhydryl groups[5]. Additionally, reagents which recognize carbohydrate moieties have been used as probes in solid-phase blotting procedures to identify and characterize glycoproteins. Solid-phase blotting and detection procedures for identifying and characterizing glycoproteins immobilized on membranes have utilized two approaches.

The first approach for detection of glycoprotein carbohydrate moieties on membranes has utilized radioactively labeled, fluorescently labeled or enzyme-labeled lectin probes[6–8]. Because lectins recognize and bind to particular sugar groups on glycoproteins, this approach is selective. A second, more general approach towards solid-phase detection of carbohydrate residues of glycoproteins has been achieved by using hydrazide probes. Hydrazide probes react with oxidized sugars on immobilized glycoproteins. Thus, glycoproteins are treated with sodium periodate which reacts with the vicinal hydroxyl groups in the glycan chains forming aldehyde groups. After

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oxidation, the aldehydes generated thus can be detected with the hydrazide probe. Many generalized hydrazide procedures for detection of glycoproteins have been developed[9–14]. These procedures differ with respect to the particular hydrazide probe, oxidation conditions and visualization reagents.

Because many proteins are glycosylated at multiple sites, thorough structural characterization of glycoproteins requires localization of glycosylation sites of the glycoprotein. This has been achieved by specific chemical or enzymatic cleavage of glycoproteins into a mixture of peptides and glycopeptides that can be subsequently separated and characterized using high-performance liquid chromatographic (HPLC) methods and ultraviolet (UV) detection. Unfortunately, UV detection alone does not permit discrimination between glycopeptides and peptides. In this report, a hydrazide detection method previously used for identifying glycoproteins in Western blots[14] has been used to simultaneously screen in one step all reversed-phase chromatography peptide fractions for the identification of glycopeptides. Briefly, all fractions in a reversed-phase endoproteinase Lys-C (cleaves proteins at lysine residues) peptide map of ribonuclease B (RNase B) were subjected to periodate treatment, labeled with digoxigenin hydrazide, blotted onto a nitrocellulose membrane and simultaneously probed with an enzyme labeled anti-digoxigenin Fab fragment to quickly identify the glycopeptides in the peptide map. Glycopeptide fractions were then further characterized by monosaccharide analysis using high-pH anion-exchange chromatography with pulsed amperometric detection (HPAE–PAD)[15].

EXPERIMENTAL

Materials

RNase B (Type III-B; Lot 17F8170) from bovine pancreas was obtained from Sigma (St. Louis, MO, U.S.A.). Monosaccharides used for standards were from Sigma or Pfanstiehl (Waukeegan, IL, U.S.A.). The 50% (w/w) NaOH solution was purchased from Fisher Scientific (San Francisco, CA, U.S.A.). Endoproteinase Lys-C (Lot 11718220-17) as well as the Glycan detection kit (Lot 11907520-03) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL, U.S.A.). HPLC-grade acetonitrile (optima) was purchased from Fisher Scientific (Rockville, MD, U.S.A.). Nitrocellulose membranes (0.45 μm) were purchased from Schleicher and Schuell (Keene, NH, U.S.A.). All other reagents used were of the highest quality commercially available. Columns used were Selectispher Concanavalin A (10 cm × 5 cm I.D.), generously provided by Perstorp Biolytica (Lund, Sweden), Vydac 218TP54 purchased from The Separations Groups (Hesperia, CA, U.S.A.) and Dionex CarboPac PA1 (250 mm × 4 mm I.D.) (Dionex, Sunnyvale, CA, U.S.A.).

Reduction and carboxymethylation of ribonuclease B

Reduction and carboxymethylation was carried out according to the procedure described by Allen[16].

Purification of ribonuclease B by Concanavalin A chromatography

The commercial preparation of RNase B was found to be contaminated with significant amounts of ribonuclease A (non glycosylated). To enrich for the

glycosylated form, 10 mg of the carboxymethylated commercial preparation were chromatographed on a Selectispher Concanavalin A column. The lyophilized glycoprotein was dissolved in binding buffer: 5 m*M* sodium acetate buffer (pH 5.2) containing 0.1 *M* NaCl, 1 m*M* $CaCl_2$ and 1 m*M* $MnCl_2$. The sample was injected onto the column in binding buffer at a flow-rate of 1 ml/min. Glycosylated RNase B was recovered from the column in a single broad peak by gradient elution (0–100 m*M* methyl mannoside over 30 min in the presence of binding buffer) at a flow-rate of 1 ml/min. Bound and unbound fractions were separately pooled, exhaustively dialyzed against water and lyophilized on a SpeedVac A290 concentrator.

SDS-PAGE

SDS-PAGE (8–25%) followed by Coomassie blue staining was performed using the Phast System (Pharmacia, Uppsala, Sweden) to compare Concanavalin A-unbound and -bound fractions to the unfractionated RNase B.

Endoproteinase Lys-C digestion

Concanavalin A-purified, reduced and carboxymethylated RNase B was dissolved in digestion buffer (25 m*M* Tris–HCl, pH 8.5, 1 m*M* EDTA). Endoproteinase Lys-C was added at a substrate-to-enzyme ratio of 100:1 (w/w). The digestion was allowed to proceed at 37°C for 18 h, at which time the digestion was terminated by freezing.

Peptide separation

The system used for peptide separations consisted of a Dionex BioLC protein system (incorporating a gradient pump module and a variable-wavelength UV–VIS detector) and an Isco Foxy fraction collector. Eluents were sparged and pressurized with helium. Peptides from an endoproteinase Lys-C digest of 750 μg of Concanavalin A-purified, reduced and carboxymethylated RNase B were eluted from the Vydac 218TP54 reversed-phase column at a flow-rate of 1.5 ml/min with a linear gradient from 0 to 40% acetonitrile containing 0.1% TFA over 1 h. Fractions of 1.5 ml were collected every minute. Every individual fraction was subsequently lyophilized using a SpeedVac A290 concentrator and stored at −20°C until reconstituted for further analysis.

Glycan detection blotting procedure for identification of glycopeptides

Individual lyophilized fractions from the peptide separation were reconstituted in 100 μl of 0.1 *M* sodium acetate buffer. From each fraction 20-μl aliquots were then probed for the presence of carbohydrate. Periodate oxidation and subsequent digoxigenin-succinyl-amidocaproic acid hydrazide labeling of the fractions were carried out in solution, prior to blotting, exactly as described by the manufacturer (Boehringer-Mannheim Biochemicals). An appropriately sized piece of nitrocellulose membrane was then cut and completely submerged in a trough of water and shaken on an orbit shaker (Lab-Line Instruments, Melrose Park, IL, U.S.A.) to allow complete wetting. Every sample fraction (already treated with periodate and digoxigenin hydrazide) was diluted to a volume of 500 μl with 10 m*M* sodium phosphate buffer, pH 8.0. The wetted nitrocellulose membrane was securely clamped between the filter support and the O-rings of the incubation chambers of a reusable microfold blotting

apparatus (V & P Scientific, San Diego, CA, U.S.A.). Diluted fractions were loaded into the top incubation chambers. Vacuum was slowly applied to trap the (glyco)-peptides onto the nitrocellulose membrane. After all the liquid had been drawn through the membrane, the membrane was removed and placed in a trough containing 10 m*M* phosphate buffer, pH 8.0 for 3 min. After 3 min, the membrane was incubated in blocking solution (Glycan detection kit) and gently shaken on an orbit shaker for 30 min. The membrane was then repeatedly washed with Tris-buffered saline, pH 6.5 (3 × 10 min) while shaking. Incubation of the membrane with the alkaline phosphatase-conjugated sheep anti-digoxigenin Fab and the staining reaction were carried out according to the manufacturer's protocol.

Hydrolysis of (glyco)peptide fractions for monosaccharide analysis

Acid hydrolysis of samples to be analyzed by HPAE–PAD was carried out in screw-cap sealed 1.5-ml polypropylene microfuge tubes as follows. To 20-μl aliquots of each reconstituted reversed-phase HPLC fraction were added 20 μl of 4 *M* TFA. Sample volume was brought up to 100 μl with 2 *M* TFA. The samples were hydrolyzed in a heating block for 6h at 100°C. The tubes were cooled and the acid was removed by lyophilization in a SpeedVac A290 concentrator.

HPAE–PAD monosaccharide analysis

The system used for the HPAE–PAD monosaccharide analysis consisted of a Dionex BioLC carbohydrate system (incorporating a gradient pump module and Model PAD-II detector). The Dionex eluent degas module was employed to sparge and pressurize the eluents with helium. In these experiments, eluent 1 was 200 m*M* NaOH and eluent 2 was water. These eluents were prepared by suitable dilution of 50% NaOH solution with high-purity water. Sample injection was via a Rheodyne 7125 valve equipped with a 50-μl sample loop and a Tefzel rotor seal to withstand the alkalinity of the eluents.

Monosaccharides [monosaccharide control samples as well as (glyco)peptide hydrolyzates] were separated on a column (250 mm × 4.6 mm I.D.) of Dionex CarboPac PA1 pellicular anion-exchange resin, equipped with an CarboPac guard column, using a flow-rate of 1 ml/min at ambient temperature. The analysis of monosaccharides[15] was carried out at an isocratic NaOH concentration of ca. 16 m*M* (*i.e.*, 8% eluent 1 and 92% eluent 2) for 17 min. A 5-min column wash with 200 m*M* NaOH (100% eluent 1) followed by a 10-min equilibration with the starting eluent was required to yield highly reproducible retention times for the monosaccharides with a *ca.* 45-min run time.

Detection of the separated monosaccharides was by PAD using a gold working electrode[13]. In order to minimize baseline distortion around the amino sugar peaks, 500 m*M* NaOH was added to the post-column effluent via a mixing tee at a flow-rate of 0.7 ml/min using the Dionex postcolumn reagent pump. The following pulse potentials and durations were used for monosaccharide analysis: E_1 = 0.05 V (t_1 = 420 ms); E_2 = 0.60 V (t_2 = 120 ms); E_3 = −0.6 V (t_3 = 60 ms). The response time of the PAD 2 was set to 3 s.

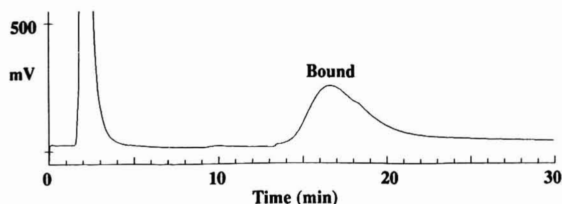

Fig. 1. Concanavalin A purification of ribonuclesase B. S-Carboxymethylated ribonuclease B (10 mg) was injected at 0.0 min. Column, high performance SelectiSpher Concanavalin A, 10 cm × 4.6 mm I.D. (Perstorp Biolytica) From 0 to 7 min, isocratic wash with binding buffer (see Experimental), followed by a 30-min linear gradient from 0 to 100 m*M* methyl mannoside at a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

The established technique of peptide mapping by reversed-phase HPLC was used to separate and isolate (glyco)peptides derived from an endoproteinase Lys-C digestion of purified bovine RNase B. Tandem use of a hydrazide dot blotting technique to screen the peptide map for glycopeptides and subsequent use of HPAE–PAD to identify the monosaccharide composition of glycopeptide hydrolyzates proved to be a quick, sensitive and reliable method for identifying glycopeptides and analyzing their glycan composition without derivitization of the carbohydrate.

Concanavalin A chromatography of reduced and carboxymethylated RNase B (Fig. 1) resulted in the identification of an unbound fraction and a bound fraction that could be eluted with methyl mannoside. Protein assay (data not shown) revealed that 30% of the applied RNase B was recovered in the bound fraction. HPAE–PAD analysis of the carbohydrate composition of the bound glycoprotein revealed a six-fold enrichment in mannose when compared to the starting glycoprotein (data not shown). SDS-PAGE and subsequent Coomassie blue staining (Fig. 2) of the commercial

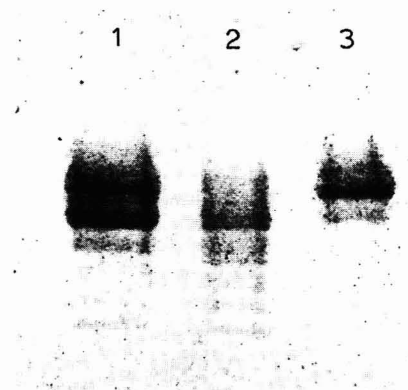

Fig. 2. Monitoring Concanavalin A purification of S-carboxymethylated ribonuclease B by 8–25% SDS-PAGE and Coomassie blue staining (Phast System, Pharmacia). Lanes: 1 = S-carboxymethylated ribonuclease B (note lower-molecular-weight band which is ribonuclease A "contamination"); 2 = pooled "unbound" fraction from Concanavalin A fractionation of S-carboxymethylated ribonuclease B; 3 = pooled "bound" fraction from Concanavalin A fractionation of S-carboxymethylated ribonuclease B.

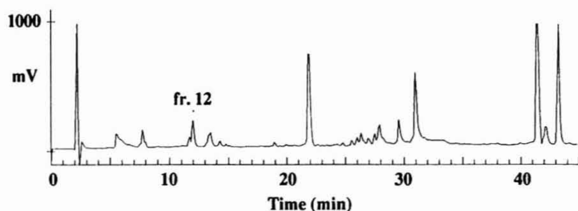

Fig. 3. Endoproteinase Lys-C reversed-phase HPLC map of Concanavalin A-purified, S-carboxymethylated ribonuclease B. A 750-μg amount of 18-h endoproteinase Lys-C digest of Concanavalin A-purified S-carboxymethylated ribonuclease B was loaded onto a Vydac 218TP54 (25 cm × 4.6 mm I.D.) column. Shown is the resultant peptide separation from a 60-min gradient of 0–40% acetonitrile in the presence of 0.1% TFA at a flow-rate of 1.5ml/min.

preparation, the Concanavalin A-bound fraction and the Concanavalin A-unbound fraction revealed a clear separation of the higher-molecular-weight glycosylated form (bound fraction) from the lower-molecular-weight RNase A contaminant (unbound fraction). Similar results have been reported by Bernard *et al.*[17].

Reversed-phase HPLC peptide mapping of reduced and carboxymethylated preparations of bovine RNase A has been reported by McWherter *et al.*[18] for cyanogen bromide, *Staphylococcus aureus* protease, tryptic and tryptic followed by chymotryptic digestions. Endoproteinase Lys-C cleavage of RNase B (cleaves specifically at lysine residues) reported here resulted in a peptide map that showed eleven major peaks with several minor peaks (Fig. 3). The theoretical sequence of the glycopeptide, based on cleavages at lysine residues, results in a predicted peptide-sequence containing six amino acid residues: Ser-Arg-Asn-Leu-Thr-Lys. Protein sequencing of the fraction 12 glycopeptide to confirm the predicted sequence remains to be determined.

Every collected fraction was evaluated for the presence of carbohydrate by performing hydrazide blotting as well as by performing HPAE–PAD analysis of hydrolyzates of each fraction. Glycan detection screening of all fractions from the endoproteinase Lys-C map revealed positive staining of a putative glycopeptide in fraction 12 (Fig. 4). The identification of a single glycopeptide fraction was consistent with the known structure of RNase B, which contains a single glycosylation site at asparagine in position 34 of its amino acid sequence[19,20]. This single glycosylation site is known to display microheterogeneity with respect to the attached oligosaccharide

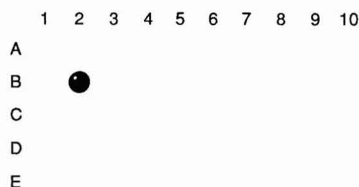

Fig. 4. Digoxigenin hydrazide blotting of periodate-oxidized reversed-phase HPLC fractions to identify glycopeptide fraction(s). Fractions treated with digoxigenin hydrazide were subsequently blotted onto a nitrocellulose membrane, probed with an alkaline phosphatase-conjugated anti-digoxigenin antibody and stained according to the manufacturer's protocol [Boehringer Mannheim Biochemicals). The only fraction found to stain positively was fraction 12 (B2); see also "fr. 12" glycopeptide peak in Fig. 3].

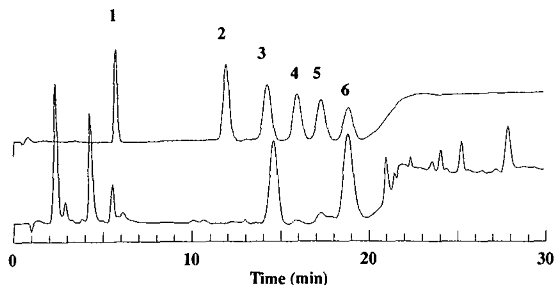

Fig. 5. High-sensitivity analysis of fraction 12 glycopeptide hydrolyzate. An aliquot of fraction 12 glycopeptide was hydrolyzed for 6 h at 100°C in 2 *M* TFA. The TFA was removed by lyophilization and the hydrolyzate was redissolved in 200 μl of water. Analysis of 150 μl of the fraction 12 hydrolyzate is shown in the lower tracing. The upper tracing is a chromatogram of 500 pmol monosaccharide standards. Peaks: 1 = fucose; 2 = galactosamine; 3 = glucosamine; 4 = galactose; 5 = glucose; 6 = mannose. Conditions for chromatography are as described under Experimental.

chains[21,22]. To verify the presence of carbohydrate in the single, positively stained peptide map fraction and the absence of carbohydrate in all other fractions, all fractions were evaluated by HPAE–PAD monosaccharide composition analysis for the presence of carbohydrate. Fraction 12 was the only fraction found by this technique to contain monosaccharides (Fig. 5). Additionally, the identification of mannose and glucosamine were consistent with the known carbohydrate monosaccharide composition of RNase B[21,22].

Other techniques for identifying attachment sites of Asn-linked sugars in glycoproteins using fast atom bombardment mass spectrometry have been described[23]. The present glycopeptide screening technique does not require expensive instrumentation. Possible problems with solid-phase hydrazide probe screening of HPLC peptide maps include the possibility that all peptides may not bind well to nitrocellulose membranes. While the quantitative evaluation of glycopeptide binding to nitrocellulose membranes remains to be evaluated; in the present report, we can state that the sensitivity of the technique minimally detected glycopeptides containing 1-nmol quantities of mannose (HPAE–PAD quantitation of mannose in an identical fraction 12 aliquot to the aliquot subjected to the hydrazide blotting procedure; data not shown).

Additionally, variations of the length and temperature of oxidation as well as the concentration of sodium periodate may affect the specificity of the hydrazide-labeling reactions the degree of labeling and ultimately the sensitivity of the procedure. None of these parameters have been examined with the present glycopeptide screening technique. Finally, complications may occur if behavior of glycopeptides in reversed-phase chromatography is influenced by carbohydrate microheterogeneity at single Asn glycosylation sites. According to Takahashi *et al.*[24], behavior of glycopeptides in reversed-phase chromatography is determined mainly by amino acid composition. Because it is known that RNase B displays microheterogeneity with respect to the oligosaccharides which are attached at asparagine residue 34 (refs. 21 and 22), future experiments will focus upon using HPAE–PAD to map these different oligosaccharides.

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CHROM. 22 328

Purification of gonadotropin releasing hormone receptors using the avidin–biotin technique

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ABSTRACT

The avidin–biotin technique has been applied to the purification of gonadotropin releasing hormone (GnRH) receptors from other solubilized membrane proteins. The following steps were involved in this approach: (a) solubilization of rat pituitary GnRH receptor with the zwitterionic detergent CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, (b) equilibration of the solubilized GnRH receptor with [biotinyl-D-Lys^6]GnRH immobilized on avidin-agarose; and (c) elution of the receptors with high salt and GnRH analogues. Following two cycles of affinity chromatography the GnRH receptor was purified to homogeneity. The overall recovery of the purified receptor was 4–10% of the initial activity in the CHAPS extract and the calculated purification was approximately 10 000 to 15 000 fold. The development of a two step affinity chromatography for the purification of GnRH receptors can be used for detailed studies on the structure and function of the receptor. These studies will advance our understanding of the molecular basis of GnRH action.

INTRODUCTION

The hypothalamic decapeptide gonadotropin releasing hormone (GnRH) stimulates gonadotropin release from the anterior pituitary and thus has a pivotal role in the regulation of reproduction[1,2]. The GnRH receptor is the initial site of action of the hormone in the mammalian pituitary and represents a family of hormone receptors that has not been investigated at the molecular level. Therefore, elucidation of the GnRH-receptor structure has theoretical and practical implications both in reproductive biology and cancer[1,2]. For most peptide hormones, such studies are greatly facilitated by developing techniques for solubilization and purification of the hormone binding protein in an active form.

The high affinity constant (10^{15} *M*) between the glycoprotein avidin and the vitamin biotin provides an important exprimental tool for a wide variety of biological applications. The avidin–biotin complex has been used as a meduator in localization,

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isolation and immunological studies[3–8]. Recently, biotinylated peptide hormones have been used for the localization and isolation of receptors on cell surfaces. Here, we report on the synthesis and the application of biotinylated GnRH to highlight the potential use of this method for GnRH receptor purification.

EXPERIMENTAL

Synthesis of [biotinyl-D-Lys^6]GnRH

[Biotinyl-D-Lys^6]GnRH was synthesized as previously described[9,10]. Briefly, [D-Lys^6]GnRH (0.6 mg; 0.45 mmol) (Peninsula, Belmont, CA, U.S.A.) was reacted with 2 molar equivalents of *d*-biotin-*p*-nitrophenyl ester (Sigma, St. Louis, MO, U.S.A.) in methanol–dimethylformamide (10:1, v/v) in the presence of 1.2 equivalents of triethylamine. After standing at 24°C for 3 h, the product was precipitated by the addition of ether and washed three times with ethyl acetate in order to remove unreacted *d*-biotin-*p*-nitrophenyl ester. The product was then purified by preparative high-voltage paper electrophoresis (Whatman No. 3 paper, 60 min at 60 V/cm) in pyridine–acetate buffer (pH 3.5); elctrophoretic mobility, 0.62.

Cell culture and assay of biological activity

Rat pituitary cell cultures were prepared as previously described[11]. After 48 h in culture, the medium was discarded, the cells were washed twice and incubated with the GnRH analogues (4 h at 37°C). The biological activity was assessed by the quantitation of luteinizing hormone (LH) released from the cells[11].

Iodination and pituitary membrane preparations

[D-Ser(t-Bu)^6, des-Gly^{10}, ethylamide]GnRH (Buserelin) was iodinated by the lactoperoxidase method[12]. Specific activity of the labeled peptide was approximately 1.0 mCi/μg, as measured by self-displacement in the pituitary radioligand receptor assay. Pituitary membranes were prepared from 25- to 28-day old Wistar derived female rats[13]. Briefly, the glands were homogenized gently with a Dounce homogenizer at 4°C in 10 m*M* Tris · HCl, pH 7.4, and centrifuged for 10 min at 1000 *g*. The supernatant was then centrifuged for 20 min at 20 000 *g*. The pellet was resuspended in 10 m*M* Tris · HCl buffer, centrifuged at 20 000 *g* for 20 min and the pellet stored at −20°C.

Solubilization of GnRH receptors

Solubilization of GnRH receptors was performed as described previously[13,14]. Briefly, pituitary membrane preparations were washed with 10 m*M* Tris · HCl, pH 7.4 by centrifugation (20 min at 20 000 *g*). The pellet was resuspended in 10 m*M* Tris buffer containing 5 m*M* 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) (Sigma), shaken for 60 min at 4°C and centrifuged (60 min at 100 000 *g*). This procedure was repeated, the supernatants were combined and used to measure binding. Usually, the solubilized receptor was kept in 1 m*M* CHAPS–10 m*M* Tris containing 10% glycerol and 1 m*M* phenylmethylsulfonyl fluoride (Sigma).

Binding assays to solubilized receptors

Solubilized receptors (25 to 50 μg protein) were incubated with labeled Buserelin

(50 000 cpm) in 0.5 ml of 10 m*M* Tris–0.1% bovine serum albumin containing 1 m*M* CHAPS for 2.5 h at 4°C. The reaction was stopped by the addition of 0.3 ml ice-cold dextran-coated charcoal [0.5 g Dextran T-70 (Pharmacia, Uppsala, Sweden) and 5.0 g activated charcoal (Norit A, Fisher, Raleigh, NC, U.S.A.) dissolved in 1000 ml of phosphate-buffered saline]. The tubes were left on ice for 10 min and then centrifuged for 20 min at 2000 *g* at 4°C. The supernatants were collected and counted in a γ-counter. Specific binding represents the bound radioactivity in the presence of 10^{-7} *M* unlabeled Buserelin subtracted from the total bound radioactivity.

Affinity chromatography on GnRH resin

Avidin–agarose (Sigma), 1.5 ml containing 2.5 g of avidin) was incubated with 10^{-6} *M* [biotinyl-D-Lys^6]GnRH in 10 m*M* Tris · HCl buffer for 5 h at 24°C. The resin was washed extensively with 10 m*M* Tris · HCl buffer (10 times, 20 ml each wash) and subsequently equilibrated with 1 m*M* CHAPS–10 m*M* Tris · HCl–10% glycerol. The resin was incubated with the solubilized receptors (1 to 2 mg) in 5 to 10 ml of 1 m*M* CHAPS–10 m*M* Tris · HCl–10% glycerol for 12 h at 4°C and poured into a column (5 cm × 0.4 cm I.D.). The eluate was collected by centrifugation and the column (kept at 4°C) was washed with 13 ml of the above buffer. The solubilized receptor was eluted by using two procedures: (i) with 0.5 *M* NaCl in 1 m*M* CHAPS–10 m*M* Tris–10% glycerol (pH 7.4) and the samples were dialyzed against 1 m*M* CHAPS–10 m*M* Tris–10% glycerol to remove excess of NaCl; or (ii) with $5 \cdot 10^{-9}$ *M* Buserelin and the samples were diluted (1:5) and dialyzed before assays. GnRH binding activity was detected by charcoal assay.

RESULTS

[Biotinyl-D-Lys^6]GnRH (Fig. 1) was prepared by chemical modification of the ε-amino group in position 6 of [D-Lys^6]GnRH with *d*-biotin-*p*-nitrophenyl ester. [D-Lys^6]GnRH was selected as the starting material for derivatization since: (i) substitution of D-amino acids in position 6 of GnRH results in more potent and metabolically stable derivatives, and (ii) the ε-amino group of lysine serves as a spacer for substitution reactions and thus the GnRH conformation is less likely to be disturbed. Table I lists the biological potency and binding affinity of GnRH, [D-Lys^6]GnRN, and the biotinylated GnRH analogue. [biotinyl-D-Lys^6]GnRH ex-

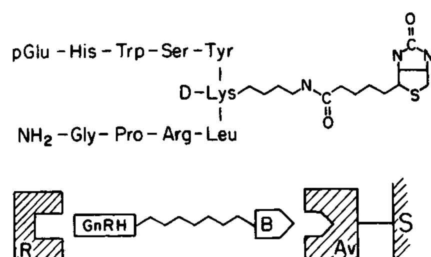

Fig. 1. Schematic representation illustrating the bifunctional ligand [biotinyl-D-Lys^6]GnRH (upper) as the mediator for absorption of GnRH receptor to avidin–Sepharose. Av = avidin; B = biotin; S = Sepharose and R = receptor.

TABLE I

BIOLOGICAL POTENCY AND BINDING AFFINITY OF GnRH, [D-Lys[6]]GnRH OR [BIOTINYL-D-Lys[6]]

IC_{50}: The concentration of unlabeled ligand at which the specific binding of labeled ligand is displaced by 50%. ED_{50}: The concentration required to stimulate 50% of the maximal LH release from pituitary cells.

Analogue	IC_{50} *(nM)*	ED_{50} *(nM)*
GnRH	10.0	0.5
[D-Lys[6]]GnRH	3.0	0.5
[Biotinyl-D-Lys[6]]GnRH	0.7	0.075

hibits a 4-fold and 14-fold higher binding affinity than that of [D-Lys[6]]GnRH and GnRH, respectively and was 7-fold more potent in stimulating LH release from pituitary cells.

Purification of GnRH receptors by affinity chromatography was performed by immobilization of [biotinyl-D-Lys[6]]GnRH on avidin–agarose (Fig. 1). The resin was equilibrated with the solubilized GnRH receptor, washed and subsequently eluted with 0.5 *M* NaCl in 1 m*M* CHAPS–10 m*M* Tris–10% glycerol (Fig. 2A). The samples were then dialyzed to remove NaCl and binding assays were conducted. Using this protocol, about 40–60% of the receptor was recovered and a purification fold of 7 to 12 was achieved. Recovery (R) of receptors was calculated according to the following equation R = (fmol[^{125}I]Buserelin bound/mg of protein) × (total mg of protein) and

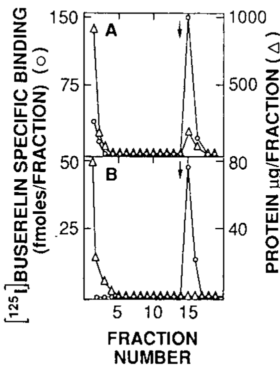

Fig. 2. Typical elution of rat GnRH receptor from affinity column. (A) [Biotinyl-D-Lys[6]]GnRH immobilized on avidin–agarose resin (1.5 ml) was incubated with the solubilized receptor (1.8 mg/6.5 ml) in 1 m*M* CHAPS–10 m*M* Tris–10% glycerol for 12 h at 4°C and poured into a column (5 cm × 0.4 cm I.D.). The supernatant was collected by centrifugation and the column was washed with 13 ml of the same buffer. The resin was then incubated (60 min at 4°C) with 2 ml of 0.5 *M* NaCl in the chromatography buffer (arrow). The supernatant was collected by centrifugation and eluated fractions (1 ml) were dialyzed. All fractions were sampled for protein and GnRH binding activity. (B) The fractions with the highest receptor activity from NaCl eluted affinity column were pooled (0.3 mg/1.3 ml) and rechromatographed in a second affinity purification step. The experimental details are as in A, except that elution of the receptor was carried out by incubating the resin (3 h at 4°C) with $5 \cdot 10^{-9}$ *M* Buserelin (arrow). The fractions (0.3 ml) were diluted (1:5), dialyzed and sampled for protein and binding activity.

CHAPS extracts were taken as 100%. Purification-fold was calculated as follows: (fmol[^{125}I]Buserelin bound/mg of protein in fraction) divided by (fmol[^{125}I]Buserelin bound/mg of protein in soluble extract). For the second purification step, the fractions exhibiting the highest binding activity from NaCl eluted column were pooled and rechromatographed. The column (after washing) was then incubated with $5 \cdot 10^{-9}$ *M* Buserelin for 3 h at 4°C in 1 m*M* CHAPS–10 m*M* Tris–10% glycerol. The eluted fractions were diluted (1:5), dialyzed and binding assays conducted (Fig. 2B). The overall recovery of the purified receptor was 4–10% of the initial activity in the CHAPS extract and the calculated purification was approximately 10 000 to 15 000 fold. Iodination of this affinity purified receptor and subsequent autoradiographic analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis revealed no other bands except that of the GnRH receptor (data not shown). This suggests that following two cycles of affinity chromatography the GnRH receptor was purified to homogeneity.

DISCUSSION

The avidin–biotin complex system has been used widely to study biological interactions that involve the specific binding between a protein and a ligand. Recently, immobilized forms of avidin have been used for the isolation of receptors. This can be accomplished either by binding of the biotinylated hormone to avidin columns, followed by subsequent interaction with the solubilized receptor or, by prior incubation of the biotinylated hormone with the solubilized receptor and then immobilization on avidin columns (shown schematically in Fig. 1). The receptor can either be eluted directly from the column or the biotinylated hormone–receptor complex can be eluted with biotin and subsequently the receptor can be dissociated from the hormone. The advantages of the method are: (i) the hormone can be attached to the support via a single defined site that is not involved in its biological function; (ii) the anchoring of the hormone to the support is unequivocal and proceeds in high yield; (iii) the chemical manipulations are performed with the free hormone and thus its effect on binding and biological activity can be readily assessed; and (iv) the technique is highly reproducible and can be readily scaled up for production of larger quantities of receptors.

In the present study, this novel procedure has been applied to the purification of GnRH receptors from other solubilized membrane proteins. The following steps were involved in this approach: (a) solubilization of rat pituitary GnRH receptor with CHAPS without alteration of binding affinity; (b) immobilization of [biotinyl-D-Lys^{6}]GnRH on avidin–agarose; (c) equilibration of the solubilized GnRH receptor with the affinity resin; and (d) elution of the receptors with high salt and GnRH analogues. Following two cycles of affinity chromatography the GnRH receptor was purified to homogeneity.

The development of a simple two step affinity chromatography for the purification of GnRH receptors permits preparation of a large quantity of the pure receptor. This can be used for detailed studies on the structure and function of the receptor, for the development of monospecific antibodies, as well as for partial amino acid sequencing. These studies will advance our understanding of the molecular basis for the action of GnRH, which has a pivotal role in the regulation of reproduction.

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CHROM. 22 348

One-step purification of trypsin and α-chymotrypsin by affinity chromatography on Eupergit–aprotinin, a novel carrier for purification of serine proteases

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ABSTRACT

Aprotinin isolated from bovine lungs was covalently immobilized on Eupergit C. The highly selective affinity adsorbent was used to purify trypsin and α-chymotrypsin from pancreatic extract in a single step. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis indicated that both enzymes were highly purified. The maximum binding capacity of Eupergit–aprotinin for bovine trypsin was calculated to be *ca.* 7.5 mg per gram of gel (wet resin).

INTRODUCTION

The natural proteinase inhibitor from bovine organs, aprotinin, is widely used as a valuable tool in biochemical and biomedical research[1–3]. Moreover, it is a very potent drug for the treatment of different clinical conditions, *e.g.*, acute pancreatitis and hyperfibrinolytic haemorrhage. In addition, aprotinin is very well suited to inhibit undesired proteolytic reactions in tissue culture[4]. An important application is the purification of various proteinases by affinity chromatography using immobilized aprotinin[5–8]. This proteinase inhibitor is an excellent candidate as a ligand for affinity chromatography[4]. In contrast to other serine proteinase inhibitors used as affinity ligands, aprotinin is able to bind trypsin and α-chymotrypsin with very high affinity.

This paper describes the immobilization of aprotinin to Eupergit C, a chemically and mechanically stable carrier, and the purification of trypsin and α-chymotrypsin from a pancreatic extract in a single step.

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EXPERIMENTAL

Pancreatin was purchased from Merck (Darmstadt, F.R.G.). All other chemicals used were commercially available. Eupergit C was a gift from Röhm Pharma (Weiterstadt, F.R.G.) and aprotinin was kindly provided from Pentapharm (Basle, Switzerland).

Aprotinin was covalently linked to Eupergit C via its amino groups to the epoxy groups of the carrier according to the standard procedure of the manufacturer[9].

Affinity chromatography

A 1-g amount of Eupergit–aprotinin (wet resin, equivalent to 1.15 ml of gel) equilibrated in 50 m*M* Tris–HCl (pH 8.0) containing 0.5 *M* sodium chloride and 1 m*M* calcium chloride (buffer A) was incubated with 5 ml of pancreatic extract (22 mg/ml) for 60 min at room temperature. The gel was poured into a chromatographic column and washed several times with the same buffer until the adsorption at 280 nm reached an adsorption baseline of 0.05. The bound proteases were eluted with 0.1 *M* hydrochloric acid (pH 1.0) containing 25 m*M* calcium chloride and collected in tubes containing 2 *M* Tris–HCl (pH 7.5). The eluent was dialysed against 50 m*M* Tris–HCl containing 1 m*M* calcium chloride and concentrated in collodion bags (SM 13200). The purity was checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)[10].

After equilibration of the gel with buffer A the gel was stored at 4°C in 0.02 *M* sodium phosphate buffer (pH 7.2) containing 0.16 *M* sodium chloride and 0.02% thimerosal (thiomersal) as a preservative.

Protein determination

Protein concentrations were determined with a bicinchoninic acid protein reagent (Pierce, Rockford, IL, U.S.A.).

RESULTS

Different amounts of aprotinin from bovine lungs immobilized on Eupergit C indicated an almost proportional trypsin binding ability up to 15 mg of aprotinin per gram of gel (wet resin) (Fig. 1). Larger amounts of aprotinin (> 15 mg) linked to 1 g of Eupergit C resulted in an unproportionally small amount of trypsin bound to the adsorbent.

In further studies, 5 mg of aprotinin were immobilized per gram of Eupergit C (wet resin). Trypsin and α-chymotrypsin were purified from pancreatic extract in a single step. Both enzymes emerged in a single protein peak from the column (Fig. 2). SDS-PAGE analysis indicated two major proteins (trypsin and α-chymotrypsin) and some low-molecular-weight autolysis fragments of these enzymes (Fig. 3).

DISCUSSION

For affinity chromatography aprotinin was immobilized on different supports, *e.g.*, Sepharose, poly(vinyl alcohol) and porous glass as reported earlier[5–8]. This

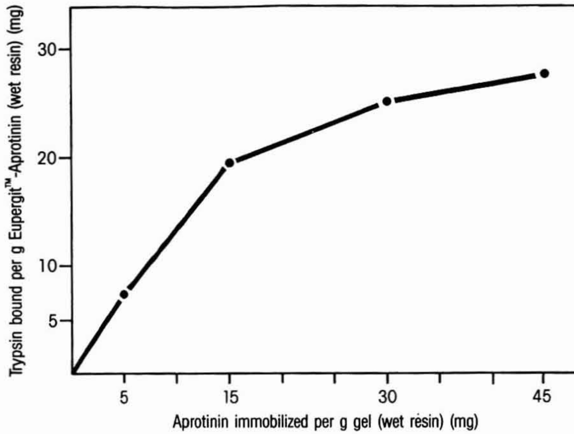
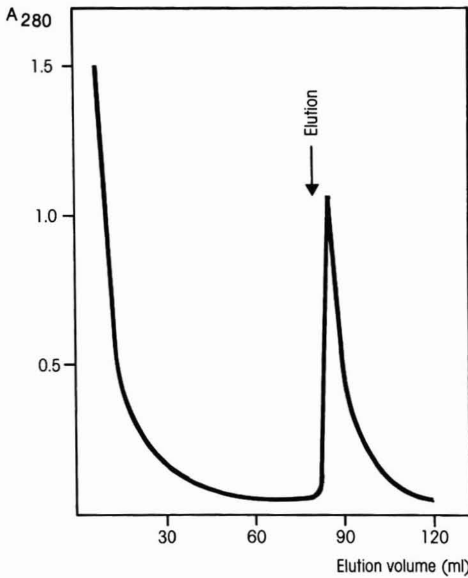


Fig. 1. Effect of different amounts of immobilized aprotinin on Eupergit to the trypsin-binding capacity of the gel.



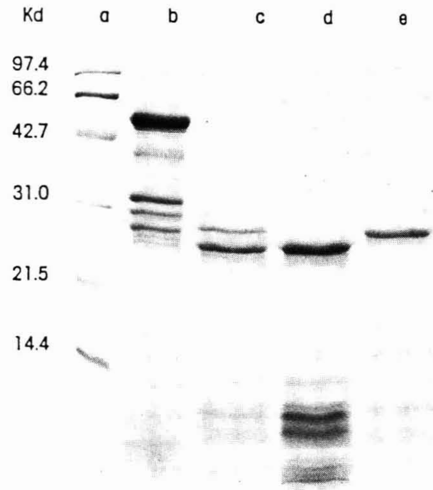


Fig. 2. Affinity chromatography of trypsin and α-chymotrypsin with Eupergit–aprotinin [5 mg of aprotinin per gram of gel (wet resin)]. Pancreatic extract (5 ml) was incubated with 1 g of gel (wet resin) and poured in a column (3 cm × 0.7 cm I.D.). Trypsin and α-chymotrypsin were eluted with 0.1 *M* hydrochloric acid (pH 1.0) containing 25 m*M* calcium chloride.

Fig. 3. SDS-PAGE analysis of Eupergit–aprotinin fractions. Lanes: (a) molecular weight standards; (b) porcine pancreatic extract; (c) trypsin and α-chymotrypsin of pancreatic extract eluted from the affinity adsorbent (Eupergit–aprotinin); (d) commercially available trypsin from Sigma eluted from the affinity adsorbent; (e) commercially available α-chymotrypsin from Sigma eluted from the affinity adsorbent. Gel concentration, 15%. Kd = kilodalton.

study was undertaken to investigate the purification of trypsin and α-chymotrypsin using Eupergit C–aprotinin, a novel carrier for affinity chromatography with different advantages resulting from its chemical (stable in all commonly used buffers between pH 1 and 9, no leakage of the ligand) and physical properties (high flow-rates of *ca.* 1000 ml/cm^2 · h at 0.5 bar).

This work showed that aprotinin covalently linked to Eupergit C is a highly selective affinity adsorbent. It has been used to purify trypsin and α-chymotrypsin from pancreatic extract in a single step. SDS-PAGE indicated that both enzymes were highly purified. The binding capacity of 1 ml of Eupergit–aprotinin [5 mg of aprotinin per gram of gel (wet resin)] for bovine trypsin was calculated to be *ca.* 7.5 mg.

The column has been used several times without any apparent decrease in capacity or selectivity. The stability of the gel is still under examination. Owing to its excellent chemical, mechanical and binding properties, Eupergit C–aprotinin is an ideal adsorbent for the purification of trypsin and α-chymotrypsin in both laboratory and industrial processes. It remains to be established whether aprotinin linked to Eupergit C could also be used for the purification of other serine proteases.

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CHROM. 22 424

Purification of the upstream element factor of the Adenovirus-2 major late promoter from HeLa and yeast by sequence-specific DNA affinity chromatography

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ABSTRACT

The purification to homogeneity of the Adenovirus-2 major late promoter (MLP) upstream element factor (UEF), a sequence specific transcription factor, which binds to upstream elements of various class B (II) genes, is reported. The protein was purified from HeLa cells and also from the yeast *Saccharomyces cerevisiae*, by using sequence-specific DNA affinity chromatography. The human (UEFh, 45 000 dalton) and the yeast (UEFy, 60 000 dalton) proteins protect the same sequences over the MLP-IVa2 intergenic region: the MLP-UE (from nucleotide −49 to −67) and the IVa2-UE (from nucleotide −98 to −122 relative to the MLP initiation site). Both proteins have a higher affinity for the MLP-UE than for the IVa2-UE.

INTRODUCTION

Transcription of protein coding genes requires the presence of distinct promoter elements in the DNA template: (1) the initiation site and the TATA-box, a highly conserved AT-rich region located 30 base pairs (bp) and 40–110 bp upstream of the transcription start site in higher and lower eukaryotes, respectively, and (2) the upstream elements which are located in the −40 to −110 region upstream from the start site (for a review, see ref. 1). *In vivo* and *in vitro* studies have shown that these promoter elements are the target of various transcription factors. While the TATA-box interacts with the TATA-box recognizing factor BTF1[2], also called TFIID[3], both the upstream elements and enhancers bind sequence specific factors (*e.g.*, Sp1, HSTF, CBP) which have some regulatory function[1].

The HeLa upstream element factor (UEF) of the Adenovirus-2-major late promoter (Ad2 MLP) had been shown to stimulate Ad2 MLP transcription both *in vivo* and *in vitro* through the binding at the MLP upstream element (MPL-UE located between positions −49 and −67 of the Ad2 MLP)[4]. Further it has also been suggested that stimulation of transcription by the sequence-specific upstream factors occurs

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through interaction with the TATA-box recognizing factor[5,6]. On the other hand, recent studies have demonstrated that the yeast TATA-box factor (BTF1y) can accurately initiate *in vitro* transcription when added to the HeLa system lacking mammalian BTF1[7,8]. These results suggest that protein–protein and DNA–protein interactions, involved in the initiation of transcription of protein coding genes, have been conserved during evolution. In a first step to understanding such mechanisms, it was necessary to identify and purify the various factors involved in the transcription machinery. We report here the purification of the UEF factor from both HeLa (UEFh) and yeast (UEFy) by using classical chromatographic techniques and also a sequence-specific DNA affinity column. Both proteins recognized the same upstream elements: the MLP-UE binding site (from −49 to −67) and the IVa2-UE binding site (from −98 to −122).

EXPERIMENTAL

Purification of the UEF from HeLa or yeast

The UEFh was purified from a HeLa whole cell extract (WCE) and the three first steps of the purification procedure (heparin–Ultrogel, DEAE-5PW, SP-5PW) were as previously described[9,10]. The SP0.35 fraction was dialysed in buffer A [50 m*M* Tris–HCl (pH 7.9)–50 m*M* KCl–8.7% glycerol–0.1 m*M* EDTA–0.5 m*M* dithiothreitol (DTT)] and incubated for 15 min at 4°C with 100 μg/ml of poly(dI–dC) (dI–dC) (Pharmacia, Uppsala, Sweden) and 4 m*M* $MgCl_2$, before being loaded onto a 1-ml sequence-specific DNA affinity column made as follows: the two 35-mer synthetic oligonucleotide strands (nucleotides −41 to −71 with respect to the Ad2 MLP start site with a GATC tetramer added at the 5′-terminus) were hybridized and ligated with T4 DNA ligase. The polymers were fixed onto a Sepharose CL-4B resin (Pharmacia) preactivated with cyanogen bromide[11]. The column was then washed successively with ten column volumes of buffer B [50 m*M* Tris–HCl (pH 7.9)–50 m*M* KCl–17.4% glycerol–5 m*M* $MgCl_2$–0.1% Nonidet P40 (NP40)–1 m*M* DTT] and five column volumes of buffer B containing 0.3 *M* KCl. The UEFh was then eluted with ten column volumes of buffer B containing 1 *M* KCl. The active 1 *M* KCl fractions (5 ml) were dialysed against buffer C [10 m*M* potassium phosphate (pH 7)–0.01 m*M* $CaCl_2$–8.7% glycerol–0.5 m*M* DTT] and loaded on a hydroxyapatite column (500 μl) (BDH, Poole, U.K.) pre-equilibrated in buffer C. The UEFh was eluted with 0.12 *M* potassium phosphate in buffer C (five column volumes). The fractions were dialysed against buffer D [50 m*M* Tris–HCl (pH7.9)– 50 m*M* KCl–25% glycerol–0.1 m*M* EDTA–0.5 m*M* DTT] and stored at −80°C. The UEFy was purified as follows. An extract of the yeast *Saccharomyces cerevisiae* S-100 (5 ml; 37 mg/ml) prepared as previously described [12] was dialysed for 12 h against buffer E [50 m*M* Tris–HCl (pH 7.9)–50 m*M* KCl–17.4% glycerol–2 m*M* $MgCl_2$–0.5 m*M* DTT–0.1 m*M* PMSF] and incubated for 15 min at 4°C with 100 μg /ml of poly(dI–dC) (Pharmacia) and 0.1% NP-40 and then for 15 min at 4°C with 1 ml of the sequence-specific DNA affinity resin. The resin was then packed in a Pasteur pipette and washed with ten column volumes of buffer F [50 m*M* Tris–HCl (pH 7.9)–50 m*M* KCl–17.4% glycerol–2 m*M* $MgCl_2$–0.1% NP-40–1 m*M* DTT), five column volumes of buffer F containing 0.2 *M* KCl and five column volumes of buffer F containing 1 *M* KCl. After dialysis against buffer F, the 1 *M* KCl fraction was incubated for 15 min at

4°C with 50 μg/ml of poly(dI–dC)(dI–dC) and reapplied to the DNA affinity column. The 1 *M* KCl eluate from this second affinity column (*ca.* 2 μg/ml) was dialysed against buffer D and stored at −80°C. Purified fractions were analysed electrophoretically on 9% sodium dodecyl sulphate—polyacrylamide gels (SDS-PAGE).

DNAse I footprinting and gel retention assays

For labelling the non-coding strand, pM677 was digested by the restriction enzyme SacII at −245, dephosphorylated with calf intestinal phosphatase (Boehringer), 5′-end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase, then digested with the restriction enzyme BamHI. The resulting SacII–BamHI (−245/+33) DNA fragment was purified on a 6% acrylamide gel. For labelling the coding strand, pM677 was linearized at the BamHI site at +33, ^{32}P-phosphorylated, digested by SacII at −245 and the fragment was purified as described above.

DNAse I footprinting reactions consisted of a 10-min preincubation at 24°C in an 18-μl reaction volume containing various amounts of the protein fraction, *ca.* 1 ng (10 000 cpm) of the 5′-end-labelled DNA fragment, 50 ng of poly(dI–dC)(dI–dC) and 4 m*M* $MgCl_2$ in buffer A. After the preincubation, 2 μl of DNAse I (10 μg/ml) (Worthington, U.K.) was added for 2 min and DNA digestion products were analysed on 8% acrylamide–8.3 *M* urea gels, followed by autoradiography.

The gel retention assay[13] consisted of a 15-min incubation step at 24°C identical with that in the footprinting assay in a 10-μl reaction volume containing 100–250 ng of poly(dI–dC)(dI–dC) (Pharmacia). A 1-μl volume of 87% glycerol was added and the mixture was electrophoresed immediately on a 4.5% polyacrylamide gel (polyacrylamide–bisacrylamide, 80:1). The electrophoresis buffer was 6.7 m*M* Tris–HCl (pH 7.9)–3.3 m*M* sodium acetate–1 m*M* EDTA. The gel was dried and autoradiographed.

RESULTS

It has generally been very difficult to purify proteins involved in the regulation of transcription to homogeneity because they usually constitute less than 0.001% of the total cellular protein. That is why specific-DNA-affinity chromatography was an attractive separation procedure for increasing the DNA-binding factor recovery[14]. To purify both UEF factors, we designed a DNA-affinity column containing a polymer of the Ad2 MLP upstream element (from nucleotide −41 to −71) (see ref. 15 and Experimental). The DNA-binding activity was detected during the purification procedure by a gel retention assay. This assay is based on the slower migration of a DNA–protein complex compared with the free DNA on a non-denaturing polyacrylamide gel[13].

Purification of the HeLa UEF

The purification scheme for the HeLa UEF is summarized in Fig. 1A and Table I. HeLa whole cell extract was applied successively to heparin–Ultrogel, DEAE-5PW and SP-5PW columns as previously described[10]. The critical step of the procedure was the use of a sequence-specific DNA affinity column prepared as described under Experimental. The SP0.35 fraction, which contains the UEF, was preincubated with a non-specific DNA competitor (polydI–dC/dI–dC) in order to prevent the interactions

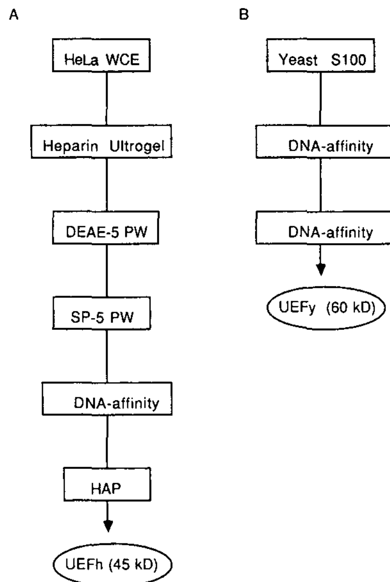

Fig. 1. Purification scheme of Ad2 MLP-UEF from (A) HeLa or (B) yeast. kD = Kilodalton.

of non-specific DNA binding proteins on the MLP-UE site, and loaded onto the affinity column. The 1 *M* KCl eluate contains two polypeptides of 40 000 and 45 000 dalton (data not shown), which co-migrates with the binding activity to the MLP-UE. These two polypeptides were separated by chromatography on a hydroxyapatite column; as shown by SDS-PAGE, the HAP0.12 fraction contained a single polypeptide of 45 000 dalton (Fig. 2A, lane 1). To provide additional evidence that the 45 000-dalton polypeptide corresponds to the Ad2 MLP-UEF, the purified polypeptide was

TABLE I

SUMMARY OF UEFh AND UEFy PURIFICATION

	Step	*Volume (ml)*	*Protein (mg/ml)*	*Total protein (mg)*	*Units*[a]	*Specific activity (units/mg)*	*Purification (factor)*	*Yield (%)*
UEFh	HeLa WCE	230	7	1610	460 000	285	–	–
	Heparin–Ultrogel	150	0.7	105	300 000	2860	10	65
	DEAE-5 PW	22	0.45	9.9	110 000	11 000	38	24
	SP-5 PW	10	0.40	4	66 000	16 500	58	14
	DNA-affinity	5	0.001	0.005	40 000	$8 \cdot 10^6$	28 000	8.7
	Hydroxyapatite	0.5	0.005	0.0025	20 000	$8 \cdot 10^6$	28 000	4.3
UEFy	Yeast S-100	5	37	185	50 000	270	–	–
	DNA-affinity 1	4	0.02	0.1	33 000	330 000	1200	66
	DNA-affinity 2	4	0.002	0.008	26 600	$3.3 \cdot 10^6$	12 000	53

[a] One unit is defined as the amount of protein that produces retention of 0.05 ng of DNA fragment in the gel retention assay.

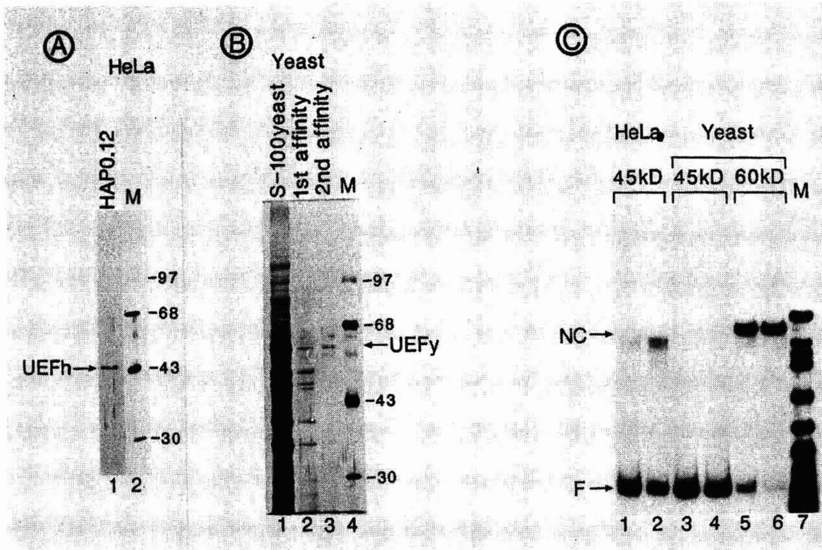

Fig. 2. (A) SDS-PAGE of purified UEFh. Lanes: 1 = 50 μl of the HAP0.12 fraction; 2 = molecular weight markers. The arrow indicates the 45-kDa polypeptide (UEFh). (B) SDS-PAGE of purification steps of the UEFy. Lanes: 1 = 0.5 μl of yeast S-100 extract; 2 and 3 = 100 μl of the 1 *M* KCl eluate of the first and second DNA affinity columns, respectively; 4 = molecular weight markers. The arrow indicates the 60-kDa polypeptide (UEFy). (C) Nucleoprotein complexes formed between the polypeptides renatured after elution from the SDS gel, and the ^{32}P-labelled SacII–BamHI fragment of pM677. Binding reactions included 100 ng of poly(dI–dC)(dI–dC), 0.1 ng of the labelled DNA and either 2–4 μl of the renatured 45 000-dalton HeLa polypeptide (lanes 1 and 2), 2–4 μl of the renatured yeast 45 000-dalton region (lanes 3 and 4) or 2–4 μl of the renatured 60 000-dalton yeast polypeptide (lanes 5 and 6). Lane 7 = size markers. NC and F indicate the nucleoprotein complexes and the free DNA, respectively.

eluted and renatured from an SDS polyacrylamide gel[16]. After renaturation, this polypeptide was able to form a specific complex with a labelled DNA containing the MLP-UE site, as detected by the gel retention assay (Fig. 2C, lanes 1 and 2). Starting from $3 \cdot 10^{10}$ HeLa cells (40 l of culture), *ca.* 2.5 μg of purified UEFh were recovered at the end of the purification (overall purification 28 000-fold; Table I). It should be noted that both polypeptides isolated by the affinity column possess the DNA-binding activity, which explains why the hydroxyapatite used to fractionate this two polypeptides did not increase the specific activity (see Table I).

Purification of the yeast UEF

The purification scheme for the UEFy is summarized Fig. 1B and Table I. A yeast S-100 extract was applied to the previously described DNA affinity matrix. After two passes on the affinity column the 1 *M* KCl eluate fraction contained a single polypeptide of 60 000 dalton (Fig. 2B, lane 3). After SDS-PAGE and renaturation, only the fraction containing the 60 000-dalton polypeptide was able to form a specific nucleoprotein complex with the labelled DNA containing the MLP-UE site (Fig. 2C, lanes 3–6). Further, a competition assay was used to check the specificity of the DNA-binding complex. The competition DNA-binding assay involved the sequential

addition of two different DNA fragments and was based on the assumption that a DNA-binding protein could bind more tightly to DNA fragments containing the wild-type UE sequence than to mutated or non-specific DNA fragments. The presence of a specific nucleoprotein complex would then be strongly reduced or eliminated, provided that the unlabelled wild-type competitor fragment was present in excess and its interaction with the factor was sufficiently stable. Preincubation of either UEFh or UEFy with a DNA fragment containing the wild-type MLP-UE prevents the formation of nucleoprotein complexes, thus demonstrating that the nucleoprotein complexes were specific for the MLP-UE. When non-specific (polydI–dC/dI–dC) or the mutated MLP-UE (which does not bind the UEF[4]) were used the formation of the complexes was not altered (data not shown). By centrifugation on a glycerol gradient, the DNA-binding activity of the UEFh and UEFy were detected in fractions corresponding to molecular weights of 45 000 and 60 000 dalton, respectively, in agreement with the observed molecular weight derived from SDS-PAGE[15,17] (data not shown).

Yeast and human UEF interact with identical DNA sequences

In order to delineate precisely the DNA region that interacts with the UEFh and UEFy, DNAse I footprint experiments were performed. On the coding strand, both the purified UEFh (Fig. 3, lane 2) and the purified UEFy (Fig. 3, lane 4) protected the MLP-UE from nucleotide −49 to −67 (with a strong DNAse I hypersensitive site at −69) and the IVa2-UE from nucleotide −98 to −120. On the non-coding

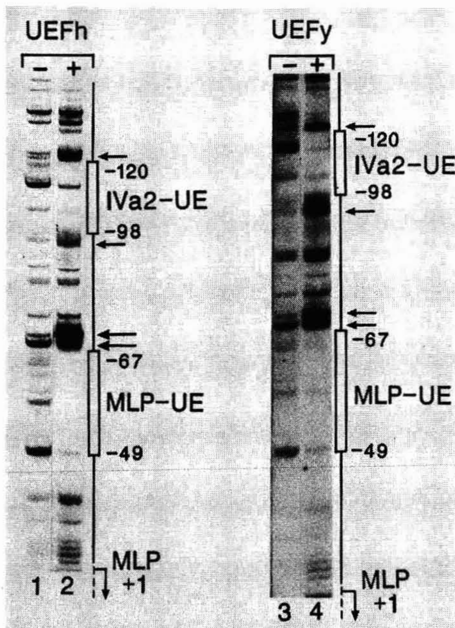

Fig. 3. DNAse I footprint on the coding strand of the MLP-IVa2 intergenic region. The ^{32}P-labelled DNA fragment (1 ng) was incubated with either UEFh (lane 2) or UEFy (lane 4) or in the absence of protein (lanes 1 and 3). The position of the MLP-UE, IVa2-UE and MLP initiation site are indicated. The arrows indicate the hypersensitives sites.

strand, the MLP-UE was protected from nucleotide −50 to −66 (MLP-UE) and from nucleotide −100 to −122 (IVa2-UE) (not shown).

The UEFy has affinities toward the two binding sites similar to those of the UEFh; they bind with a higher affinity to the MLP-UE ($K_d = 10^{-11}$) than to the IVa2-UE ($K_d = 10^{-10}$) (data not shown). Moreover, no cooperativity was apparent in the simultaneous binding of UEFy to these two sites (see also refs. 15 and 18).

DISCUSSION

By the use of a sequence-specific DNA affinity column, we purified to homogeneity two related proteins from HeLa (UEFh) and yeast (UEFy). The affinity chromatographic step improved the purification process, especially with the yeast protein; indeed, starting with the crude yeast extract, the purified UEFy was obtained by two passes over the affinity column with a relatively high recovery (50%) compared with the UEFh purification process (see Table I). This procedure was efficient both in time and in yield of protein, and the column was used more than ten times without a decrease in the capacity. In addition, we demonstrated that both UEFh and UEFy recognized the MLP-UE and IVa2-UE with identical DNAse I footprinting patterns, although these two proteins have different molecular weights. The similarity in the DNA-binding properties raises the possibility that the UEFy possesses the same transcription stimulatory property as the UEFh. We therefore tested the stimulatory effect of the UEFy in various *in vitro* transcription systems. Using the complete mammalian basic system (BTF1, BTF2, BTF3, STF, RNA polymerase B)[10], we were not able to detect a stimulation of *in vitro* transcription from the Ad2 MLP by the purified UEFy. It has been suggested that protein–protein interaction between the UEF and the TATA-box factor (BTF1) could be a prerequisite for transcription stimulation by the UEF. We therefore replaced mammalian BTF1 by its yeast counterpart (BTF1y) to allow protein–protein interaction between these two yeast proteins. However, this exchange did not elicit stimulation of transcription by addition of UEFy. There are several possibilities for this lack of stimulation in our previously designed transcription system. First, because of its higher molecular weight (60 000 *versus* 45 000 dalton for the UEFh), UEFy is not able to interact with the mammalian transcription machinery and may require a complete yeast transcription system or an additionnal yeast factor to stimulate Ad2 MLP *in vitro* transcription. It is also conceivable that stimulation of MLP transcription by the UEFy requires a precise adjustment that is not fullfilled in our present heterologous *in vitro* system. Indeed, we have recently demonstrated that the purified UEFy was able to stimulate *in vitro* transcription of the Ad2 MLP in a yeast crude nuclear extract[17], which strongly suggests that the UEFy has in yeast an analogous function to the UEFh in HeLa. However, detection of the stimulatory activity was possible only when the factor was included in a homologous system.

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Review

Progress in affinophoresis

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1. INTRODUCTION

Coupling of an affinity ligand to a soluble polyionic polymer such as succinylpolylysine makes it a specific mobile carrier in an electric field for substances having affinity to the ligand. The conjugate has a high electrophoretic mobility with a large electrokinetic force and therefore specifically changes the electrophoretic mobility of a substance that associates with the ligand. The conjugate is called an affinophore and the technique of electrophoresis using an affinophore is called affinophoresis[1] (Fig. 1).

The usefulness of biospecific affinity in the separation of biological molecules has become widely recognized since the development of affinity chromatography[2]. In

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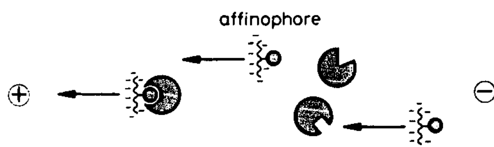

Fig. 1. The principle of affinophoresis.

the field of electrophoresis also, immobilization of affinity ligands on agarose or polyacrylamide gel has opened up new horizons for the separation of proteins and the determination of biospecific affinity[3–5]. However, the feasibility of an alternative type of matrix, the "mobile" matrix, had not been realized until the development of affinophoresis.

The key to the successful application of biospecific affinity to the separation of biological macromolecules or to biochemical analyses is the use of good matrices devoid of non-specific binding. From this point of view, the use of polyionic polymers as affinity matrices seemed dubious, since ionic polymers could be regarded as "spiders' webs" catching all substances having opposite charges. The success of affinophoresis, however, showed that there is ample scope for the use of polyionic polymers as mobile affinity matrices.

The use of mobile matrices gives affinophoresis several unique features. First, it is applicable to substances which have little or even no electrophoretic mobility, such as a protein at its isoelectric point. Second, it is applicable to suspended particles such as cells, because affinophoresis does not necessarily require an insoluble support.

In this review, selected results obtained with this distinctive method are presented and some of its basic aspects are discussed as a guide to its potential applications.

2. RELATED TECHNIQUES

Affinophoresis is a type of affinity electrophoresis, which is a method of analysis or separation of biological substances based on the change in electrophoretic mobility due to the formation of complexes with other substances by specific affinity. Affinity electrophoresis can be classified into four categories according to two criteria: whether both interacting substances are mobile (mobile system) or one of them is immobile (immobile system), and whether one of the interacting substances is linked or not to a matrix, either mobile or immobile (Table 1).

Cross-electrophoresis[6], which was used to detect protein–protein, protein–nucleic acid affinity, etc., is the root of affinity electrophoresis and is a representative of "mobile" affinity electrophoresis without matrices. The interaction between glycoproteins and lectins was determined by using this system[7]. Crossed immunoelectrophoresis[8] and gel shift assay for the analysis of nucleic acid–protein interactions[9] also come into this category, although they utilize changes in other parameters in addition to the pure electrophoretic mobility, *i.e.*, the solubility and the size of the complexes, respectively. When one of the interacting substances is inherently insoluble, the technique should be called "immobile" affinity electrophoresis without matrices. The electrophoresis of some plant lectins in starch gel[10] corresponds to this peculiar case.

TABLE 1

AFFINITY ELECTROPHORESIS CLASSIFIED ACCORDING TO TWO CRITERIA

From ref. 50.

Criterion	*Mobile system*	*Immobile system*
Without matrices	Cross-electrophoresis[6] Affinity electrophoresis of glycoproteins in the presence of lectins[7] Crossed immunoelectrophoresis[8] Gel shift assay[9] Antigen-specific electrophoretic cell separation[16]	Electrophoresis of lectins in starch gel[10]
With matrices	Affinophoresis[1,26,28,32,46,47,49,50] (mobile matrices: succinylpolylysine, polyacrylyl-β-alanyl-β-alanine, diethylaminoethyl-dextran)	Affinity electrophoresis with immobilized ligands[3–5,11–13] (immobile matrices: polyacrylamide gel, agarose gel)

Immobile matrices, such as polyacrylamide gel[3,5] or agarose gel[4], on which one of the interacting substances is coupled, were introduced together with the name "affinity electrophoresis" as the counterpart of affinity chromatography. "Immobile" affinity electrophoresis with matrices has been applied for quantitative studies of many affinity systems[11,12]. Separation of a polyclonal antibody into distinct monoclonal antibody species and the simultaneous determination of their affinity constants so far represents the summit of the achievements with this method[13]. Affinophoresis should be categorized as "mobile" affinity electrophoresis with matrices. Affinophoresis and affinity electrophoresis with immobilized ligands can thus be categorized as affinity electrophoresis with matrices.

Apart from the biospecific affinity, the micelle of dodecyl sulphate has also been successfully used as a mobile carrier in electrophoresis, *i.e.*, polyacrylamide gel electrophoresis of protein in the presence of the detergent[14] and electrokinetic chromatography for lipophilic molecules[15].

In order to separate cells based on the difference in surface antigens, a technique called antigen-specific electrophoretic cell separation (ASECS) has been developed[16]. In this method, the electrophoretic mobility of specific cells is decreased by treatments with homologous antibody and second antibody. ASECS could be classified as a type of mobile affinity electrophoresis without matrices.

3. AFFINOPHORESIS OF PROTEINS

3.1. Affinophores and affinophoresis

Affinophores have been prepared by using three types of matrices, *i.e.*, diethylaminoethyldextran, polyacrylyl-β-alanyl-β-alanine and succinylpolylysine. Examples of affinophoresis with these affinophores will provide a good introduction to the method. Affinophoresis can be carried out in free solution in principle, but the use of an insoluble gel support greatly facilitates its application, as in the case of ordinary

electrophoresis of proteins. Agarose gel of about 1% is porous enough for free electrophoresis of affinophore–protein complexes and can be easily impregnated with an affinophore by adding the affinophore to a warm solution of agarose before casting a gel.

3.1.1. Cationic affinophore based on dextran. The first affinophore prepared was a cationic type bearing an affinity ligand for trypsins[1]. Dextran was chosen for the base material because it has little tendency to bind proteins by non-specific interactions and because of the availability of size-fractionated polymers from commercial sources. The second point is important for free electrophoresis of an affinophore–protein complex in the support gel. For the derivatization of dextran, an alkylation reaction under conditions similar to those used in Hakomori's methylation methods for saccharides was utilized[17–19]. Dextran (average molecular weight 10 000) dissolved in dimethyl sulphoxide was treated with sodium methylsulphinyl carbanion and then alkylated with N-chloroacetyl-6-aminohexanoic acid and diethylaminoethyl bromide. The reaction introduced ω-carboxyl spacers for the subsequent coupling of affinity ligands and cationic charges of diethylaminoethyl groups onto the dextran. A competitive inhibitor for trypsin, *m*-aminobenzamidine[20], was coupled with the aid of a water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide][21] to the spacer. The affinophore consisted of dextran of about 60 glucose residues, carrying about 10 affinity ligands and about 25 positive charges (the sum of those of the ligands and the diethylaminoethyl groups) on average.

The affinophore was effective for the affinophoresis of trypsins from three different sources. At pH 7, the migration of cationic trypsins from bovine and *Streptomyces griseus*[22] was increased and that of anionic trypsin from *Streptomyces erythreus*[23] was decreased. As expected from the principle of affinophoresis, *Streptomyces erythreus* trypsin was carried towards the cathode at its isoelectric point, pH 4, in the presence of the cationic affinophore (Fig. 2). These effects of the affinophore were completely suppressed by adding a competitive inhibitor, leupeptin[24], to the gel or were not observed for trypsins irreversibly inhibited with tosyllysine chloromethyl ketone (TLCK)[25]. The mobility of chymotrypsin, which has a similar structure but differs in substrate specificity from trypsin, was not affected[26]. The results indicate that the alteration of the mobility depends on the specific affinity between the active site of trypsins and the affinity ligands on the affinophore.

The affinophore was deeply stained by acidic dyes generally used for the detection of proteins in support gels, such as Coomassie Brilliant Blue R250 or Amido Black 10B. After the affinophoresis, the gel was dehydrated with acetone and the proteins were detected by reaction of fluorescamine[27] with the amino groups of the proteins to form fluorescent derivatives. Another point to note is that this affinophore is slightly adsorbed by agarose gel. These aspects will be discussed below.

3.1.2. Anionic affinophore based on polyacrylic acid derivative. An affinophore can be either cationic or anionic. The second affinophore prepared was an anionic affinophore for trypsin[28]. Radical polymerization of acrylyl-β-alanyl-β-alanine provided an anionic affinophore matrix, in which carboxyl groups exist at the tips of hydrophilic spacers protruding from the polymer backbone. The polymerization reaction was carried out in the presence of thioglycolic acid to reduce the degree of polymerization. Although the degree of polymerization of the polymer has not been determined, gel chromatography of the polymer through a column of Sepharose

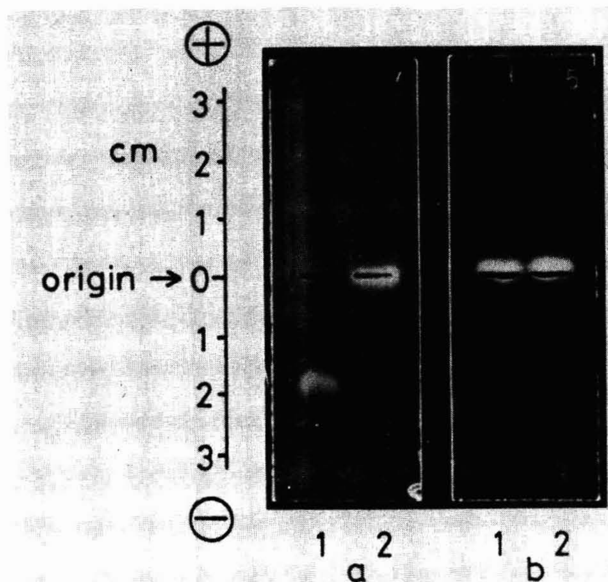

Fig. 2. Affinophoresis of *Streptomyces erythreus* trypsin at its p*I*. Electrophoresis of *Streptomyces erythreus* trypsin (4 μg, lane 1) and the trypsin inhibited with TLCK (4 μg, lane 2) was carried out for 1 h, at a constant current of 1.2 mA per plate in 0.1 *M* sodium acetate buffer (pH 4.0). Agarose gel plates (0.8%, 76 mm long × 26 mm wide × 0.65 mm thick) were prepared in (a) the presence or (b) the absence of the cationic benzamidine–affinophore. Proteins were detected after reaction with fluorescamine, as fluorescent bands. (From ref. 1.)

CL-6B indicated that the hydrodynamic volumes of most of the polymer molecules are considerably smaller than that of immunoglobulin G, which can move almost freely through 1% agarose gel. *p*-Aminobenzamidine was coupled to one-fifth of the carboxyl groups of the polymer by the use of water-soluble carbodiimide. This affinophore was also stained with Coomassie Blue dye, but it was no longer stainable after the change of the ionic groups from carboxyl to sulphonic acid, which was accomplished by the coupling of aminomethanesulphonic acid to the carboxyl groups by the use of the carbodiimide.

The mobilities of *S. griseus* and bovine trypsins were greatly changed in the presence of the anionic affinophore (Fig. 3). The dose–response relationship showed that *S. griseus* trypsin has a higher affinity for the affinophore than bovine trypsin does. This is consistent with the observation that the inhibition constant of benzamidine for *S. griseus* trypsin is 1.0 μM and that for bovine trypsin is 15 μM[29,30]. The slight anodic shift of TLCK-treated *S. griseus* trypsin and TLCK-treated bovine trypsin was once ascribed to a non-specific ionic effect of the polymer. However, this is not likely considering the high potency of the phosphate buffer used in the experiments for suppressing such ionic interactions (see below). Some other type of low-affinity interaction may be operating. Migration of *S. erythreus* trypsin, an anionic trypsin, was not influenced by the affinophore. Although the reason for this ineffectiveness was not studied, it seems possible that the ionic repulsion between the protein and the

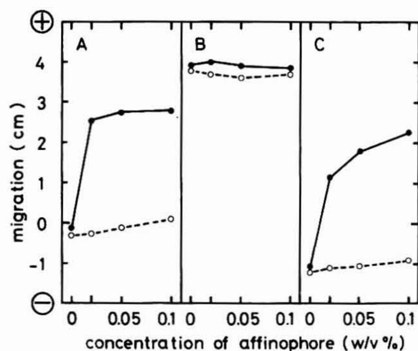

Fig. 3. Dependence of the migration of trypsins on the concentration of the anionic benzamidine–affinophore. Electrophoresis of (●) trypsins and (○) those inhibited with TLCK (4 μg each) was carried out at a constant current of 100 mA per plate for 40 min on an agarose gel plate (1%, 125 mm long × 80 mm wide × 1 mm thick) containing the indicated concentration of the affinophore and 0.1 *M* sodium phosphate buffer (pH 7). Proteins were detected by staining with Coomassie Brilliant Blue R250. A solution of the affinophore at a concentration of 0.1% (w/v) contained 0.46 m*M* *p*-aminobenzamidine. A, *S. griseus* trypsin; B, *S. erythreus* trypsin; C, bovine trypsin. (From ref. 28.)

affinophore interferes with the specific interaction. Alternatively, the difference in the mobilities of the protein and its complex with the affinophore might be too small. *S. griseus* trypsin in pronase, a mixture of proteases[31], was separated from other proteins by the affinophoresis (Fig. 4). In this way, the affinophoresis of trypsins was realized with either a cationic or an anionic affinophore.

3.1.3. Anionic affinophore based on polylysine. Although polyacrylyl-β-alanyl-β-alanine is an excellent matrix for an anionic affinophore, the synthesis of the monomer is laborious for biochemists who are interested in particular applications of affinophoresis, and the degree of polymerization cannot readily be determined. Poly-L-lysine is commercially available as a size-fractionated polymer. Although the polymer might be usable as a cationic matrix as it is, it was used after succinylation as an anionic matrix in order to avoid the problems associated with the use of a cationic affinophore, *i.e.*, adsorption on agarose gel and staining by dyes. Poly-L-lysine with an average degree of polymerization of 190 was succinylated and L-tryptophan methyl ester was coupled to one-fifth of the succinyl groups as the precursor of an affinity ligand for chymotrypsin and its derivatives[32]. This affinophore was also weakly stained with Coomassie Blue dye, but the coupling of aminomethanesulphonic acid to the residual carboxyl groups overcome this problem. Finally, the ester moiety of the ligand was removed by alkali treatment. The affinity ligand was tryptophan with a free carboxyl group and was thus a product-type ligand for chymotrypsin.

Two chemically modified derivatives of chymotrypsin were subjected to affinophoresis by using the tryptophan–affinophore together with chymotrypsin and chymotrypsinogen[32]. Phenylmethanesulphonylchymotrypsin (PMS-chymotrypsin) was produced by affinity labelling of the hydroxyl group of the active centre serine residue of chymotrypsin with phenylmethanesulphonyl fluoride (PMSF) and thus the substrate-binding pocket was occupied by the phenyl ring of the reagent[33]. Anhydrochymotrypsin is a product of a β-elimination reaction of phenylmethanesulphonic acid

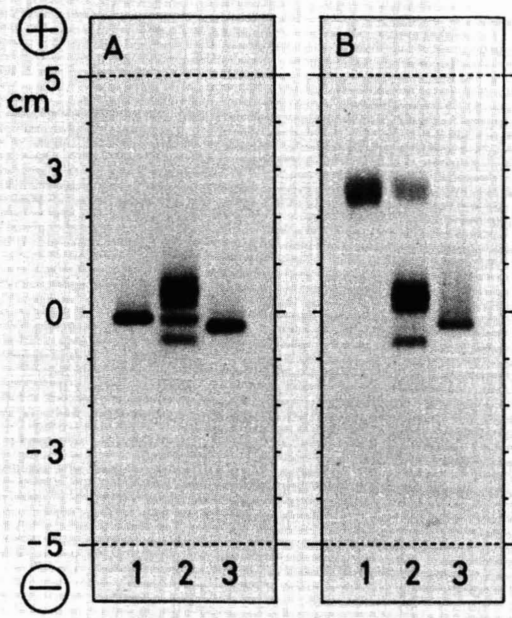

Fig. 4. Separation of *S. griseus* trypsin from pronase by affinophoresis. Electrophoresis of *S. griseus* trypsin (4 μg, lane 1), pronase (16 μg, lane 2) and the trypsin inhibited with TLCK (4 μg, lane 3) was carried out in (A) the absence or (B) the presence of the anionic benzamidine–affinophore (0.02%, w/v). Other conditions as in Fig. 3. Both sides (20 mm wide) of the gels were cut off and the central parts of the gel plates are shown. (From ref. 28.)

from PMS-chymotrypsin and has a dehydroalanine residue instead of serine at the active centre of chymotrypsin[34]. The transformation of chymotrypsin to its anhydro derivative enhances its affinity toward its own product-type ligands, *e.g.*, the affinity ligand of the tryptophan–affinophore[35,36]. The original mobilities of these proteins were very small with the electrophoresis buffer of 0.1 *M* sodium phosphate (pH 7.2) (Fig. 5). Addition of the affinophore to the gel greatly increased the mobility of anhydrochymotrypsin towards the anode and moderately increased that of chymotrypsin. For chymotrypsinogen and PMS-chymotrypsin, the effect of the affinophore was very small and it is consistent with the defectiveness of the substrate binding site of these proteins.

3.2. Some considerations on affinophoresis

Some discussion of the theoretical basis of affinophoresis would be helpful for the understanding of the method and useful for its further development.

3.2.1. Mobility. Suppose an affinophore (A) and a protein (P) are in the following equilibrium:

$$\mathrm{A} + \mathrm{P} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{AP} \tag{1}$$

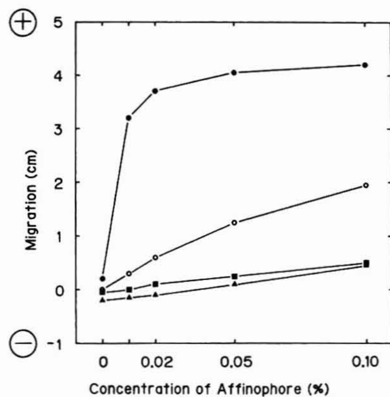

Fig. 5. Dependence of the migration of chymotrypsin and related proteins on the concentration of the tryptophan–affinophore. Electrophoresis of proteins (4 μg each) was carried out at a constant current of 100 mA per plate for 30 min on the agarose gel plate (1%, 125 mm long × 80 mm wide × 1 mm thick) containing the indicated concentration of the affinophore and 0.1 *M* sodium phosphate buffer (pH 7.2). Proteins were detected by staining with Coomassie Brilliant Blue R250. A solution of the affinophore at a concentration of 0.1% (w/v) (15 μM) contained 0.56 m*M* tryptophan. (○) Chymotrypsin; (●) anhydrochymotrypsin; (■) PMS-chymotrypsin; (▲) chymotrypsinogen A. (From ref. 32.)

For an affinophore bearing a number of ligands, complexes of higher order, AP_2, AP_3, etc., should also be formed. For simplicity, it is assumed here that only the one-to-one AP complex is formed. This situation would be realized in the presence of an excess of affinophore over a protein. The change in the electrophoretic mobility in affinophoresis is based on the difference between the original mobility of the protein (m_0) and that of the AP complex (m_c). The mobility of a particle in an electrophoresis experiment is the velocity reached when the frictional drag just balances the electric force, *i.e.*, $QE = kv$, when a particle of charge Q migrates at velocity v in an electric field E with frictional constant k. On application of an electric field, the time required for a protein with a molecular weight of $1.2 \cdot 10^5$ to reach a constant velocity was calculated to be of the order of 10^{-13} min[37]. On the other hand, the rate constants of dissociation of enzyme–substrate complexes (k_{-1}), which would be comparable to that of the AP complex, are known to be 10^2–10^6 min^{-1} in general[38]. In other words, at the fastest, the dissociation reaction occurs once in 10^{-6} min for a single protein molecule under conditions that allow almost all the protein to form the complex with the affinophore. Hence the dissociation–association reaction is very slow compared with the time required for the change in electrophoretic velocity associated with the reaction. Consequently, the mobility of proteins subjected to affinophoresis is microscopically discontinuous, changing between m_0 and m_c in each dissociation and association reaction. The observed mobility of the protein (m) is an average as follows:

$$m = \frac{P_f}{P_t} \cdot m_0 + \frac{P_c}{P_t} \cdot m_c \qquad (2)$$

where P_f and P_c are the concentration of the free protein and that of the protein complexed with the affinophore, respectively, and P_t is the total concentration of the

protein, *i.e.*, $P_t = P_f + P_c$. The equilibrium obeys the mass action law and the equilibrium constant (K_d) is given by

$$K_d = \frac{P_f[A]}{P_c} \tag{3}$$

where [A] is the concentration of the free affinophore. From eqns. 2 and 3, the following equation describing the change in mobility of a protein subjected to affinophoresis is obtained:

$$m - m_0 = (m_c - m_0)\frac{[A]}{K_d + [A]} \tag{4}$$

This equation is a rectangular hyperbola (Fig. 6) similar to the Henri–Michaelis–Menten equation of enzyme kinetics. The observed mobility of the protein (m) reaches that of the complex (m_c) at infinite concentration of the affinophore. Two important points concerning the effectiveness of affinophores are clearly shown. First, the difference between the original mobility of the protein and the mobility of the complex determines the maximum effect, but the mobility of the affinophore itself does not directly relate to the effectiveness. Second, the concentration of the affinophore should be comparable to or greater than the dissociation constant.

The double reciprocal plot of $1/(m - m_0)$ *versus* $1/[A]$ is linear and corresponds to the Lineweaver–Burk plot of enzyme kinetics:

$$\frac{1}{m - m_0} = \frac{K_d}{m_c - m_0} \cdot \frac{1}{[A]} + \frac{1}{m_c - m_0} \tag{5}$$

The intercept on the $1/(m - m_0)$ axis is $1/(m_c - m_0)$ and that on the $1/[A]$ axis is $-1/K_d$. An identical equation was derived for affinity electrophoresis through different approaches[39,40].

The results of the affinophoresis of chymotrypsin and anhydrochymotrypsin presented in Fig. 5 were plotted according to eqn. 5 (Fig. 7). Straight lines were fitted

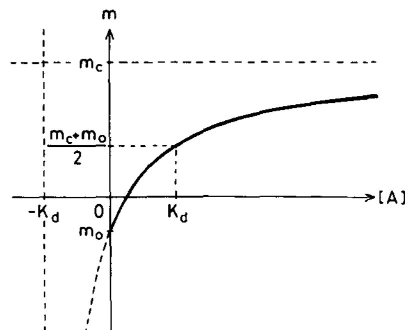

Fig. 6. Plot of m *versus* [A] according to eqn. 4; m is the observed mobility of the protein and [A] is the concentration of the affinophore.

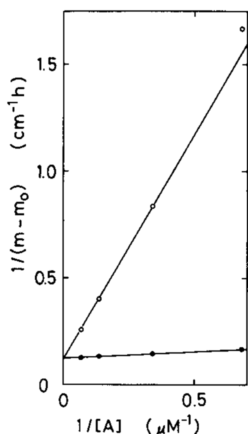

Fig. 7. Double reciprocal plot of the results of the affinophoresis of (O) chymotrypsin and (●) anhydrochymotrypsin according to eqn. 5.

and the values of $m_c - m_0$ and K_d were obtained as 8.8 cm h^{-1} and 18.1 μM for chymotrypsin and 8.4 cm h^{-1} and 0.60 μM for anhydrochymotrypsin, respectively (Fig. 7). In this case, the concentration of the affinophore was calculated simply by dividing the concentration of the lysine residue, which can be determined by amino acid analysis after hydrolysis, by the average degree of polymerization of the base polymer.

3.2.2. Number of ligands. At a rough estimate, the dissociation constant for an affinophore bearing n ligands $[K_d(n)]$ should decrease from that for the affinophore bearing a single ligand $[K_d(1)]$ by a factor of $1/n$, since there are n ways of complex formation, *i.e.*, $K_d(n) = K_d(1)/n$. The estimated number of ligands on the tryptophan–affinophore was 38. The value of $K_d(1)$ can then be calculated to be 690 μM for chymotrypsin and 23 μM for anhydrochymotrypsin by multiplying the K_d values obtained above by 38. The dissociation constants with acetyl-L-tryptophan at pH 6.0 (4°C) were reported to be 400 μM for chymotrypsin and 14 μM for anhydrochymotrypsin[35]. These values are roughly comparable to the $K_d(1)$ values calculated above. Hence increasing the number of ligands on an affinophore would be effective in raising its affinity for a protein.

For proteins having two or more binding sites for the ligand, the number of ligands on a single affinophore molecule would have another special effect. If each binding site of the protein can simultaneously bind to the ligands on such a flexible polymer, the interaction should be very strong, just like the avidity or bonus effect of an antibody–antigen interaction.

3.2.3. Size of affinophore. The electrophoretic mobility of a linear polyionic polymer such as polyacrylic acid is known to be independent of its degree of polymerization above a certain ionic strength[41]. The affinophore should be large enough to retain its high mobility on association with a protein. When an insoluble gel support for electrophoresis is used, its structure should be such as to allow unrestricted migration.

3.2.4. Heterogeneity of affinophore. Polymers used as the matrices in affino-

phoresis are heterogeneous in size, which would make the electrophoretic mobility of the AP complex heterogeneous. When an affinophore and protein are in equilibrium with rapid association–dissociation, each protein molecule undergoes many association–dissociation reactions with many different affinophore molecules in the course of affinophoresis. Thereby, the mobility of the AP complex approaches an average value, and thus the heterogeneity of the affinophore does not result in diffusion of protein bands in the direction of electrophoresis. If the value of k_{-1} is assumed to be 10^2 min^{-1}, a value which is low compared with k_{-1} proposed for usual enzyme–substrate complexes[38], an affinophoresis time of 30 min will allow a protein to bind about 3000 different affinophore molecules, and this number is sufficiently large for close approach to an average mobility.

For a binding equilibrium with extremely slow dissociation, *e.g.*, the biotin–avidin system in which k_{-1} is $2.4 \cdot 10^{-6}$ min^{-1} (ref. 42), heterogeneity of the affinophore might cause the diffusion of the protein bands in the direction of the electrophoresis.

3.2.5. Ionic interaction. It is desirable that the non-specific ionic interaction of proteins with an affinophore matrix should be as small as possible. To examine the ionic interaction between proteins and soluble polyionic polymers, electrophoresis of proteins was carried out on 1% agarose gel plates in the presence of succinylpolylysine (average degree of polymerization 120)[43]. Proteins migrated as distinct bands even in the presence of the polyionic polymer, but the mobility of cationic proteins was influenced. By using 0.1 *M* tris(hydroxymethyl)aminomethane (Tris)–acetic acid buffer (pH 7.9, ionic strength 0.06), the mobility of lysozyme (chicken), cytochrome *c* (horse) and chymotrypsinogen A (bovine) was greatly changed (about 80% of the maximum change) even at 10 μM polymer (corresponding to 1.2 m*M* lysine residue), whereas that of anionic proteins was not affected.

The relationship between the mobility of a protein and the concentration of the polymer was identical with that for affinophore–protein interaction, *i.e.*, it can be described as a rectangular hyperbola. If the formation of a distinct complex is assumed for the ionic interaction between cationic proteins and succinylpolylysine as in the case of specific interaction, the extent of ionic interaction can be estimated by using a dissociation constant as for affinophoresis. As expected, the ionic interaction is sensitive to the ionic strength of the electrophoresis buffer.

By using 0.1 *M* sodium phosphate buffer (pH 7, ionic strength 0.18), the ionic interaction of ribonuclease A (bovine), chymotrypsinogen A and cytochrome *c* with up to 100 μM polymer was nearly completely suppressed. Lysozyme still showed some interaction under these conditions. If an affinophore molecule bears 20 ligands, 100 μM of it would be sufficient to cause half of the maximum mobility change for a specific protein with the intrinsic dissociation constant of 2 m*M* for the ligand. For a high-affinity system, buffers of lower ionic strength can be used, and this is desirable for rapid completion of electrophoresis.

The ionic interaction can be positively utilized in "mobile ion-exchange electrophoresis". Succinylpolylysine is usable as a soluble ion exchanger for the separation of proteins. Haemoglobin and glycated haemoglobin were separated by using dextran sulphate as a soluble ion-exchanger[44].

3.3. Practical guide for the preparation of affinophores

At present, polylysine is the most convenient base polymer for affinophores, as it is commercially available as fractionated polymers differing in degree of polymerization, and the reactivity of its amino group is very useful for derivatization to the affinophore. Although polylysine with an average degree of polymerization of 120 or 190 has been used, the relationship between the size of the polymer and the effectiveness of an affinophore has not been studied. Large affinophores may be suitable for the affinophoresis of a protein of high molecular weight and small ones for affinophoresis in a small-pore gel such as polyacrylamide gel.

An affinophore can be either cationic or anionic in principle. However, as mentioned above, the use of cationic affinophores poses some practical problems in agarose gel electrophoresis. First, cationic affinophores are adsorbed on agarose, probably owing to an ionic interaction with sulphate or carboxyl groups on agarose, and second, they are stained with a dye such as Coomassie Blue and hence they interfere with the detection of proteins in the gel. When the ligand was benzamidine or tryptophan, even anionic affinophores with carboxyl groups were stained by Coomassie Blue dye. Although the factors determining the affinity of the dye have not been fully identified, the importance of cationic or aromatic functional groups was suggested by the results of an experiment using synthetic polyamino acids[45]. A change in the ionic group from carboxylate to sulphate made the affinophores no longer stainable with the dye. The tryptophan–affinophore in which succinyl groups were fully modified with aminomethanesulphonic acid slightly interfered with the staining of proteins by the anionic dye, probably because of ionic repulsion. Partial coupling of aminomethanesulphonic acid (about one fifth of succinyllysine residues or some excess over a cationic ligand) may thus be advisable.

The following procedure requires ligands that have an amino or a carboxyl group available for coupling, *i.e.*, these groups should not be a determinant of specificity (Fig. 8). The coupling reactions are mainly based on the formation of amide bonds by means of a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide[21]. The amount of the polymer can be determined by amino acid analysis

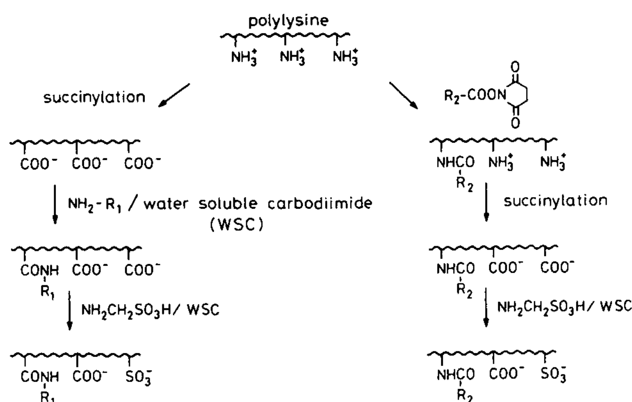

Fig. 8. Preparation of anionic affinophores based on polylysine. NH_2–R_1, an affinity ligand having an amino group available for coupling; R_2–COOH, an affinity ligand having a carboxyl group available for coupling.

after hydrolysis[32]. The coupling of amino ligands is carried out in an aqueous solution (pH 4.5–5) of succinylpolylysine and a ligand (10–20 mol-% of succinyllysine residue) by adding the carbodiimide (50 mol-% of succinyllysine residue). Aliphatic amines with high pK_a values are less reactive than arylamines, amino acid esters or aminomethanesulphonic acid under these conditions. In this instance, the use of 0.2 *M* morpholinopropanesulphonic acid buffer (pH 7) is recommended. The coupling reaction should be monitored by following the disappearance of the free ligand. For a carboxyl ligand, direct coupling to polylysine by means of the carbodiimide did not afford satisfactory results, probably because of the high pK_a value of the ε-amino group of the lysine residues. In this case, the carboxyl group of the ligand should be activated beforehand as the N-hydroxysuccinimide ester. After the coupling of the activated ligand to polylysine in 0.1 *M* sodium phosphate buffer (pH 7.5), the polymer is succinylated. Aminomethanesulphonic acid is coupled by means of water-soluble carbodiimide as for amino ligands at pH 4.5–5.

Polylysine and succinylpolylysine are both highly soluble in water. The reaction can be carried out at high concentration (20 mg/ml) and thus is completed very rapidly with high coupling yields. The carbodiimide can be used in excess over the ligand, as most of the excessively activated carboxyl groups are hydrolysed back to carboxyl groups[21]. After the reactions, polymers can be readily purified by dialysis. The affinophores prepared based on succinyl polylysine had mobilities of 1.0–1.3 relative to bromophenol blue at pH 8.

3.4. *Two-dimensional affinophoresis*

One-dimensional affinophoresis gives satisfactory results for samples of simple composition. For complex samples, two-dimensional affinophoresis makes the identification of a specific protein very easy. The first electrophoresis is carried out without an affinophore and the second is carried out at right-angles to the first under identical conditions except for the presence of an affinophore. Non-specific proteins should lie on a diagonal line and the specific protein should be found away from the line, as its mobility is changed in the second electrophoresis by the affinophore. Even if the change in mobility is not large, the deviation can be readily observed.

An extract of pancreatin, a dry preparation of porcine pancreatic juice, was subjected to two-dimensional affinophoresis with the anionic benzamidine–affinophore based on polyacrylyl-β-alanyl-β-alanine[46]. After the first electrophoresis in a slab of 1% agarose gel, the gel containing the affinophore was formed on the side of the separated proteins and the second electrophoresis was carried out so that the affinophore would migrate over the protein samples (Fig. 9). Such a two-dimensional affinophoresis can be completed within 1.5–2 h. Coomassie Blue staining revealed that a doublet spot was separated from the diagonal line (Fig. 10A). Prior to protein staining, a sheet of filter-paper impregnated with a fluorogenic substrate for trypsin was placed on the gel for a short period in order to absorb a part of the solution in the gel. Incubation of the paper developed a double fluorescent spot at the position corresponding to the separated spots revealed by protein staining (Fig. 10B). When the matrix polyionic polymer polyacrylyl-β-alanyl-β-alanylaminomethanesulphonic acid was used instead of the affinophore at an equivalent concentration, deviation of trypsin from the diagonal line was not observed. *S. griseus* trypsin in pronase was also separated by two-dimensional affinophoresis[46].

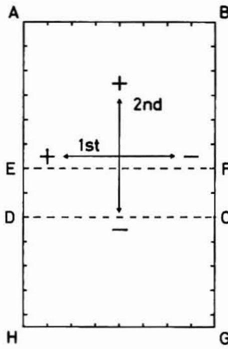

Fig. 9. Construction of gels for two-dimensional affinophoresis. The first-dimensional electrophoresis was carried out in a square gel without an affinophore (the square ABCD, 80 × 80 mm). A sample solution was applied in a hole at 25 mm from edge CD. Prior to the second-dimensional electrophoresis, part of the gel (the rectangle CDEF) was cut away and the gel containing an affinophore was formed (the rectangle EFGH). The gels were 1 mm thick. The edges of the diagram are notched at 10 mm intervals. (From ref. 46.)

The extracts of legume seeds were subjected to two-dimensional affinophoresis with an anionic affinophore bearing α-D-mannoside as an affinity ligand in order to separate mannose-binding lectins[47]. The affinophore was prepared by coupling *p*-aminophenyl-α-D-mannoside (10 mol-% of lysine residue) and aminomethane-

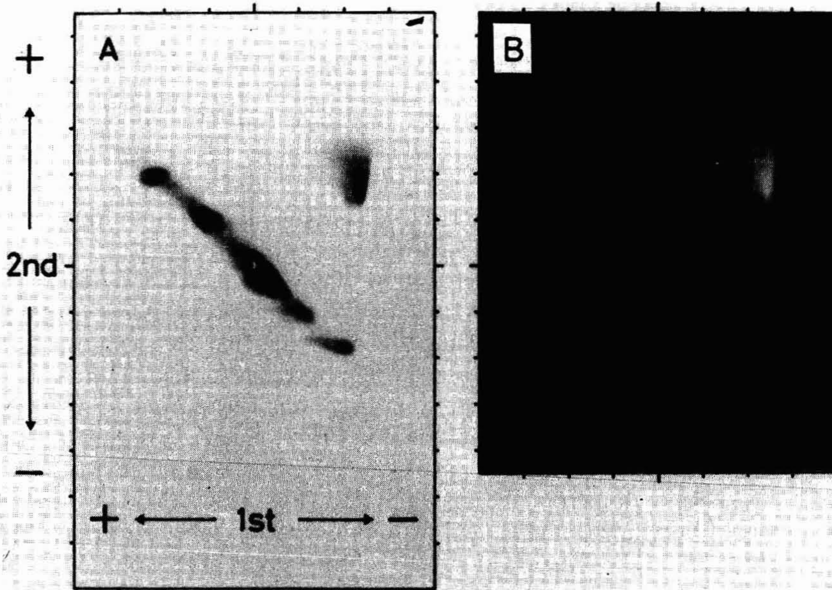

Fig. 10. Separation of porcine trypsin from pancreatin by two-dimensional affinophoresis. The extract (2 μl) of pancreatin was applied in a hole 5.5 cm from the top, 4 cm from the sides. Electrophoresis was carried out for 20 min at 60 mA per plate (about 25 V cm^{-1}) in each direction by using 0.1 *M* Tris–acetic acid buffer (pH 7.85). The concentration of the affinophore was 0.02%. (A) Coomassie Brilliant Blue R250 staining; (B) detection of trypsin activity with a fluorogenic substrate. (From ref. 46.)

sulphonic acid (20 mol-% of lysine residue) to succinylpolylysine (degree of polymerization 120).

Pea seed contains a lectin specific for D-mannose or D-glucose[48]. The lectin has a molecular weight of about 50 000 and has two sugar-binding sites. Two-dimensional affinophoresis of the extract of pea seed separated a spot from the diagonal line (Fig. 11A). Immuno-staining of the protein blotted onto a nitrocellulose membrane after the affinophoresis with anti-pea lectin antibody showed that the spot was the lectin (Fig. 11B). Blotting of separated proteins from the agarose gel is very easily performed and 10 ng of the lectin could be detected. In the presence of a free ligand, methyl-α-D-mannoside, the spot did not migrate away from the diagonal line (Fig. 11C and D). This shows the specificity of the affinophoresis. A lectin was also separated from the extract of fava bean in the same way. In the case of jack bean, a similar experiment resulted in the formation of a dense precipitate in the second electrophoresis. The problem was resolved by decreasing the amount of the sample to one-tenth. The abundance of the lectin, concanavalin A, in the bean might create conditions favourable for the formation of the specific aggregate of the affinophore and the lectin.

Anti-hapten antibody in rabbit serum was separated by two-dimensional affinophoresis directly from antiserum[49]. The hapten was a tripeptide with a blocked amino terminus, N-(dibenzyloxyphosphinoyl)-L-alanyl-L-prolyl-L-proline. The affinophore was prepared by reaction of the N-hydroxysuccinimide ester of the N-blocked tripeptide with polylysine having an average degree of polymerization of 190. The polymer was then succinylated and the coupling was carried out with aminomethanesulphonic acid. The content of the ligand was 15 mol-% of lysine residue and that of aminomethanesulphonic acid was about 20 mol-%. The specific anti-hapten antibody separated by the affinophoresis was detected by Coomassie Blue staining and immunostaining.

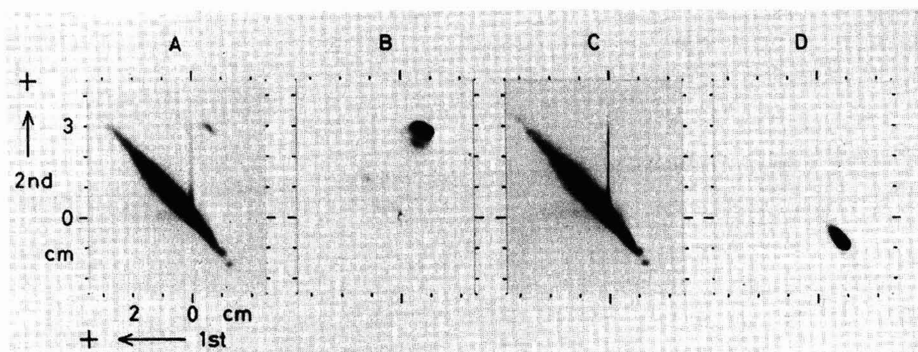

Fig. 11. Two-dimensional affinophoresis of the extract of pea seed with the mannose–affinophore. The sample was applied at the position 0 and electrophoresis was carried out for 30 min at 25 V cm^{-1} (40–50 mA per plate) in each direction by using 0.1 *M* Tris–acetic acid buffer (pH 7.9) in (A and B) the absence or (C and D) the presence of 0.1 *M* methyl-α-D-mannoside. The concentration of the affinophore was 5.2 μM (58 μM for the ligand). A and C, 2 μl of the extract were applied and stained with Coomassie Brilliant Blue R250; B and D, 2 μl of the 10-fold diluted extract were applied and immunostaining was carried out after blotting onto a nitrocellulose membrane. Only the central part of gels containing proteins is shown. (From ref. 47.)

4. AFFINOPHORESIS OF CELLS

The difference in the electrophoretic mobilities of different cells is not always large enough to allow a clear separation by the usual electrophoresis. The alteration of the mobility of specific cells by affinophoresis might therefore be useful for the separation of specific cells by electrophoresis. Each cell population expresses distinct surface antigens and thus affinophoresis specific for the surface antigens should be effective.

4.1. Attachment of affinophore to surface antigens

For the preparation of an affinophore, the direct chemical conjugation of an antibody to an ionic polymer is a straightforward approach in principle. A preliminary attempt showed that direct coupling by using water-soluble carbodiimide resulted in a considerable loss of the binding ability of proteins, probably because of the formation of multiple bonds. As an alternative, an indirect method was emoloyed[50]. The procedure consists in four steps of treatment of cells with an antiserum, biotinylated second antibody, avidin and biotinylated succinylpolylysine as an affinophore (Fig. 12). The biotin–affinophore was prepared by reaction of the N-hydroxysuccinimide ester of *d*-biotin with polylysine, followed by succinylation. The anionic affinophore was used in order to avoid the expected non-specific ionic adsorption on the negatively charged cell surface. The procedure is readily applicable to different types of cells simply by changing the antiserum. The affinophoresis of cells was carried out at a non-equilibrium state of binding, unlike the case with proteins, owing to the low dissociation rate of the antigen–antibody system and avidin–biotin system.

4.2. Affinophoresis of red blood cells

Affinophoresis of red blood cells (RBCs) from rabbits, humans and rats was carried out and the electrophoretic mobility of cells was determined by using an automated cell electrophoresis analyser, CEP-1 (Shimadzu, Kyoto, Japan)[51]. Electrophoresis of all of the RBCs was accelerated when a homologous antiserum was used (Fig. 13). The largest effect, a 2.9-fold acceleration, was observed for rabbit RBCs,

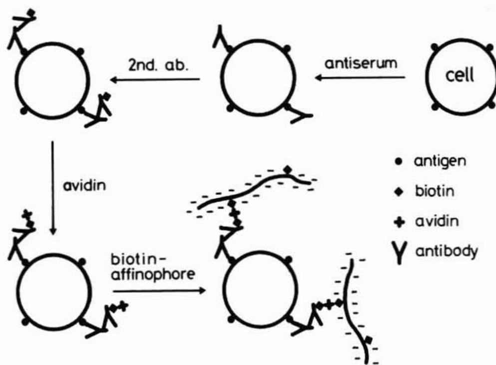

Fig. 12. Coupling of biotin–affinophore to cell. (From ref. 50.)

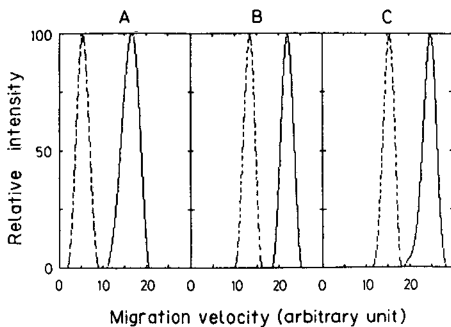

Fig. 13. Affinophoresis of red blood cells. The results of the electrophoresis of non-treated cells (broken lines) and those of affinophoresis (solid lines) are superimposed for each species. Electrophoresis was carried out with an automated cell electrophoresis analyser in 10 m*M* sodium phosphate buffer (pH 7.2) containing 0.13 *M* NaCl and 5 m*M* KCl at 25°C. The effect of electroosmotic flow is corrected. A, Rabbit; B, human; C, rat. (From ref. 50.)

which have the lowest original mobility among the three. The effect of affinophoresis depended wholly on the specificity of the antiserum used, since no acceleration was observed when a non-immune serum was used instead of the homologous antiserum. The affinophoresis increased the mobility of RBCs of human and rat 1.7- and 1.6-fold, respectively. The largest amount of antiserum should be used for the largest effect, provided that cell aggregates are not formed. The other three reagents, biotinylated second antibody, avidin and biotin–affinophore, can be used in amounts that give the plateau levels of the dose–response relationship.

A larger affinophore, with an average degree of polymerization (DP) of 1150, was more effective than a smaller one (DP of 270). For an affinophore of a given size, the lower the biotin content the more effective it was. The most effective affinophore had an average of seven biotinyl residues on one succinylpolylysine molecule with an average DP of 1150.

To test the feasibility of affinophoresis for specific cell separations, two types of RBCs were mixed and subjected to affinophoresis by using either of the two corresponding antisera. Electrophoresis of an untreated mixture of rabbit and human RBCs gave two peaks (Fig. 14, A1). The peaks were assigned by comparing the data with those obtained for each single species presented in Fig. 13: the peak of lower velocity for the rabbit and that of higher velocity for humans. Affinophoresis of the same mixture after treatment with anti-human RBC serum resulted in specific acceleration of human RBCs and thus the distance between the two peaks was extended (Fig. 14, A3). The use of anti-rabbit RBC serum gave a single peak, probably because of the specific acceleration of rabbit RBCs and the consequent overlapping with human RBCs (Fig. 14, A2). Similar results were obtained for the combination of rabbit and rat RBCs (Fig. 14, C). The mixture of non-treated human and rat RBCs gave a single peak because of the small difference in the original migration velocities of the two (Fig. 14, B1). By using either antiserum, the affinophoresis gave two peaks (Fig. 14, B2, B3). The peak which has the higher velocity in each histogram must be that of the RBCs homologous with the antiserum used. The clearer separation of the peaks attained with the anti-rat RBC serum accords with this interpretation, as rat

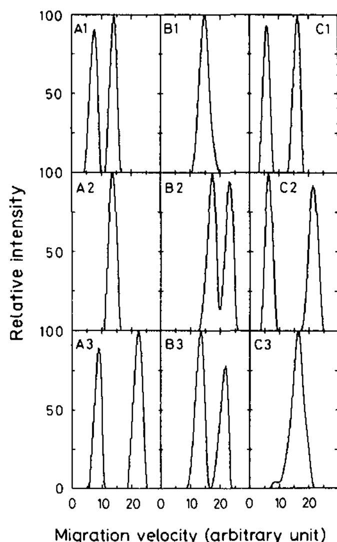

Fig. 14. Affinophoresis of mixed red blood cells. RBCs from two spcies were mixed and electrophoresis was carried out before or after treatment for affinophoresis. Other conditions were the same as in Fig. 13. A, Rabbit and human; A1, no treatment; A2, affinophoresis with an anti-rabbit RBC serum; A3, affinophoresis with an anti-human RBC serum. B, Human and rat; B1, no treatment; B2, affinophoresis with an anti-human RBC serum; B3, affinophoresis with an anti-rat RBC serum. C, Rat and rabbit; C1, no treatment; C2, affinophoresis with an anti-rat RBC serum; C3, affinophoresis with an anti-rabbit RBC serum. (From ref. 50.)

RBCs originally showed a higher velocity than human RBCs. The last results show the great potential value of affinophoresis in specific cell separations, which cannot be achieved by ordinary electrophoresis.

5. PROSPECTS

For the affinophoresis of proteins, insoluble supports for electrophoresis other than agarose gel may offer specific advantages. The use of a cellulose acetate membrane would further simplify the procedure. If an affinophore is reasonably small, the use of polyacrylamide gel and discontinuous buffer systems[52] could afford a higher resolution than the agarose gel system. Affinophoresis would make a good combination with capillary electrophoresis.

For a preparative application of affinophoresis, two-step electrophoresis would be effective. This is identical with two-dimensional affinophoresis in principle. First electrophoresis is carried out without the affinophore and then the fraction containing the target substance is subjected to a second electrophoresis under identical conditions except for the presence of the affinophore. The target substance would be transported at the anodic side of the other substance if an anionic affinophore is used. A discontinuous buffer system, *e.g.*, chloride ion as the leading ion and glycine as the trailing ion, may be useful in some instances. If experimental conditions are chosen in order that an affinophore–protein complex is transported at the interface of ions, it would be possible to retrieve the target substance in a highly concentrated state.

At present, a preparative electrophoresis apparatus that is usable for cell separation is still costly and its operation is not straightforward. As electrophoresis is simple in itself, the development of apparatus that is simpler and easier to operate than the present version is likely. In that event affinophoresis should become a method of choice for separating specific cells, in addition to ASECS.

Affinophoresis is a versatile method and the results described here are only a starting point. The scope for future developments is enormous.

6. ACKNOWLEDGEMENT

The author is grateful to Professor Ken-ichi Kasai for his helpful advice on the manuscript.

7. ABSTRACT

The use of polyionic polymers as mobile affinity matrices in electrophoresis has led to the development of a specific separation method for biological substances, affinophoresis. The conjugate of a polyionic polymer and an affinity ligand is called an affinophore. Electrophoresis of proteins in the presence of an affinophore results in a change in the mobility of a specific protein due to the difference between the mobility of the protein and that of the protein–affinophore complex. Polylysine is useful as a base polymer of affinophores and has been used successfully as an anionic matrix after succinylation. Affinophoresis of proteases, lectins and antibodies has been carried out in agarose gel and the mobility of the protein having affinity to each ligand was specifically changed. Two-dimensional affinophoresis, in which an affinophore was included only in the second-dimensional electrophoresis, was effective for the separation of the components of a complex mixture of proteins even if the change of mobility was not large. Red blood cells were successively treated with homologous antiserum, biotinylated second antibody, avidin and biotinylated succinylpolylysine as an affinophore. Specific acceleration of the homologous cells to the antiserum was observed even when the affinophoresis was applied to mixed red blood cells from different species.

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Effect of polyethylene glycol on the non-specific adsorption of proteins to Eupergit C and agarose

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ABSTRACT

Non-specific adsorption of serum proteins to Eupergit C (EC) and agarose during the process of immunoaffinity chromatography often leads to contamination of the specifically eluted antigens to be purified. This effect was studied by application of serum samples to a β-mercaptoethanol-blocked EC (EC-βME) column followed by analysis of proteins eluted with various elution buffers. Inclusion of polyethylene glycol (PEG 400 or 1500) in the loading buffer reduced the non-specific adsorption of proteins to EC but had an adverse effect on agarose. Covalent attachment of amino-PEG to EC and to epoxy-activated Sepharose mimicked the effect of PEG in solution with EC and resulted in a marked reduction in non-specific adsorption of serum proteins. Inclusion of PEG in the loading buffer during immunopurification of a serum protein (immunoglobulin G) or seminal plasma protein (human decidua protein hDP71) resulted in a marked improvement in the purity of these proteins eluted from the respective columns by ammonium acetate (pH 10).

INTRODUCTION

During the last decade, affinity chromatography has become a major tool in the purification of biologically active proteins[1]. The interaction between the immobilized ligand and the protein(s) to be purified is usually highly specific, resulting in a high separation power. Thus, protein purification may be achieved by a small number of purification steps. One of the major difficulties which occurs with this method, however, is the contamination of the affinity-purified protein(s) of interest by foreign proteins which are non-specifically adsorbed to the matrix during the loading step and are later eluted during the elution step as contaminants of the product.

Non-specific adsorption of proteins has been shown to increase with the hydrophobicity of the matrix[2]. In addition, different proteins tend to be adsorbed to a given matrix to different extents, depending on their hydrophobicity, size and shape and their concentration in the solution[2]. This type of adsorption is characterized by a hysteretic behaviour[3]. When a protein is purified from a crude solution (*e.g.*, serum

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or ascites fluid), some of the more hydrophobic proteins present in the preparation may be adsorbed non-specifically and reversibly to the matrix and later eluted under the specific elution conditions, contaminating the purified protein(s) of interest.

In an attempt to reduce non-specific adsorption of foreign proteins to Eupergit C and agarose, we have studied the effect of water-soluble polymers, particularly polyethylene glycol (PEG), on such adsorption. PEG is an amphipathic reagent that readily associates with protein surfaces. The interaction of proteins with PEG has been shown to increase the solubility in organic solvents[4], decrease the immunogenicity[5], prolong the lifetime in blood circulation[6] and confer protection against proteolytic degradation[7]. PEG has been also used as a ligand in the hydrophobic chromatography of proteins[8].

PEG 400 and 1500 are not toxic to humans[9] and are even included in skin ointments for human use[10]. Therefore, it is expected that their inclusion in the loading buffer of injectable proteins to be immunopurified will not be hazardous to humans.

In this paper, we describe studies on the reduction of the non-specific adsorption of proteins to the surface of Eupergit C after treatment of the matrix with PEG.

EXPERIMENTAL

Polyethylene glycols 200, 400 and 1500 were purchased from Fluka (Buchs, Switzerland). Ammonium sulphate fractionated goat anti-rabbit immunoglobulin G (IgG) was obtained from Bio-Makor Biochemicals (Rehovot, Israel), agarose (Type I) from Sigma (St. Louis, MO, U.S.A.) and epoxy-activated Sepharose from Pharmacia (Uppsala, Sweden). Eupergit C was obtained from Rohm-Pharma (Darmstadt, F.R.G.). Amino-polyethylene glycol 1500 was kindly provided by Prof. Kula of the Institute for Enzyme Technology Jülich, F.R.G.

Preparation of blocked Eupergit C column

A 2-mg amount of Eupergit C (150-μm beads) were extensively washed with phosphate-buffered saline (PBS) (pH 7.4) and then incubated with 20 ml of 0.2 *M* β-mercaptoethanol (βME) (pH 8.0) for 4 h at room temperature. The beads were then packed into a stainless-steel column (10 × 0.6 cm I.D.) (Knauer, Bad Homburg, F.R.G.). The packed column was connected to a high-performance liquid chromatographic (HPLC) system (Gilson, Villiers-le-Bel, France; Model 303) equipped with a Model 111B UV detector and an HP Model 3390 integrator (Hewlett-Packard, Sunnyvale, CA, U.S.A.). Prior to use the column was extensively washed alternatively with PBS (pH 7.4) and with 0.2 *M* ammonium acetate (pH 10.0).

Agarose column

One gram of agarose was packed into a 3 × 1.2 cm I.D. plastic column which was connected to a peristaltic pump (Pharmacia) and a Gilson Model 111B UV detector equipped with an LC optical cell. Prior to the application of serum samples to the column it was thoroughly washed with PBS (pH 7.4), 0.2 *M* ammonium acetate buffer (pH 10.0) and again with PBS (pH 7.4).

Amino-polyethylene glycol-modified Eupergit C and agarose

Eupergit C beads (2 g) were extensively washed with PBS (pH 7.4) and once with

1 *M* potassium phosphate buffer (pH 7.4). Excess of buffer was decanted and 1.0 g of amino-PEG 1500, dissolved in 5 ml of the same buffer (with gentle heating), was mixed with the beads for 18 h at 4°C. Unbound amino-PEG was washed off with PBS (pH 7.4.).

Similarly, an amino-PEG derivative of agarose was prepared by reaction of amino-PEG (1.0 g) with 2 g of epoxy-activated Sepharose.

The amino-PEG derivatives of Eupergit C and Sepharose were packed into columns and used as described above.

Adsorption of serum proteins

Normal horse serum (NHS) (0–1 ml) was applied to the Eupergit C or the agarose columns. The two columns were washed at a flow-rate of 1.0 or 0.5 ml/min, respectively, with PBS (pH 7.4) or PBS containing 1% PEG (200, 400 or 1500) until all the non-adsorbed proteins were washed off. Elution of adsorbed proteins was achieved by washing the columns with either a solution of 10% sodium dodecyl sulphate (SDS) in 8 *M* urea or 0.2 *M* ammonium acetate buffer (pH 10.0). The eluting material was monitored by following the absorbance at 280 nm. The amount of protein eluted was determined as the area under the protein peaks (in A_{280} units) derived by peak integration. Normal serum contains *ca.* 80 A_{280} units/ml. In some experiments the fraction of proteins adsorbed to the matrix and later eluted with ammonium acetate buffer (pH 10) was collected, dialysed against PBS at 4°C for 16 h and reapplied to the PBS-washed Eupergit C–βME column.

SDS polyacrylamide gel elctrophoresis (PAGE)

SDS-PAGE was performed with 10% gels according to the procedure of Laemmli[11].

Immunopurification of human decidua protein (hDP71)

hDP71 was purified on a Eupergit C-based immunoaffinity column as described previously[12].

Immunopurification of rabbit IgG

Ammonium sulphate fractionated goat anti-rabbit IgG [2 ml of PBS containing 5.2 mg of antibody (Ab) and 20 mg of total protein] was coupled to 2 g of Eupergit C beads as described previously[13,14]. One gram of the beads was incubated with βME for 18 h at 4°C and 1 g was treated with amino-PEG as described above.

IgG of normal rabbit serum (50 ml) was fractionated by ammonium sulphate precipitation (37% saturation). IgG solution (2 mg in 50 μl of PBS) was mixed with 0.5 ml of normal horse serum and loaded onto each of the anti-IgG columns in PBS or in PBS containing 1% PEG 400. Unbound protein was washed off the column with the respective buffer at a flow-rate of 1 ml/min, then the bound IgG was eluted with 0.2 *M* ammonium acetate buffer (pH 10.0). Samples of the eluates were analysed for contaminating proteins by SDS-PAGE.

RESULTS AND DISCUSSION

Adsorption of serum proteins to Eupergit C and to agarose

Eupergit C is a polymeric matrix bearing oxirane groups which are capable of covalently binding proteins via their amino, thio or hydroxy moieties[15]. Owing to the slightly hydrophobic nature of the matrix, non-specific adsorption of proteins may occur in the process of affinity purification, especially when crude biological fluids are loaded onto the column. In order to distinguish between non-specific adsorption and covalent binding of proteins to Eupergit C beads, reactive oxirane groups on the surface of the beads were blocked by reaction with βME, thus eliminating the possibility of covalent binding of proteins. The modified Eupergit C beads were packed into an HPLC column. Aliquots of normal horse serum were applied to the column and washed with PBS until no protein was eluted. Washing of the column with an elution buffer [0.2 *M* ammonium acetate buffer (pH 10.0) or 1% SDS in 8 *M* urea] resulted in the elution of non-specifically adsorbed proteins (Figs. 1b and 2). The mechanism of protein adsorption to the matrix seems complicated; when the non-adsorbed protein (Fig. 1, peak I) was collected and reapplied to the same column, a certain amount (similar to the amount of protein adsorbed to the column at the first run) was adsorbed to the matrix and later eluted with 0.2 *M* ammonium acetate buffer

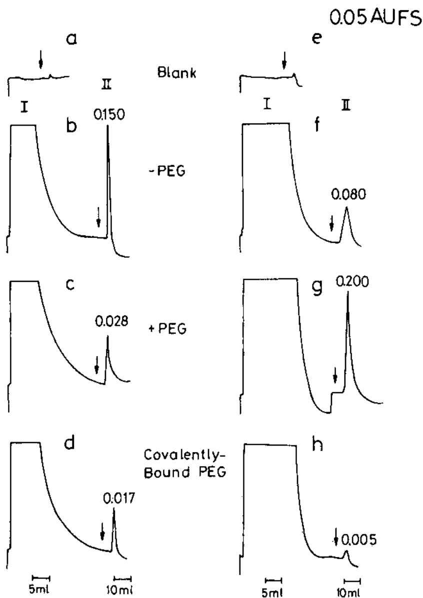

Fig. 1. Adsorption of serum proteins on Eupergit C derivatives (HPLC patterns), onto intact agarose and onto Sepharose 6B-bound PEG (LC patterns). Normal horse serum (0.5 ml) was applied to the columns in PBS (pH 7.4) or PBS containing 1% PEG 400 as described under Experimental. After removal of the non-adsorbed material (peak I), the adsorbed material (peak II) was eluted with 0.2 *M* ammonium acetate buffer (pH 10.0). The absorbance of the eluent was monitored at 280 nm. (a–d) β-Mercaptoethanol-blocked Eupergit C C30N; (e–g) intact agarose; (h) epoxy-activated Sepharose 6B modified with amino-PEG 1500. (a, e) Blank runs, no serum applied; (b, f) control runs in absence of PEG; (c, g) runs in presence of 1% PEG 400; (d, h) runs on covalently bound amino-PEG columns. Numbers on peak II denote peak area in $A_{280\ nm}$ units. Arrows indicate the application of the elution buffer, 0.2 *M* ammoniom acetate (pH 10.0).

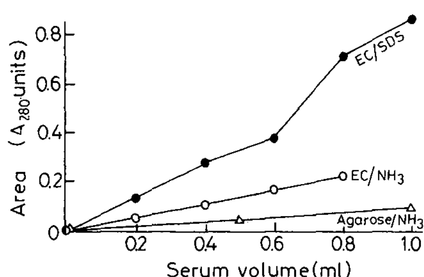

Fig. 2. Dependence of protein adsorption from serum by β-mercaptoethanol-blocked Eupergit C and intact agarose on the amount of serum loaded onto the columns. ●, Protein eluted from Eupergit C with SDS–urea buffer; ○, protein eluted from Eupergit C with 0.2 *M* ammonium acetate buffer (pH 10.0); △, protein eluted from agarose with 0.2 *M* ammoniom acetate buffer (pH 10.0).

(pH 10.0). In contrast, when the protein previously adsorbed to the matrix (peak II) was reapplied to the same column, no protein was re-adsorbed and eluted by the elution buffer.

The amount of serum proteins adsorbed to the matrix and later eluted by the elution buffers was directly proportional to the amount of serum applied to the column (about 0.4% of the protein loaded; Fig. 2). The amount of protein adsorbed on the column increased with increasing NaCl concentration in the loading buffer; in 0.1 *M* sodium phosphate buffer (pH 7.4) the absorption was about 50% of that in PBS, whereas increasing the salt concentration to 1 *M* resulted in the adsorption of about 2% of the protein applied to the column. This observation indicates that the adsorption is of a hydrophobic, rather than ionic, nature.

The adsorption of serum proteins to the agarose beads was followed using a similar approach. Aliquots of NHS were applied to the agarose column in PBS (pH 7.4) and the amount of protein eluted from the column on washing with 0.2 *M* ammonium acetate (pH 10.0) was determined. As shown in Fig. 1, the amount of protein adsorbed to the agarose beads was lower than that of the proteins adsorbed to the Eupergit C beads. Still, the adsorption of proteins was directly proportional to the amount of serum applied to the column (Fig. 2).

Of all the proteins that may be adsorbed to an immunoaffinity column, only those which are eluted under the specific conditions used to elute the protein of interest may lead to contamination of the product. Although those proteins which may be eluted only with SDS–urea treatment may cause other difficulties (*e.g.*, affect column performance), they will not cause product contamination. Therefore, we decided to concentrate our efforts on those proteins which elute under the specific elution conditions, namely with 0.2 *M* ammonium acetate buffer (pH 10.0).

As shown above, when serum is applied to the Eupergit C–βME and the agarose columns, about 0.4% and 0.2% of its proteins, respectively, are adsorbed to the matrices and eluted with 0.2 *M* ammonium acetate buffer (pH 10.0). Only a few of the proteins of the normal serum were adsorbed to Eupergit C–βME beads, as shown by SDS-PAGE of the ammoniom acetate-eluted fraction (data not shown). It is pertinent to note that when bovine serum albumin (20 mg in 0.5 ml of PBS, which is approximately equivalent to the amount of albumin in 0.5 ml of serum) was applied to the same column under similar conditions, no protein was apparently adsorbed to the matrix.

Effect of PEG on protein adsorption to Eupergit C and agarose

In an attempt to reduce the adsorption of serum proteins to Eupergit C, we examined the effect of the addition of organic polymers to the loading buffer (PBS, pH 7.4) on protein adsorption. When poly(vinyl alcohol) (M_r = 25 000, 1%) was included in the loading buffer, a dramatic increase (about 10-fold) in the adsorption of serum proteins to the Eupergit C–βME column was observed (data not shown). In contrast, inclusion of PEG in the loading buffer at a final concentration of 1% reduced the amount of adsorbed material. As shown in Fig. 3, PEG 1500 and 400 were more effective than PEG 200. The protective effect of PEG 1500 was more pronounced at low ionic strength (<0.15 *M* NaCl). In the presence of 1 *M* NaCl almost no effect of PEG on protein adsorption was observed (data not shown).

After the Eupergit C column had been washed once with PBS containing 1% PEG, a "memory effect" was observed; in the following run, even if PEG was omitted from the loading buffer, the column behaved as if PEG was still present and the amount of protein adsorbed was very low. This effect lasted for 3–5 runs before the column returned to "normal" behaviour (Fig. 4).

In contrast to the protective effect of PEG against protein adsorption observed with Eupergit C beads, an adverse effect of PEG on protein adsorption to agarose was observed. Inclusion of PEG in the loading buffer resulted in a 20-fold increase in protein adsorption to the agarose beads. No "memory effect" was observed in this instance. When PEG was omitted from the loading buffer, adsorption was at the "normal" level, regardless of whether or not the column had been washed with PEG in the previous run.

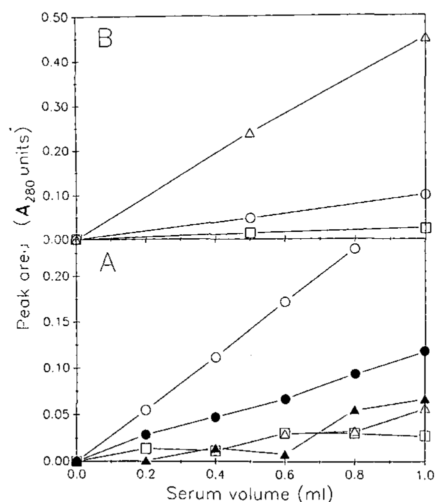

Fig. 3. Effect of polyethylene glycol on the non-specific adsorption of proteins to β-mercaptoethanol-blocked Eupergit C (A) and intact agarose (B). PEG of various molecular weight was included at a concentration of 1% in the loading buffer of different aliquots of normal horse serum. Adsorbed protein was eluted from the columns by 0.2 *M* ammonium acetate buffer (pH 10.0). ○ = No PEG included in the loading buffer; ● = PEG 3; △ = PEG 400; ▲ = PEG 1500; □ = covalently bound amino-PEG, no PEG included in the loading buffer.

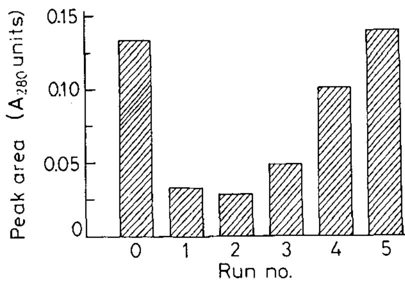

Fig. 4. Memory effect of PEG 400 on protein adsorption to β-mercaptoethanol-blocked Eupergit C in successive runs. Run 1 was carried out in the presence of 1% PEG 400 in the sample loading buffer. Runs 0 and 2–5 were carried out in the absence of PEG.

Immunoaffinity purification in the presence of PEG

As shown above (Figs. 1c and 3), inclusion of PEG in the loading buffer resulted in a decrease in the amount of proteins adsorbed to a Eupergit C–βME column. It was still necessary to show that PEG has a protective against non-specific adsorption of proteins to the matrix in an immunoaffinity purification process. This was demonstrated with the immunopurification of rabbit IgG from serum using Eupergit C-immobilized polyclonal anti-IgG and of the human decidua protein hDP71 from seminal plasma using Eupergit C-immobilized monoclonal antibodies DEC21[12].

Rabbit IgG (ammonium sulphate fraction) was first "contaminated" by mixing it with normal horse serum and then loaded onto the Eupergit C-conjugated anti-IgG column in PBS (pH 7.4), in the absence or presence of 1% PEG 400. As shown in Fig. 5A, the IgG fraction loaded on the matrix in the absence of PEG was eluted

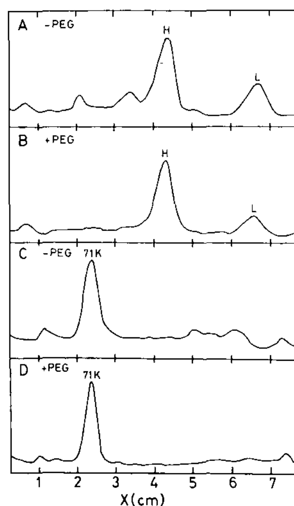

Fig. 5. Immunoaffinity purification of rabbit IgG (A and B) and hDP71 (C and D) using Eupergit C-immobilized goat anti-rabbit IgG and monoclonal anti-hDP71 (DEC21), respectively. Patterns of SDS-PAGE of the materials eluted in peak II of runs carried out in the absence (A and C) or presence of 1% PEG 400 (B and D) in the sample loading buffer are shown.

contaminated with a few other protein bands (containing about 10–20% of the total protein, by peak integration). In contrast, when PEG 400 (1%) was included in the loading buffer the IgG was eluted much purer.

Similar results were obtained with the immunopurification of hDP71 from crude cell-free seminal plasma. As shown in Fig. 5B, in the absence of PEG in the loading buffer the hDP71-containing fraction eluted from the column with 0.2 *M* ammonium acetate buffer (pH 10.0) also contained several minor protein bands, as revealed by SDS-PAGE. When PEG 400 was included in the loading buffer, however, the hDP71 eluted from the column was essentially pure.

Preparation and properties of covalently linked PEG–Eupergit C and –agarose

As described above, in order to reduce non-specific adsorption of proteins onto Eupergit C, PEG 400 or 1500 should be included in the loading buffer. Although PEG is considered to be non-toxic and non-immunogenic, in some instances one may wish to avoid the inclusion of any foreign material in the loading buffer during an immunoaffinity purification process. Therefore, we examined the possibilily of modifying the surfaces of Eupergit C and agarose with covalently bound PEG in an attempt to achieve protection against non-specific adsorption of proteins to the modified matrices.

PEG-modified Eupergit C and agarose were prepared by covalent binding of diamino-PEG 1500 to Eupergit C and to expoxy-activated agarose. As shown in Figs. 1d and h and 3, the reduction of non-specific adsorption of proteins by the modified matrices was similar to, or even slightly better than, the reduction observed when PEG was included in the loading buffer with the non-modified matrices. Interestingly, in contrast to the increase in protein adsorption observed when PEG 400 was included in the loading buffer of the agarose column (see Fig. 1), the covalent linking of PEG 1500 to the agarose resulted in a decrease in protein adsorption. Possibly two different mechanisms are involved. When the serum samples are injected into the agarose column in the presence of PEG, its proteins may react with PEG and their surface properties may be altered. In contrast, when PEG is reacted with the insoluble matrix it may create a modified matrix which possesses different adsorption properties.

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Chemistry and preparation of affinity ligands useful in immunoglobulin isolation and serum protein separation

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ABSTRACT

A number of synthetic affinity gels having high affinity for immunoglobulins and albumin have been prepared by first reacting hydroxyl groups of a polymer with pentafluoropyridine and 4-dimethylaminopyridine in an anhydrous polar organic solvent and then reacting the gel further with nucleophiles such as ethyleneglycol or glycine in basic aqueous solutions. Immunoglobulins can be adsorbed to the gel in either high-salt or low-salt buffers, while albumin can only be adsorbed under low-salt conditions. The identity of the eluted proteins was analyzed by gradient polyacrylamide gel electrophoresis and enzyme-linked immunosorbent assay techniques. Human, goat, mouse and rabbit serum proteins were fractionated on these gels by using different adsorption and desorption conditions. The possible structures of the ligand are discussed. The results showed that the chromatographic behavior of these new gels with synthetic, low-molecular-weight ligands was remarkably similar to that of the more complex immunoglobulin binding gel such as immobilized Protein A or Protein G.

INTRODUCTION

The interaction between specific sites of a protein and a solid matrix forms the basis of protien separation by adsorption chromatography. Known mechanisms for interaction between sites on a protein molecule and ligand sites of the chromatographic matrix may involve ionic, hydrophobic, hydrogen bonding, Van der Waals, charge-transfer, covalent or salt-promoted interaction[1,2]. These interactions may operate singly or, alternatively, two or more of them may operate simultaneously to give rise to a so-called mixed-mode chromatography[3]. Highly specific binding between a specific protein and a bio-affinity ligand has been amply demonstrated in protein purification by affinity chromatography[4–6]. The mechanism for such a specific binding may involve one or more of the above listed interactions. Affinity ligands of non-biological origin have also been used successfully as group-selective protein adsorbent. For example, dye ligand chromatography that uses Cibacron Blue F3GA as the affinity ligand has been effective in purifying a large number of nucleotide-

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binding and other proteins[7]. Recently Porath and co-workers[8–16] developd a novel and rapid method for selectively purifying antibody by salt-promoted chromatography on a "thiophilic" adsorbent. This adsorbent was prepared by reacting a hydroxyl group carrying polymer such as Sepharose sequentially with divinylsulfone and mercaptoethanol. In view of the structural simplicity of the ligand in thiophilic gel with a molecular weight of less than 400, the selectivity of thiophilic gel toward antibody is indeed remarkable. This is in contrast to the much more complex structure of other antibody binding ligands such as Protein A which has a molecular weight of about 42 000. The exciting results from Porath's group have given us much impetus to search for low-molecular-weight ligands capable of performing or mimicking the selectivity of such complex immunoglobulin binding proteins as Protein A or Protein G.

We wish to describe a class of affinity gels prepared by using synthetic low-molecular-weight ligands capable of selectively binding immunoglobulins in either low- or high-salt buffers and albumin under low-salt conditions. These gels were able to bind immunoglobulins from different animal species with high degree of selectivity and avidity. Their binding selectivities are very reminiscent of those of Protein A or Protein G. The affinity gels were obtained by first reacting Sepharose Cl-4B or other hydroxyl carrying polymers with pentafluoropyridine (PFP) and 4-dimethylaminopyridine (DMAP) in an anhydrous polar organic solvent such N,N′-dimethylformamide (DMF) or acetonitrile and then reacting the intermediate gel formed with nucleophiles in aqueous solution. Patent applications dealing with processes described herein have been filed.

MATERIALS AND METHODS

Chemicals

Sepharose Cl-4B was from Pharmacia (Uppsala, Sweden) and Fractogel TSK HW 75F from Toyo Soda (Tokyo, Japan). PFP and DMAP were purchased from Aldrich (Milwaukee, WI, U.S.A.), potassium sulfate, glycine, ethylene glycol, diethanolamine, citric acid, Tween 20, hydrogen peroxide, human immunoglobulin G (IgG), human albumin, goat serum and *p*-nitrophenyl phosphate were from Sigma (St. Louis, MO, U.S.A.), DMF and acetone from J. T. Baker (Phillipsburg, NJ, U.S.A.), human serum was from Irvine Scientific (Santa Ana, CA, U.S.A.), mouse serum and rabbit serum were from Pel-Freez (Roger, AK, U.S.A.), rabbit anti-human albumin, rabbit anti-human albumin HRP conjugate and *o*-phenylenediamine from Dako (Carpenteria, CA, U.S.A.), goat anti-human IgG and goat anti-human IgG alkaline phosphatase conjugate were from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

Instrumentation

The effluent from the chromatographic column was monitored continuously at 280 nm with an LKB 2238 Uvicord SII and the pH of the effluent with an LKB 2195 pH/ion monitor. Fractions were collected with an LKB 2070 Ultrorac II fraction collector. Electrophoretic analyses were performed on 10–15% polyacrylamide gradient gels by using Phast system from Pharmacia. Vmax Kinetic microplate reader (Molecular Devices) was used for serum proteins enzyme-linked immunosorbent assay (ELISA).

Gel preparation

Preparation of PFP-substituted intermediate gel. Sepharose Cl-4B gel (100 ml) was washed with 5 × 100 ml distilled water. The washed gel was suspended in 100 ml distilled water in a 2-l beaker mounted on a shaker rotating at 100 rpm. To the gel 1 l dry acetone was added over 30 min. The gel was filtered and resuspended in 1 l dry acetone and was tumbled at room temperature for 15 min. The gel was filtered and 300 ml dry DMF were added to the gel and the gel was tumbled for 5 min. After filtering the gel was suspended in 100 ml of DMF containing 27.5 mmol DMAP. To the gel suspension were further added 250 ml DMF containing 25 mmol PFP. The gel was tumbled at room temperature for 2 h. Then the gel was washed with 1 l DMF and 2 × 1 l acetone. The washed, substituted gel can be stored in 200 ml acetone at 4°C for several weeks.

Preparation of PFP-O gel. A 10-ml volume of PFP-substituted intermediate gel was washed with 100 ml distilled water and 100 ml 0.1 *M* sodium hydrogencarbonate, pH 9.0. The gel was suspended in an equal volume of 0.1 *M* sodium hydrogencarbonate, pH 9.0 containing 10% ethylene glycol and tumbled at room temperature for 24 h. The gel was washed with 100 ml of 0.1 *M* sodium hydrogencarbonate, pH 9.0 and resuspended in twice its volume of 0.1 *M* sodium hydroxide and tumbled for 14 h at room temperature. The gel was washed with 100 ml distilled water, 100 ml 1 *M* sodium chloride, 100 ml distilled water and then 100 ml phosphate-buffered saline (PBS). When not in use the gel was stored in PBS at 4°C.

Preparation of PFP-glycine gel. A 10-ml volume of PFP-substituted intermediate gel was washed with 100 ml distilled water and 100 ml 0.1 *M* sodium hydrogencarbonate, pH 9.0. The gel was suspended in an equal volume of 1 *M* glycine in 0.1 *M* sodium hydrogencarbonate, pH 9.0 and the gel was tumbled at room temperature for 24 h. The gel was washed with 100 ml 0.1 *M* sodium hydrogencarbonate, pH 9.0 and resuspended in twice its volume of 0.1 *M* sodium hydroxide and tumbled for 14 h at room temperature. The gel was washed with 100 ml distilled water, 100 ml 1 *M* sodium chloride, 100 ml distilled water and then 100 ml PBS. When not in use the gel was stored in PS at 4°C.

Chromatographic procedures

Binding in presence of low salt concentration. A 4-ml volume of the gel packed in a disposable column (9 × 0.8 cm) was washed with about 20 ml of 20 m*M* sodium phosphate, pH 7.4. Filtered serum (1 ml) was appropriately diluted with 20 m*M* sodium phosphate buffer, pH 7.4 and passed through the column at a flow-rate of 0.25 ml/min. The column was washed with the same buffer at a flow-rate of 1 ml/min and then bound proteins were eluted first with 10 m*M* sodium phosphate, pH 7.4 containing 0.5 *M* potassium sulfate and then with 0.1 *M* glycine buffer, pH 5.0, 4 and at last 2.8 at the same flow-rate of 1 ml/min. Fractions of 3 ml were collected for each wash and the absorbance at 280 nm was read for each fraction. For PFP-O gel, the concentration of albumin and IgG in some selected fractions was determined by using the specific sandwich ELISA method.

Binding in presence of high salt concentration. A 4-ml volume of the gel packed in a disposable column was washed with about 20 ml of 10 m*M* sodium phosphate, pH 7.4 containing 1.5 *M* sodium chloride. A 1-ml volume of filtered serum was diluted 10-fold in 10 m*M* phosphate buffer, pH 7.4 containing 1.5 *M* sodium chloride and

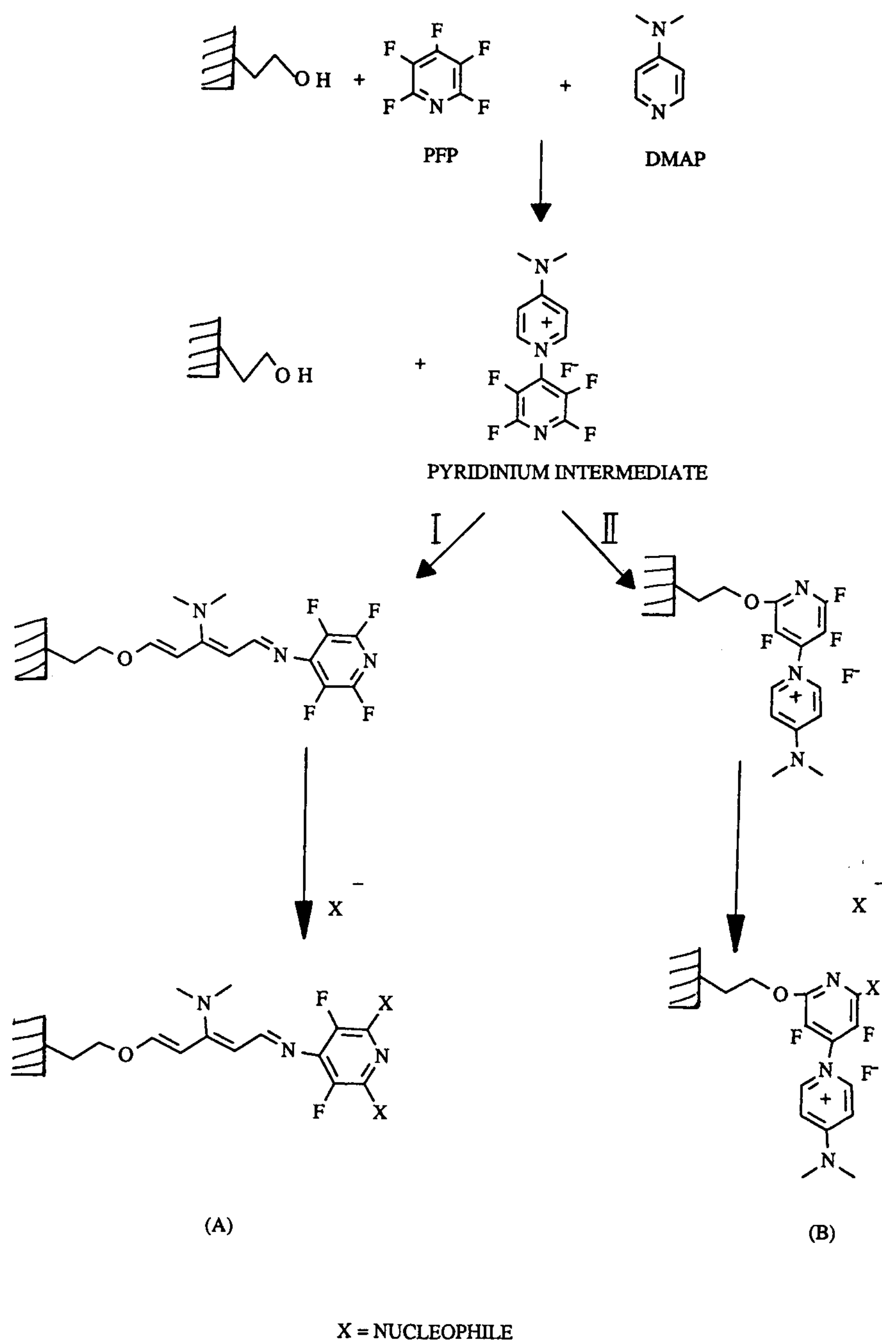

Fig. 1. Postulated synthetic steps for the preparation of the affinity gel.

passed through the column at a flow-rate of 0.25 ml/min. The column was washed with the same buffer at a flow-rate of 1 ml/min and bound proteins were eluted by washing the gel with 0.1 *M* glycine buffer pH 3.5 at the same flow-rate. Fractions of 3 ml were collected and the absorbance at 280 nm was read for each fraction.

RESULTS

The reaction of Sepharose Cl-4B with PFP and DMAP in organic solvent gave an intermediate product which upon further reaction in aqueous solution with nucleophiles (ethylene glycol or glycine) resulted in gels with unique selectivity toward immunoglobulins and albumins from several animal species. The synthetic routes and possible structures of the ligand of immunoglobulin-binding gels are shown in Fig. 1. Elemental analysis of the gel gave an N:F ratio of 3:2 which is consistent with both structures. The ligand density of the gel was calculated to be 1–13 mmol per g dry gel.

The results of fractionating human serum which has been diluted with 20 m*M* phosphate buffer pH 7.4 on PFP-O gel are shown in Fig. 2. Almost all of the UV absorbing materials from the diluted serum were adsorbed on the gel. Six major fractions were obtained by eluting the adsorbed materials sequentially with 10 m*M* phosphate, pH 7.4 containing 0.5 *M* potassium sulfate, 0.1 *M* glycine buffer, pH 5, 4 and 2.8. From the electrophoresis of the eluted fractions under either reducing or non-reducing conditions (Fig. 3A and B), it was observed that fractions in peaks I and II that were eluted by a buffer solution with 0.5 *M* K_2SO_4 contained mostly albumin and possibly transferrin. Fractions in peak III, eluted by lower ionic strength buffer of pH 5, contained IgG. Using the same buffer, a smaller quantity of IgG was also eluted in fractions of peaks IV. Fractions in peaks V and VI, elued respectively with buffers of pH 4 and 2.8 were shown to contain a number of other serum proteins. The identity of albumin and IgG was further confirmed by ELISA for specific protein determinations

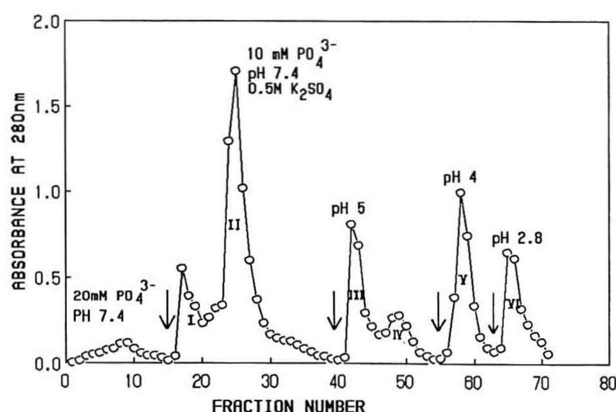

Fig. 2. Fractionation of human serum on PFP-O gel. Gel volume was 4 ml. Serum was diluted 10-fold with 20 m*M* sodium phosphate, pH 7.4 and was applied at a flow-rate of 0.25 ml/min at room temperature. Fractions of 3 ml were collected. Elutions were carried out with different buffers at the fraction indicated by arrows. I and II were fractions eluted with 10 m*M* sodium phosphate, pH 7.4 containing 0.5 *M* potassium sulfate. III and IV contained fractions eluted with 0.1 *M* glycine, pH 5. V contained fractions eluted with 0.1 *M* glycine, pH 4 and VI contained fractions eluted with 0.1 *M* glycine, pH 2.8.

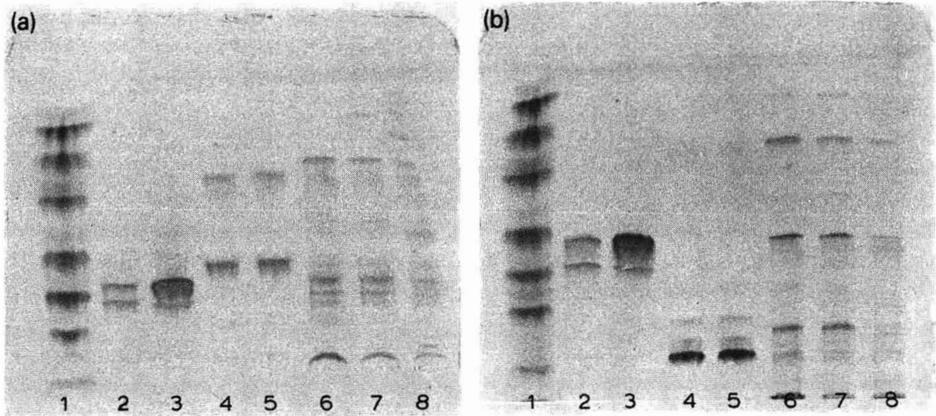

Fig. 3. Sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis under reducing (A) and non-reducing (B) conditions of fractions shown in Fig. 2. Lanes: 1 = molecular weight marker reference proteins; 2 = peak I of Fig. 2; 3 = peak II of Fig. 2; 4 and 5 = peak III of Fig. 2; 6 and 7 = peak V of Fig. 2; 8 = peak VI of Fig. 2.

(Fig. 4). Albumin was found mainly in the high-salt, flow-through fractions, *i.e.* in peak II and only a small amount in peak I, while IgG was found almost exclusively in peaks III and IV with a much smaller quantity in peak V. The recovery of isolated IgG was estimated to be 55%. Attempts were made to simplify the isolation of immunoglobulins from the serum by applying serum which has been diluted with a high-salt buffer. Fig. 5 showed results of such an experiment. Two major peak fractions were obtained, the first peak was the high-salt (1.5 *M*), flow-through fractions which contained mostly albumin and some minor other serum proteins. The second peak, eluted with 0.1 *M* glycine, pH 3.5, contained the major portion of the IgG and some other serum proteins. The recovery of IgG was greater than 80%. From the

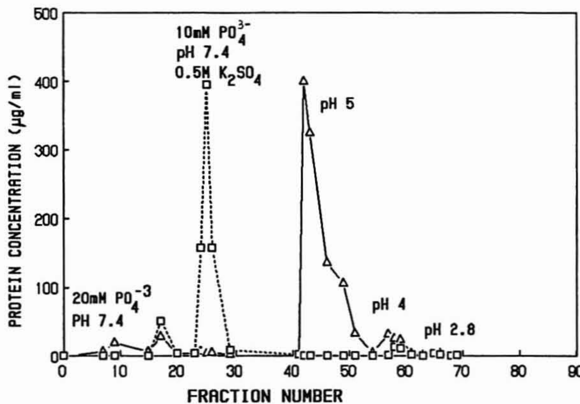

Fig. 4. Sandwich ELISA for human serum albumin and IgG in fractions obtained from chromatography of human serum on PFP-O gel (Fig. 2). □ = Albumin; △ = IgG.

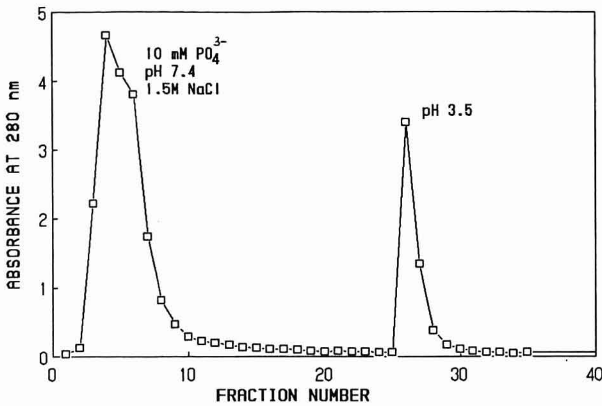

Fig. 5. Fractionation of human serum on PFP-O gel under high salt loading condition. Gel volume was 4 ml. Serum was diluted 10-fold with 10 m*M* sodium phosphate, pH 7.4 containing 1.5 *M* sodium chloride and was applied at a flow-rate of 0.25 ml/min at room temperature. Fractions of 3 ml were collected. Elution was carried out with 0.1 *M* glycine, pH 3.5.

electrophoresis (Fig. 6) of fractions shown in Fig. 5, it was clear that IgG isolated by using this high-salt procedure was not as pure as that obtained by the procedure used in Fig. 2. Goat, mouse and rabbit sera, all prediluted in high-salt buffer (10 m*M* phosphate, pH 7.4 containing 1.5 *M* sodium chloride) have been individually chromatographed on a column of PFP-O gel and were fractionated into two major fractions. The recovery of IgG isolated from sera was greater than 60%. In every case, the second fraction was the IgG-rich fraction. The chromatograms are shown in Figs. 7, 9 and 11, respectively. The electrophoretic patterns of these results are respectively shown in Figs. 8, 10 and 12. A similar chromatogram (Fig. 13) was obtained when rabbit serum was fractionated on a glycine-substituted gel (PFP-glycine gel). The first peak fraction was rich in albumin and the second was rich in IgG.

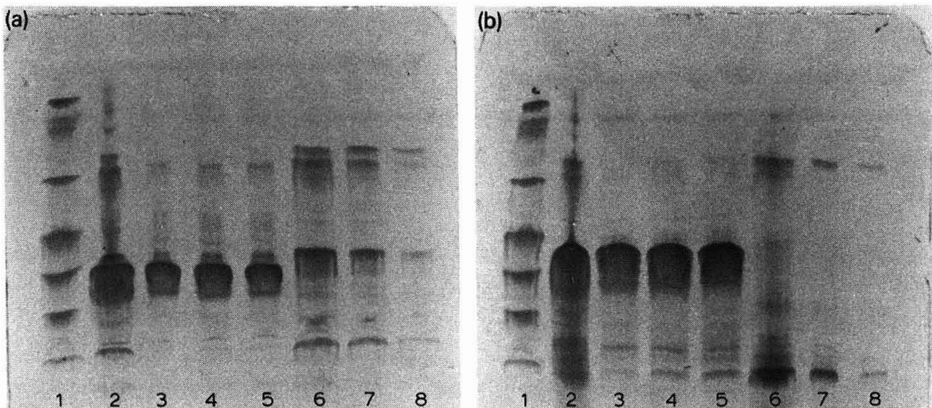

Fig. 6. Sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis under reducing (a) and non-reducing (b) conditions of fractions shown in Fig. 5. Lanes 1 = molecular weight marker reference proteins; 2 = unfractionated whole serum; 3, 4 and 5 = unbound, flow-through fractions; 6, 7 and 8 = fractions eluted with 0.1 *M* glycine, pH 3.5.

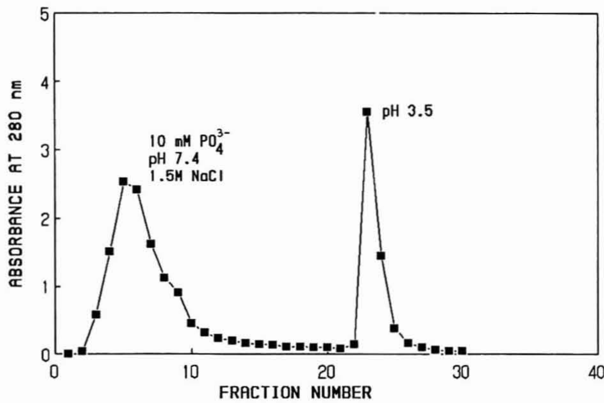

Fig. 7. Fractionation of goat serum on PFP-O gel under high salt loading condition. Gel volume was 4 ml. Serum was diluted 10-fold with 10 m*M* sodium phosphate, pH 7.4 containing 1.5 *M* sodium chloride and was applied at a flow-rate of 0.25 ml/min at room temperature. Fractions of 3 ml were collected. Elution was carried out with 0.1 *M* glycine, pH 3.5.

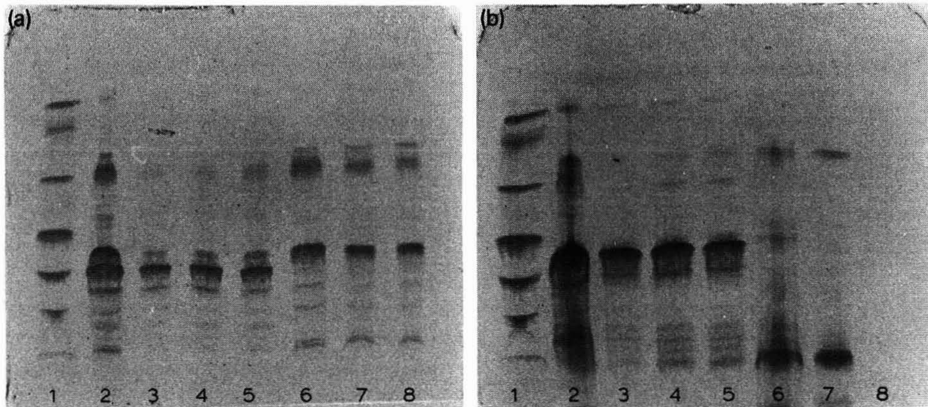

Fig. 8. Sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis under reducing (a) and non-reducing (b) conditions of fractions shown in Fig. 7. Lanes: 1 = molecular weight marker reference proteins; 2 = unfractionated whole serum; 3, 4 and 5 = unbound, flow-through fractions; 6, 7 and 8 = fractions eluted with 0.1 *M* glycine, pH 3.5.

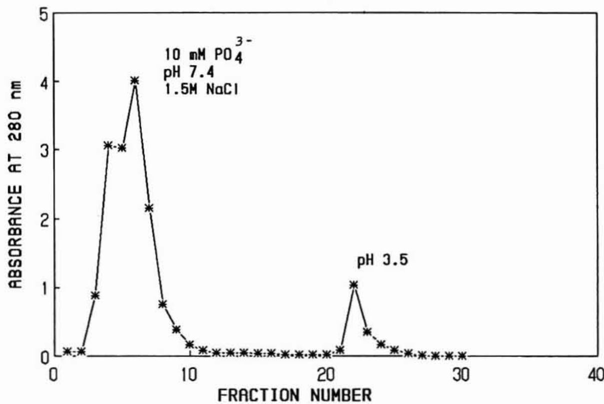

Fig. 9. Fractionation of mouse serum on PFP-O gel under high salt loading condition. Gel volume was 4 ml. Serum was diluted 10-fold with 10 m*M* sodium phosphate, pH 7.4 containing 1.5 *M* sodium chloride and was applied at a flow-rate of 0.25 ml/min at room temperature. Fractions of 3 ml were collected. Elution was carried out with 0.1 *M* glycine, pH 3.5.

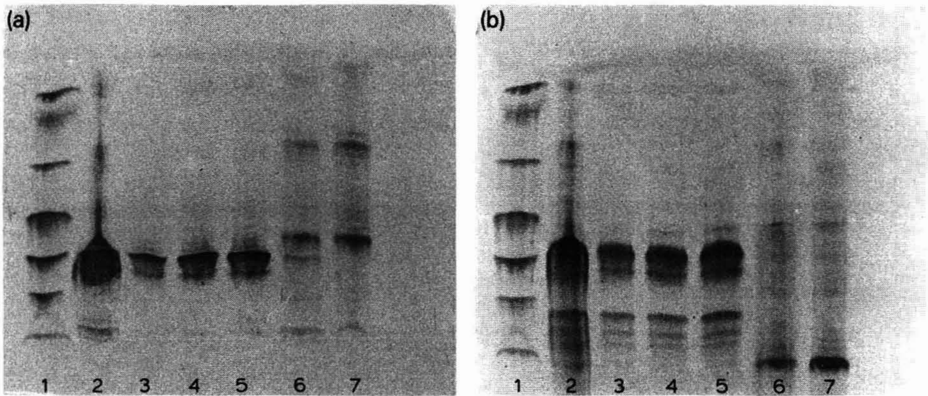

Fig. 10. Sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis under reducing (a) and non-reducing (b) conditions of fractions shown in Fig. 9. Lanes: 1 = molecular weight marker reference proteins; 2 = unfractionated whole serum; 3, 4 and 5 = unbound, flow-through fractions; 6 and 7 = fractions eluted with 0.1 *M* glycine, pH 3.5.

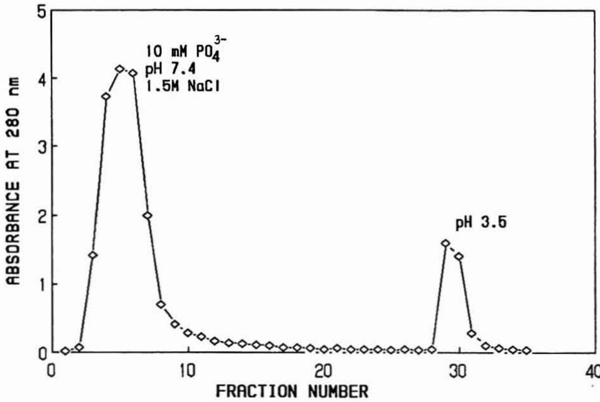

Fig. 11. Fractionation of rabbit serum on PFP-O gel under high salt loading condition. Gel volume was 4 ml. Serum was diluted 10-fold with 10 m*M* sodium phosphate, pH 7.4 containing 1.5 *M* sodium chloride and was applied at a flow-rate of 0.25 ml/min at room temperature. Fractions of 3 ml were collected. Elution was carried out with 0.1 *M* glycine, pH 3.5.

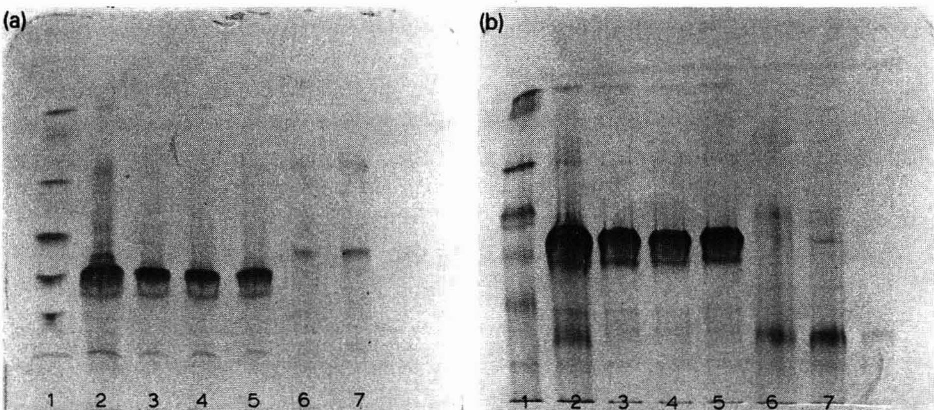

Fig. 12. Sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis under reducing (a) and non-reducing (b) conditions of fractions shown in Fig. 11. Lanes: 1 = molecular weight marker reference proteins; 2 = unfractionated whole serum; 3, 4 and 5 = unbound, flow-through fractions; 6, 7 and 8 = fractions eluted with 0.1 *M* glycine, pH 3.5.

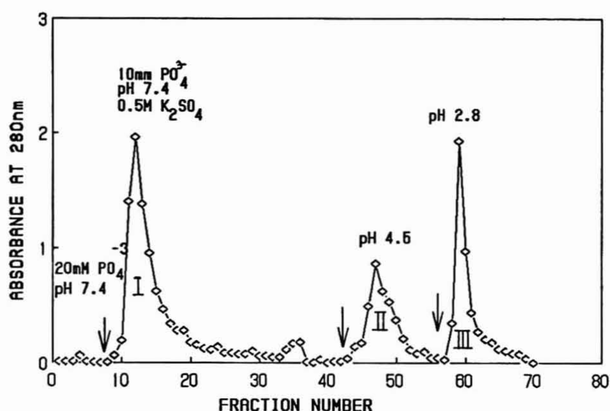

Fig. 13. Fractionation of rabbit serum on PFP-glycine gel. Gel volume was 4 ml. Serum was diluted 100-fold with 20 m*M* sodium phosphate, pH 7.4 and was applied at a flow-rate of 1.25 ml/min at room temperature. Fractions of 3 ml were collected. Elutions were carried out with different buffers at the fraction indicated by arrows. I contained fractions eluted with 10 m*M* sodium phosphate, pH 7.4 containing 0.5 *M* potassium sulfate. II contained fractions eluted with 0.1 *M* glycine, pH 4.5. III were fractions eluted with 0.1 *M* glycine, pH 2.8.

DISCUSSION

A number of chromatographic matrices capable of selectively binding immunoglobulin and albumin from serum have been prepared by first reacting the hydroxyl groups of a polymeric matrix with PFP and DMAP and then with nucleophiles. The postulated synthetic steps leading to the formation of the affinity gels are shown in Fig. 1. The first step involved the reaction of PFP and DMAP to form a pyridinium intermediate. The second step involved the reaction of the hydroxyl group of the gel with the pyridinium intermediate. There are two possible points of attack by the hydroxyl group on the pyridinium ion. One possible point of nucleophilic attack (route I) would be on the 2′ position of the pyridinium ring with the consequence of forming an open-chain, extended conjugated system. Such a gel will have the ligand structure depicted in Fig. 1A. The second attack (route II) by the hydroxide ion can occur at position 2 of the fluoropyridine ring with the resultant formation of a gel having a structure as shown in Fig. 1B. We cannot at this time make a definitive conclusion from these two possible alternatives regarding the chemical structure of the ligand on the gel. The use of pyridine compounds allows us to have some degree of control on the ionization state of the ligand by changing the pH of the buffer. It is also an extension of our previous work on the use of 2-fluoro-1-methylpyridinium salts as hydroxyl group activating agent in affinity chromatography[17,18].

The most noticeable property of these gels is their remarkably high protein binding capacity. Almost all of the serum proteins were bound to the gel when serum diluted by 20 m*M* phosphate buffer, pH 7.4 was passed through the gel column (Figs. 2 and 13). Another unique property of the gel is the adsorption of albumin at low-salt and its desorption at high-salt buffer. Immunoglobulins on the other hand were adsorbed at either low or high salt concentrations and were desorbed only by lowering the pH (Figs. 2, 5, 7, 9, 11 and 13). High degree of selectivity in the binding of serum

albumin and IgG by the gel was demonstrated by gradient polyacrylamide gel electrophoresis and ELISA (Figs. 3, 4, 6, 8 and 10). Further work is in progress to fine-tune the binding selectivity of the gel toward albumin and IgG by selecting optimal buffers for the binding and elution conditions.

The chromatographic behavior of both PFP-O and PFP-glycine gels differs from the thiophilic gel described by Porath and co-workers[8–16] in that (i) the structure of the ligand contains no element of sulfur, (ii) high salt concentration is not a necessary condition for protein adsorption (in fact albumin was desorbed at high salt concentration) and (iii) the desorption of IgG is obtained by lowering the pH rather than reducing the salt concentration in the eluting buffer.

The mechanism of protein adsorption to PFP-O or PFP-glycine gel is not known yet. The adsorption mechanism is not consistent with anyone of the known interaction alone, *i.e.* pure ion-exchange, pure hydrophobic or other interactions. It is, however, possible that more than one interaction might be operative as in the case of mixed-mode chromatography[3]. By looking at the two possible structures for the ligand of either PFP-O or PFP-glycine gel, it is not difficult to imagine the operationof more than one interaction in the adsorption of proteins to the gel. It is likely that ion-exchange, hydrophobic and charge-transfer types of interaction are simultaneously involved.

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Site-directed immobilization of proteins

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ABSTRACT

To determine if immobilization chemistry can be used to orient antibody on a support so that the bivalent binding potential can be fully utilized, we developed three activated matrices that couple to different functional groups on the molecule. When AminoLink Gel was used to couple antibody randomly through primary amino groups, the molar ratio of immobilized antibody to recovered antigen averaged 1:1. Iodoacetyl groups on SulfoLink Gel couple through sulfhydryls in the hinge region of the antibody molecule, in theory leaving the antigen binding site available. However, the antibody-to-antigen molar ratio was only slightly improved. Hydrazide groups on CarboLink Gel couple to aldehyde groups generated by oxidation of carbohydrate moieties that are located primarily on the Fc portion of the antibody molecule. The molar ratio of immobilized antibody to purified antigen using CarboLink Gel reached the optimum of 1:2. CarboLink Gel is most effective at orienting antibody for better antigen purification capability.

INTRODUCTION

Immobilized antibody has been used extensively as a reagent for the isolation of other biological molecules[1]. This may be of particular value in systems where there is no naturally occurring ligand.

Antibody structure has been characterized using a number of analytical techniques[2]. The immunoglobulin G (IgG) molecule is composed of two light and two heavy chains held together by disulfide bonds (Fig. 1A). There are two antigen sites on each molecule formed by the variable regions of one light and one heavy chain. When the antibody molecule is cleaved enzymatically with pepsin, one major and several smaller fragments are produced (Fig. 1B)[3]. The antigen binding fragment, termed $F(ab')_2$, retains both binding sites. The remainder of the molecule is called Fc for "fragment crystallizable".

A commonly used immobilization chemistry is cyanogen bromide (CNBr) activation[4]. Coupling randomly through primary amino groups scattered throughout the antibody molecule, this matrix has the potential to couple at or near the critical antigen binding regions, blocking those sites[5].

For the present study, three activated matrices were developed that couple to

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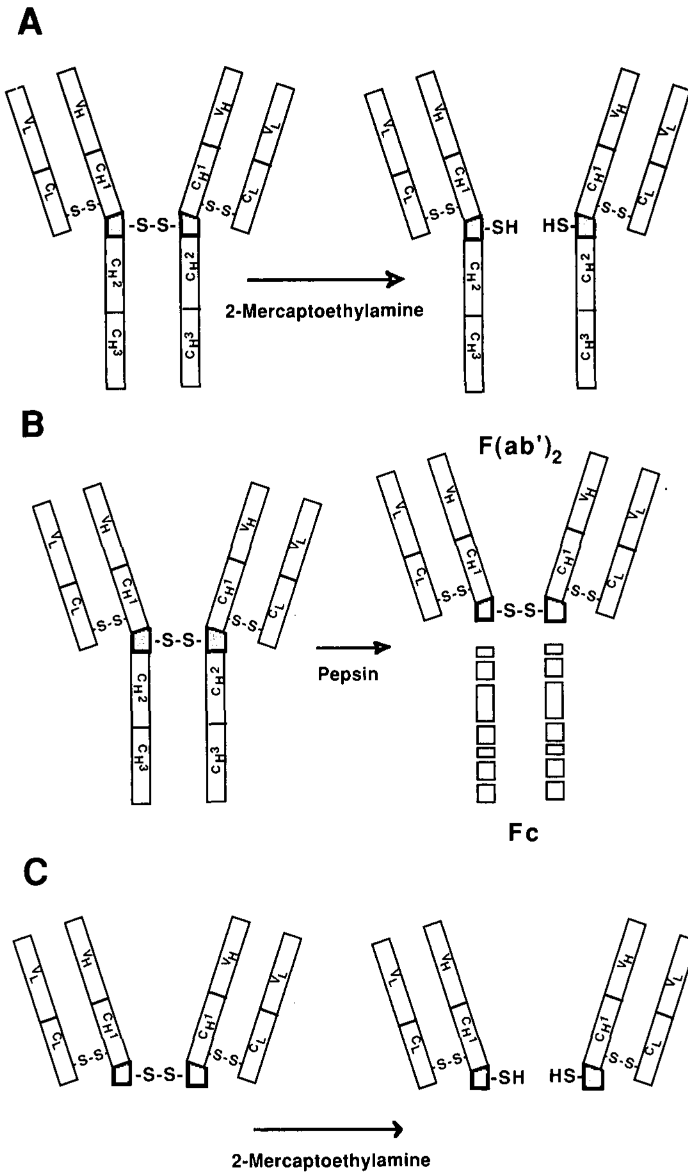

Fig. 1. Production of antibody fragments. (A) The antibody molecule is composed of two light and two heavy chains held together by disulfide bonds. There are two antigen binding sites on each molecule formed by the variable regions of one light and one heavy chain. (B) Enzymatic cleavage of antibody with pepsin yields one major and several smaller fragments. The antigen binding fragment, termed $F(ab')_2$, retains both binding sites. The remainder of the molecule is called Fc (crystallizable fragment). (A and C) Whole antibody and $F(ab')_2$ fragments treated with 2-mercaptoethylamine are preferentially reduced in the hinge region between heavy chains, yielding two antigen binding fragments.

amino, sulfhydryl or oxidized carbohydrate groups located in unique distribution patterns on the antibody molecule. Functional assays were performed to determine if immobilization chemistry could be used to preferentially bind and position the antibody molecule so that both antigen binding sites are accessible after coupling.

EXPERIMENTAL

Proteins

Bovine serum albumin (BSA), avidin, and trypsin were all obtained from Pierce (Rockford, IL, U.S.A.). Sheep anti-BSA was purchased from Bethyl Labs. (Montgomery, TX, U.S.A.) and thioredoxin was purchased from Calbiochem (San Diego, CA, U.S.A.). Human serum albumin (HSA), collagen (type VI), chorionic gonadotropin, fetuin, ovalbumin, α_1-acid glycoprotein, pepsin, ceruloplasm, aldolase, β-lactoglobin, arginine[8] vasopressin, α_1-antitrypsin, cytochrome *c*, myoglobin, α-lactalbumin, transferrin, hemoglobin, ribonuclease A, lysozyme, bovine IgG, human IgG, and rabbit anti-HSA were all purchased from Sigma (St. Louis, MO, U.S.A.).

Activated matrices

The activated gels and accompanying buffers were all obtained from Pierce. All columns contained 2 ml of activated gel. AminoLink Gel is 4% beaded agarose, periodate oxidized to yield aldehyde groups which react with primary amine groups[6]. SulfoLink Gel is 6% beaded agarose substituted with a 12-carbon spacer arm containing a terminal iodoacetyl group which can react preferentially with a free sulfhydryl group on the ligand[7,8]. CarboLink Gel is 6% beaded agarose substituted with a 23-atom spacer arm containing a terminal hydrazide group which can react with an aldehyde group on the oxidized glycoprotein[9,10].

Sample preparation for AminoLink Gel

The protein (1–20 mg) was dissolved or diluted in 2 ml AminoLink Coupling Buffer (0.1 *M* phosphate, pH 7.0).

Coupling to AminoLink Gel

The sample was added to a 2-ml AminoLink column that was equilibrated with AminoLink Coupling Buffer. The working reducing solution was prepared by dissolving 32 mg sodium cyanoborohydride in 0.5 ml distilled water, and 0.2 ml was added to the protein solution in the column. The column was gently mixed for 2 h at room temperature and then incubated for an additional 4 h at room temperature without mixing. The column was washed with 6 ml of AminoLink Coupling Buffer to remove unbound material.

Blocking of excess reactive sites on AminoLink Gel

The protein-coupled column was equilibrated with AminoLink Quenching Buffer (1.0 *M* Tris–HCl, pH 7.4). An additional 2 ml of AminoLink Quenching Buffer and 0.2 ml of the working reducing solution were added to the column and it was mixed at room temperature for 30 min. The column was then washed sequentially with 16 ml of 1.0 *M* sodium chloride and 16 ml of 0.05% sodium azide.

Sample preparation for SulfoLink Gel

Whole antibody or $F(ab')_2$ fragments (1–10 mg) were treated with 2-mercaptoethylamine to reduce the disulfide bonds (Fig. 1A and C). The protein was dissolved or diluted in 1 ml of SulfoLink Sample Preparation Buffer (0.1 *M* sodium phosphate, 5 m*M* ethylenediaminetetraacetate, disodium salt, pH 6.0). This solution was added to 6 mg of 2-mercaptoethylamine and incubated at 37°C for 1.5 h. The sample was brought to room temperature and excess reductant was removed using a 15-ml polyacrylamide desalting column (Pierce) equilibrated with SulfoLink Coupling Buffer (50 m*M* Tris, 5 m*M* ethylenediaminetetraacetate, disodium salt, pH 8.5).

Coupling to SulfoLink Gel

The reduced protein solution (2 ml) was added to a 2-ml SulfoLink column that was equilibrated with SulfoLink Coupling Buffer. The column was gently mixed for 15 min at room temperature and then incubated for an additional 30 min at room temperature without mixing. The column was washed with 6 ml of SulfoLink Coupling Buffer to remove unbound material.

Blocking of excess reactive sites on SulfoLink Gel

L-Cysteine–HCl (2 mg) was dissolved in 2 ml of SulfoLink Coupling Buffer and added to the protein-coupled column. The column was gently mixed for 15 min at room temperature and then incubated for an additional 30 min at room temperature without mixing. The column was then washed sequentially with 16 ml of 1.0 *M* sodium chloride and 16 ml of 0.05% sodium azide.

Sample preparation for CarboLink Gel

Carbohydrate moieties on the protein were oxidized to form aldehyde groups. The protein (0.5–10 mg) was dissolved or diluted in 1 ml CarboLink Coupling Buffer (0.1 *M* phosphate, pH 7.0). The sample was added to 5 mg of sodium metaperiodate and allowed to incubate at room temperature for 30 min, protected from light. Excess oxidizing agent was removed on a 5-ml Sephadex G-25 M (Pharmacia, Piscataway, NJ, U.S.A.) desalting column equilibrated with CarboLink Coupling Buffer.

Coupling to CarboLink Gel

The oxidized protein solution (2 ml) was added to a 2-ml CarboLink column that was equilibrated with CarboLink Coupling Buffer. The column was gently mixed for 6 h at room temperature. The column was washed with an additional 6 ml of CarboLink Coupling Buffer to remove unbound antibody and then washed sequentially with 16 ml of 1.0 *M* sodium chloride and 16 ml of 0.05% sodium azide.

Affinity purification

Phosphate-buffered saline (PBS, 0.01 *M* sodium phosphate, 0.15 *M* sodium chloride, pH 7.4) was used to equilibrate all columns and to dissolve the proteins. A 1-ml sample was applied to the columns which were then allowed to incubate at room temperature for 1 h. The columns were washed with 16 ml of PBS and fractions containing bound protein were eluted using ImmunoPure Elution Buffer (Pierce).

Column regeneration

As soon as possible after affinity purification, columns were washed with 16–20 ml of PBS and stored with a degassed solution of 0.05% sodium azide in water.

Antibody fragments

$F(ab')_2$ fragments of intact antibody (Fig. 1B) were generated using ImmunoPure $F(ab')_2$ Preparation Kit (Pierce). Briefly, 10 mg of human IgG were dissolved in 1 ml of digestion buffer (20 m*M* sodium acetate, pH 3.0) and incubated with 0.125 ml of immobilized pepsin for 4 h at 37°C. The digested sample was applied to a 2.5-ml Protein A column to remove undigested IgG and Fc fragments. The $F(ab')_2$ fragments in the eluent were dialyzed against SulfoLink Sample Preparation Buffer.

Coupling efficiency

Coupling efficiency was determined by comparison of the protein concentration in the unbound material to that in the starting sample. All columns were run in duplicate. Standard error was less than 10%. Protein concentration was determined either by absorbancy at 280 nm or by an assay system for protein using bicinchoninic acid as a detection reagent[11] (BCA Protein Assay, Pierce).

RESULTS AND DISCUSSION

AminoLink Gel is agarose, activated to yield aldehydes which react with primary amine groups to form Schiff bases. Reductive amination forms a stable covalent linkage with minimal leakage of the ligand[6]. The data presented in Table I show that AminoLink Gel is able to immobilize a wide variety of proteins. The coupling efficiency of proteins varying in size and isoelectric point (p*I*) averaged 80% when using a 20-mg sample. In general, the higher the p*I* of the protein, the greater the coupling efficiency. The molecular weight of the protein, however, appeared to have no effect. The coupling efficiency of immunoglobulins from a number of species averaged 80–90%.

TABLE I

COUPLING EFFICIENCY OF PROTEINS USING AMINOLINK GEL

A 20-mg amount of each protein was applied to 2 ml of AminoLink Gel as described in Experimental.

Protein	*pI*	*Molecular weight*	*Coupling efficiency (%)*
Ovalbumin	4.7	45 000	52
α_1-Antitrypsin	4.0	–	66
Fetuin	3.3	–	74
IgG (bovine)	–	150 000	79
Cytochrome *c*	9.0–9.4	11 700	79
Myoglobin	6.8–7.8	16 900	84
α-Lactalbumin	5.2	35 000	84
Transferrin	5.9	85 000	88
Hemoglobin	6.8	64 500	91
Ribonuclease A	9.5	13 700	92
Lysozyme	11.0	14 000	96

On the antibody molecule, primary amine groups are not localized, but occur throughout the molecule. Therefore, antibody can be immobilized near the antigen binding region as well as the Fc end, resulting in either total or partial loss of antigen binding capability.

Fig. 2 shows an affinity chromatographic profile using an AminoLink column coupled with 6 mg of anti-HSA. Another AminoLink column, without immobilized antibody but with reactive sites blocked, was included as a control to determine any non-specific binding. All columns were loaded with 5 mg of HSA. Fig. 2 shows that there is minimal non-specific binding to the blank control column. Approximately 2 mg of HSA were recovered from the antibody-coupled columns. The molar ratio of immobilized antibody to bound antigen never exceeded 1:1, indicating that half of the antigen binding sites on the molecule were not accessible. This supports the concept that coupling through amino groups results in random orientation of the molecule.

However, AminoLink chemistry offers an advantage over CNBr activation chemistry. The leakage of immobilized proteins from CNBr-activated Sepharose 4B is well documented[6,12–15]. The stable covalent bond formed with AminoLink Gel results in minimal leakage of the ligand, ensuring reuse of the column and low contamination of the purified sample.

Iodoacetyl groups on SulfoLink Gel react with free sulfhydryl groups to form a stable thio ether linkage. Antibody treated with 2-mercaptoethylamine is preferentially reduced in the hinge region between heavy chains, yielding two antigen binding fragments (Fig. 1)[16]. Immobilization at the hinge region sulfhydryls should increase the potential of leaving the antigen binding site unobstructed. Table II shows that reduced human IgG, either whole molecule or $F(ab')_2$ fragments, had an average coupling efficiency to SulfoLink Gel of 90%.

Fig. 3 shows an affinity chromatographic profile using SulfoLink Gel coupled with 5 mg of anti-HSA. A 2-ml SulfoLink column, without antibody but with reactive

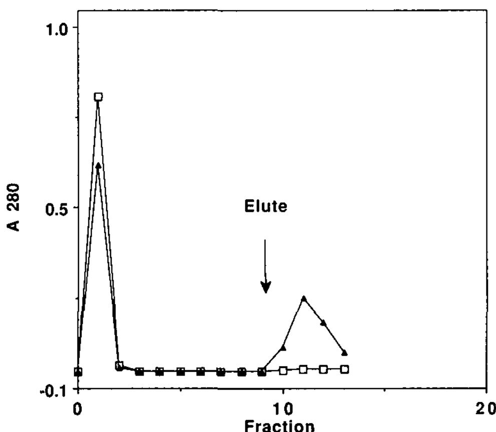

Fig. 2. Affinity purification of HSA using AminoLink Gel. A column (▲) containing 2 ml AminoLink Gel was coupled with 6 mg of anti-HSA as described in the Experimental section. Another AminoLink column (□), without immobilized antibody but with reactive sites blocked, was included as a control to determine any non-specific binding. For affinity chromatography, all columns were loaded with 5 mg of HSA. Columns were eluted and 2-ml fractions were collected.

TABLE II

IMMOBILIZATION OF IgG TO SULFOLINK GEL

Reduced human IgG (1–10 mg) and 4 mg of reduced $F(ab')_2$ fragments were applied to 2 ml of SulfoLink Gel according to the protocol described in Experimental.

Human IgG	*Coupling efficiency (%)*
Reduced whole molecule	90
Reduced $F(ab')_2$	88

sites blocked, was used to test for non-specific binding. All columns were loaded with 5 mg of HSA. Fig. 3 shows that, with this matrix also, there is minimal non-specific binding. Approximately 2.6 mg of HSA were bound and recovered from the antibody-coupled columns. The molar ratio of immobilized antibody (whole molecule) to recovered antigen ranged from 1:1 to 1:1.2. Restated, 50–60% of the antigen binding sites on the reduced antibody molecules were able to bind antigen.

Although polyacrylamide gel electrophoresis reveals that the predominant species following reduction with 2-mercaptoethylamine is the half molecule containing an antigen binding site, the presence of single light and heavy chains is also found. This may account, in part, for the less than optimum binding capacity.

Table III shows the coupling efficiency of several sulfhydryl- and/or disulfide-

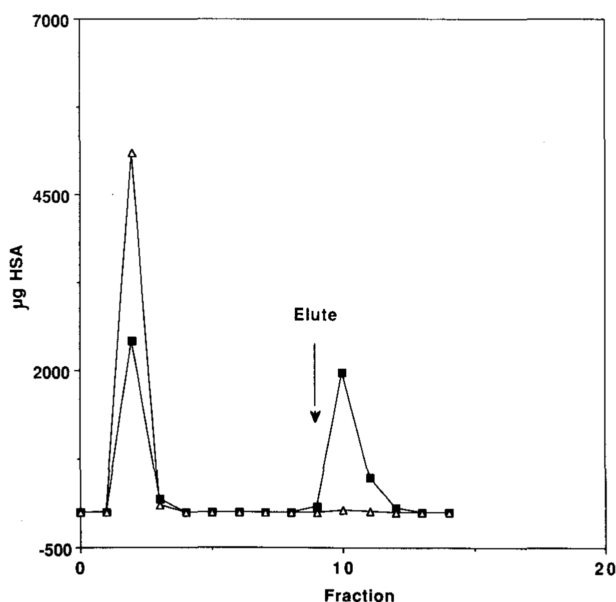

Fig. 3. Affinity Purification of HSA using SulfoLink Gel. Approximately 5 mg of anti-HSA were immobilized to 2 ml of SulfoLink Gel. (■) Another SulfoLink column, (△), without immobilized antibody but with reactive sites blocked, was included as a control to determine any non-specific binding. All columns were loaded with 5 mg of HSA for affinity chromatography. Columns were eluted and,2-ml fractions were collected.

TABLE III

COUPLING EFFICIENCY OF PROTEINS TO SULFOLINK GEL

Protein[a]	*Molecular weight*	*–SH group per molecule*[b]	*S–S group per molecule*[b]	*Coupling efficiency (%)*
Ceruloplasm	150 000	1–3	–	75
Aldolase	147 000	7–28	–	87
BSA	66 000	0.7	17	25
HSA	66 000	0.7	17	72
Ovalbumin	45 000	3–4	1	40
β-Lactalbumin	36 000	2	2	63
Trypsin	24 000	0	6	13
Thioredoxin	11 700	2	1	20
Arginine[8] vasopressin	1084	0	1	90

[a] A 2-mg amount of the reduced form of the peptide, arginine[8] vasopressin, and 8 mg of reduced protein were applied to 2 ml of SulfoLink Gel as described in Experimental.
[b] Values for sulfhydryl content reported in the literature[17,18].

containing proteins of varying size. All proteins were reduced prior to immobilization. Coupling efficiency cannot be strictly correlated with the number of potential free sulfhydryls in the reduced form of the protein. The accessibility of the sulfhydryls would be a contributing factor. As was seen with AminoLink Gel, there is no correlation with the size of the molecule although the best coupling efficiency was seen with the peptide arginine[8] vasopressin.

The coupling chemistry of SulfoLink Gel is also suitable for the purification of anti-peptide antibodes. Most peptide sequences are synthesized with a terminal cysteine residue added for immunogen conjugation. The free sulfhydryl provides a reactive group with which to couple the peptide to a carrier protein for immunization. This same reactive group can be used to couple the peptide to SulfoLink Gel in the same orientation as that used for the immunogen conjugate[19]. The spacer arm on the matrix will also reduce steric hindrance and facilitate the isolation of peptide-specific antibodies.

CarboLink Gel is agarose, activated to contain hydrazide groups which react with oxidized carbohydrate moieties. On the antibody molecule, carbohydrate residues are located primarily on the Fc end. The coupling efficiency of immunoglobulins to CarboLink Gel was variable and slightly lower than that found with the other activated matrices, averaging 70%. One possible reason is that the degree of glycosylation and the composition of the carbohydrate residues are not consistent within a polyclonal response and yield a varying number of reactive aldehyde groups following periodate oxidation[9,20]. Table IV shows that the coupling efficiency of several other glycoproteins to CarboLink Gel was also variable but generally good.

Coupling to antibody through the Fc portion of the molecule should increase the chances of retaining full antigen binding ability following coupling. This is supported by the data shown in Fig. 4. Approximately 0.5 mg of anti-BSA were coupled to 2 ml of gel, and 2 mg of BSA were applied for affinity purification. As a control for non-specific binding, a 2-ml column containing no antibody was also loaded with BSA. The elution profile shows that, with this matrix also, there is minimal

TABLE IV

COUPLING EFFICIENCY OF PROTEINS USING CARBOLINK GEL

A 2-mg amount of protein was applied to 2 ml of CarboLink Gel as described in Experimental.

Protein	*Molecular weight*	*Coupling efficiency (%)*
Collagen (type VI)	163 000	63
Human IgG	150 000	74
Avidin	66 000	95
Chorionic gonadotropin	59 000	91
Fetuin	48 700	87
Ovalbumin	45 000	86
α_1-Acid glycoprotein	44 100	90
Pepsin	34 000	47

non-specific binding. Approximately 0.5 mg of BSA were bound and recovered from the antibody-coupled column. This matrix consistently demonstrated the optimum molar ratio of immobilized antibody to bound antigen of 1:2. The increased efficiency in antigen binding by antibody immobilized through carbohydrate moieties is supported by the work of others[21].

Immobilized antibody has become a commonly used reagent for the isolation and purification of other molecules. However, antibody with specificity for the particular antigenic determinant(s) of the molecule of interest must be generated. In some cases, only small amounts of antibody are produced. For example, there may be a limited supply of purified antigen, antigenic fragments or synthetic peptides with which to stimulate an immune response, resulting in a low yield of polyclonal

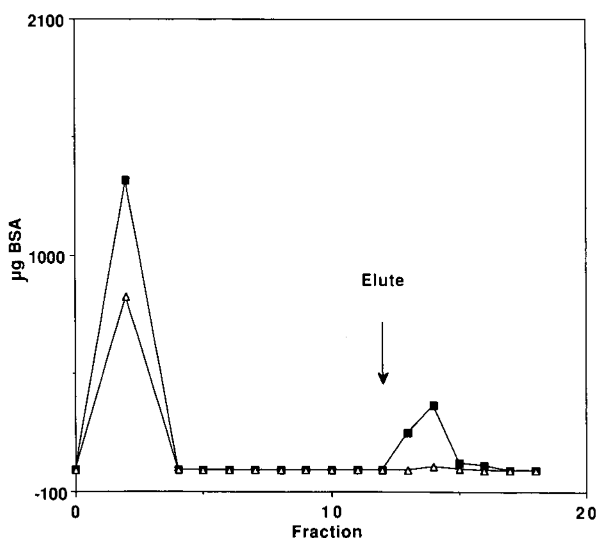

Fig. 4. Affinity Purification of BSA using CarboLink Gel. Approximately 0.5 mg of anti-BSA were coupled to 2 ml of CarboLink Gel, and 2.0 mg of BSA were applied for affinity chromatography (■). Control columns (△) containing no antibody were also loaded with BSA. Columns were eluted and 1-ml fractions were collected.

antibody. Adequate quantities of monoclonal antibody may not be available due to the loss or mutation of the hybridoma. In these circumstances in particular, it is desirable to take advantage of the full binding capability of the antibody by using a matrix capable of correctly orienting the molecule.

Equally important is the ability to reuse the support. All of the matrices provided stable linkage as demonstrated by the fact that they were used and regenerated ten times with minimal loss of affinity purification capability (data not shown).

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Enhanced activity of immobilized dimethylmaleic anhydride-protected poly- and monoclonal antibodies

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ABSTRACT

The effect of reversible protection of the free amino groups of poly- and monoclonal antibodies by dimethylmaleic anhydride on their binding activity following immobilization onto various carriers was studied. The treatment with dimethylmaleic anhydride resulted in a 1.6–1.8-fold increase in the activity of immobilized goat anti-mouse immunoglobulin antibody immobilized onto different epoxy containing carriers and a 3–10.7-fold increase in the activity of immobilized monoclonal antibodies specific for carboxypeptidase A. The increase in activity was most pronounced at low antigen to carrier loads and over a wide range of modifier to protein ratios. The application of reversible protection of antibodies may permit the development of highly active immobilized antibody preparations for use in immunoaffinity purification.

INTRODUCTION

Immobilization of antibodies (or other biologically active proteins) onto solid matrices often leads to partial or complete loss of their antigen-binding activity, as a result of either (a) chemical modification of amino residues essential for activity, (b) steric hindrance caused by the attachment of the antibody to the matrix at a region close to its active site or (c) conformational changes and/or restriction of the intramolecular mobility imposed by multi-point interaction with the matrix.

The susceptibility of individual antibody molecules to loss of antigen-binding activity by mechanism (a) or (b) would depend on their chemical composition. These effects may therefore vary between different antibody molecules. On the other hand, as the number and distribution of free amines are roughly similar in most antibody molecules, it is logical to expect that most if not all of them would suffer a loss of activity due to multi-point attachment of the amines to the carrier. This problem may be more severe with carriers that have a high density of reactive groups, such as Eupergit C.

Eupergit C is a polymethylmethacrylamide-based polymer bearing oxirane

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groups which may bind proteins mainly via reaction with their amino groups. Thiol moieties, which may also react with oxirane groups of Eupergit C, are less recurrent in the molecules of most proteins.

Recently, the use of dimethylmaleic anhydride (DMA) for enhancement of the activity of antibodies conjugated with methotrexate has been described[1]. It was suggested that DMA treatment served to protect amino acid residues essential for antibody activity.

We hypothesized that reversible protection of some free amines of the antibody molecule by DMA prior to its immobilization onto Eupergit C might serve on the one hand to protect essential amino acid residues in the antigen-binding site of the antibody, and on the other to reduce the number of attachment points between antibody and carrier. Thus, the activity of the immobilized antibody may be enhanced. The results of experiments described in this paper tend to support this hypothesis.

EXPERIMENTAL

Materials

Immunoaffinity-purified goat anti-mouse Fc antibodies were obtained from Biomakor (Nes-Ziona, Israel). Two products based on Eupergit C were used (1) CB6200, which are 6 mm in diameter poly(methyl methacrylate) beads, coated with non-porous 1-μm diameter, Eupergit C beads; and (2) standard Eupergit C, which is 150-μm diameter porous beads. Both products were obtained from Rohm (Darmstadt, F.R.G.). Epoxy-activated Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). Poly(vinyl alcohol)-epoxy (PVA-epoxy) was obtained from Riedel-de Haën (Seelze, F.R.G.). All other materials were purchased from Sigma (St. Louis, MO, U.S.A.) unless specified otherwise.

Monoclonal antibodies

CB6F1 mice were immunized with two injections, ten days apart, of 10 μg of carboxypeptidase A (CPA) in 0.2 ml of phosphate-buffered saline (PBS) emulsified in an equal volume of Freund's complete adjuvant. After a further 20 days, their spleen cells were fused with NSO myeloma cells[2] as described previously[3]. The resulting hybridoma cultures were first tested by enzyme-linked immunosorbent assay (ELISA) for secretion of antibodies recognizing CPA. In a second screening, the cultures were tested by reverse ELISA (as described below) for their ability to immobilize CPA without affecting its enzymic activity.

A similar approach was utilized for the production of another antibody specific for the enzyme horseradish peroxidase (HRP), which was named mAb HRP2, except that the reverse ELISA was used as the only screening procedure.

Ascites fluid was produced in CB6F1 mice following priming with Freund's incomplete adjuvant as described previously[4].

All the mAbs used in this study were purified by the use of protein A–Sepharose (Bio-Rad Labs.) as described previously[5].

ELISA

The wells of microtitre ELISA plates were coated with the protein being tested.

They were exposed for 16 h to a solution containing 1 μg of protein per well in 0.1 ml of 0.1 *M* carbonate buffer (pH 9.6) at 4°C. After washing several times with tap water, mAb (hybridoma culture media or ascites fluids diluted in skim milk containing 1% fat) was added (0.1 ml per well). After 1 h at 37°C, the wells were washed five times with tap water. To each well, 0.1 ml of goat anti-mouse immunoglobulin G (IgG–HRP complex (Bio-Rad Labs.), diluted 1:3000 with skim milk (1% fat), was added and the plates were incubated at 37°C for 1 h. After washing several times with tap water, 0.1 ml of *o*-phenylenediamine reagent was added to each well and the colour allowed to develop. The *o*-phenylenediamine reagent contained 2 mg of 1,2-phenylenediamine in 1 ml of 50 m*M* sodium citrate buffer (pH 5.0) containing 0.08% hydrogen peroxide. The activity was determined by measuring of the rate of increase in absorbance at 492 nm compared with a non-specific absorbance at 405 nm in a EAR400 ELISA Reader (SLT, Grodig, Austria).

Reverse ELISA

Microtitre ELISA plates were coated with rabbit anti-mouse Ig antibodies (Sigma) (10 μg per ml of PBS, 100 μl per well, for 16 h at 4°C) and washed several times with tap water. Hybridoma culture fluids were added to the wells (100 μl per well) the plates were incubated for 1 h at 37°C and washed several times with tap water. CPA (100 μl, 10 μg per ml of PBS) was added to the wells and the plates were incubated for 1 h at 37°C and washed several times with PBS. CPA substrate (100 μl per well of PBS) was added to the wells and the plates were incubated for 1h at 37°C. CPA activity was measured by the ninhydrin method on the ELISA plates as follows: to 0.1 ml of fresh ninhydrin reagent (3% ninhydrin in methyl Cellosolve) were added 0.05 ml of $2 \cdot 10^{-4}$ *M* sodium cyanide in 3.8 *M* sodium acetate (pH 5.3), sample was then added and the mixture was heated at 95°C for 20 min, cooled and read in ELISA reader at 550 nm.

A similar reverse ELISA procedure was used for the determination of the activity of the mAb HRP2 except that the substrate used was the *o*-phenylenediamine substrate solution (above) as described for the regular ELISA.

Immobilization of antibodies on Eupergit C, PVA-epoxy and epoxy-activated Sepharose 6B

DMA method. DMA [dissolved in dimethylformamide (DMF)] was added to antibodies [5–10 μg per sample, concentration 0.3–1.3 mg/ml, 5–30 μl, in 25 m*M* sodium borate buffer (pH 9.5) + 1 *M* NaCl] at a 400 molar excess, keeping the final DMF concentration in the antibody solution below 5%. The solution was incubated for 30–60 min at 0°C. The antibodies were then added to carrier beads [0.5 mg in 5 μl of 1 *M* potassium phosphate (pH 7.5)] in a Beckman tube and incubated for 4 h at room temperature and then 16 h at 4°C. Excess oxirane groups were blocked by incubating the carrier beads–antibody conjugate with 2-mercaptoethanol (2ME) solution (final concentration 0.2 *M*) for 4 h at room temperature. The beads were then washed with PBS and the DMA was hydrolysed by incubation at acidic pH [50 m*M* citrate buffer (pH 5.5) for 1 h at room temperature], followed by washing with PBS.

Standard method. The method is identical with the above DMA method except that the antibodies were not reacted with the DMA prior to their immobilization and the DMA was not hydrolysed by the acid treatment after blocking with 2ME solution.

Immobilization of antibodies onto CB6200 beads

Standard method. A 10-μg amount of anti-Fc antibodies was bound to the bead in 0.3 ml of 1 *M* potassium phosphate buffer (pH 7.5). The beads were incubated with the antibody for 16 h at 4°C and washed with PBS. The beads were then blocked with 2ME (0.2 *M* for 4 h at room temperature) and finally washed with PBS.

DMA method. The pretreatment of antibodies with DMA, binding of the antibodies to the bead, blocking with 2ME and hydrolysis of DMA were as described above.

Determination of the activity of anti-CPA antibodies

CPA was added to the Eupergit C–antibody conjugate (at more than 2 mol per mole of input antibody) in PBS + 0.5 *M* NaCl and incubated for 1 h at room temperature with shaking. The non-bound CPA was washed with PBS. Hippuryl-L-phenylalanine (10 m*M* in PBS + 0.5 *M* NaCl, 50 μl per sample) was added to the sample and incubated for 10 min with shaking. A sample was removed for determination of the amount of product by the ninhydrin method[6].

Determination of the activity of anti-mouse Fc antibodies immobilized on CB6200 beads

MAb HRP2 was applied to the beads (10 μg of antibody per bead in 0.3 ml of low-fat milk) and incubated for 1 h at room temperature. Following washing with water, HRP was added to the beads (5 μg per bead in 0.3 ml of low-fat milk) the beads were incubated for 1 h at room temperature and washed with PBS. The beads were then transferred into the wells of a 24-well plate (Costar) and to each well was added 1 ml of *o*-phenylenediamine substrate solution. The plates were placed on a horizontal rotating table, mixed at 200 rpm for 5 min and then the reaction was stopped by the addition to each well of 0.5 ml of 4 *M* HCl. The colour developed was analysed by the ELISA reader as described above.

Determination of the activity of the anti-mouse Fc antibodies immobilized on Eupergit C standard, PVA-epoxy and epoxy-activated Sepharose 6B

Anti-mouse Fc antibodies were immobilized on 5 mg of carrier. MAb HRP2 (50 μg in 25 μl of milk) was then added, the tubes were incubated for 1 h at room temperature and then the carrier beads were washed with PBS. To each tube was added HRP (50 μg of enzyme in 25 μl of milk) and the tubes were incubated for 1 h at ambient temperature, with shaking, and the carrier beads were then washed with PBS. The beads were dispersed in 1 ml of PBS and a 50-μl sample was transferred into a fresh tube and mixed with 1 ml of *o*-phenylenediamine substrate solution. Colour development was stopped after 1 min by the addition of 0.5 ml of 4 *M* HCl. The colour was analysed by the ELISA reader as described above.

RESULTS

The immobilization of goat anti-mouse Fc antibodies was selected as a model system for the study of the effect of DMA on the activity of immobilized polyclonal antibodies. The immobilized anti-mouse Fc antibodies were used for the binding of mAb HRP2 and the amount of the HRP enzyme which was bound by the immobilized mAb served for the determination of the activity of the anti-mouse Fc antibodies. The

TABLE I

EFFECT OF DMA ON THE ACTIVITY OF POLYCLONAL ANTI-MOUSE Fc ANTIBODIES IMMOBILIZED ON DIFFERENT CARRIERS

Carrier	*Antibody activity*[a]		*Improvement factor*
	Standard method	*DMA method*	
CB6200	1.060	1.949	1.84
	–	0.341[b]	0.32
Eupergit C standard	0.740	1.214	1.64
Epoxy-activated Sepharose 6B	0.023	0.040	1.74
PVA-epoxy	0.086	0.156	1.81

[a] Antibody activity is expressed as A_{492} resulting from the activity of horseradish peroxidase which is bound to mAb HRP2 which is bound by the immobilized anti-mouse Fc antibodies. Assay was performed as described under Experimental. Background resulting from the direct immobilization of mAb HRP2 or horseradish peroxidase on the carrier was subtracted.

[b] Blocking with 2-mercaptoethanol was performed after hydrolysis of the bound DMA.

results of these experiments are summarized in Table I. As can be seen, reversible protection of the antibodies with DMA allowed the formation of more active immobilized anti-mouse Fc antibodies. Similar activity improvement factors were obtained for the different carriers used.

When the DMA was removed from the immobilized antibodies prior to elimination of unreacted oxirane groups, a decrease in activity of antibodies immobilized on Eupergit C was observed (Table I). A similar result was obtained when excess of DMA was removed from the system (without hydrolysing the bound DMA) before the unreacted oxirane groups were eliminated (data not shown).

Immobilization of mAbs specific for the enzyme CPA which do not inhibit enzymic activity was selected as a model system for the study of the effect of DMA treatment on the activity of immobilized mAbs. The activity of the immobilized

TABLE II

ACTIVITY OF ANTI-CPA MONOCLONAL ANTIBODIES IMMOBILIZED ON EUPERGIT C BY THE STANDARD AND DMA METHODS

Monoclonal antibody	*Antibody activity*[a]		*Improvement factor*
	Standard method	*DMA method*	
CPA1	0.040	0.362	9.0
CPA8	0.060	0.216	3.6
CPA9	0.082	0.242	3.0
CPA14	0.102	0.300	3.0
CPA18	0.030	0.322	10.7

[a] Antibody activity is expressed as A_{550} resulting from the activity of carboxypeptidase A bound by the immobilized antibody. CPA activity was measured by the ninhydrin method as described under Experimental. Background resulting from the activity of carboxypeptidase A directly immobilized on Eupergit C was subtracted.

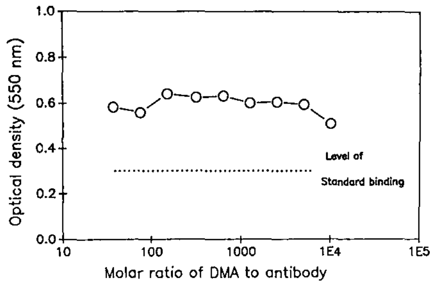

Fig. 1. Effect of varying the ratio of DMA per antibody on the activity of immobilized monoclonal antibody CPA8. The graph depicts the binding activity of the immobilized antibody as manifested by the activity of the CPA bound to the antibody.

antibodies was evaluated through measurements of the enzymic activity of CPA bound by the antibody (for details see Experimental). Five different mAbs were tested in this assay. As can be seen in Table II, antibodies immobilized after protection with DMA possessed a higher CPA binding activity compared with the standard immobilization method.

The optimum ratio of DMA to protein necessary for achieving the above response was examined in another experiment. MAb CPA8 was immobilized on Eupergit C following pretreatment with different doses of DMA. As can be seen in Fig. 1, a similar effect of the DMA treatment on the activity of the immobilized antibody was observed over a wide range of DMA to protein ratios.

The effect of the DMA treatment on the activity of antibodies immobilized at different loads on Eupergit C was also studied. As can be seen in Fig. 2, maximum improvement in the activity of immobilized mAb CPA8 was observed at low antibody loads and was less pronounced at high antibody loads.

DISCUSSION

Several possible mechanisms may explain the increase in activity of antibodies immobilized on Eupergit C, epoxy-activated Sepharose 6B and PVA-epoxy achieved by employing reversible protection by DMA.

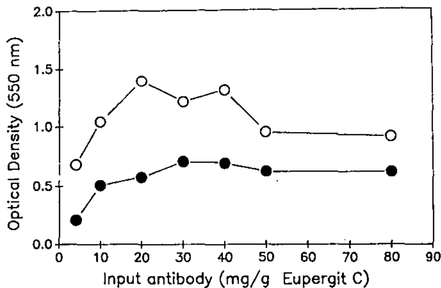

Fig. 2. Immobilization of different doses of (○) DMA-treated and (●) untreated monoclonal antibody CPA8 on Eupergit C. The graph depicts the binding activity of the immobilized antibody as manifested by the activity of the enzyme CPA bound by the antibody.

Blocking of most of the free amines on treatment of the protein with DMA would result in the formation of fewer bonds between the protein and matrix, down to a single bond. This would result in immobilization of antibodies having as native a conformation and intramolecular mobility as possible and therefore having antigen-binding properties close to those of free antibodies.

Failure to eliminate the residual oxirane groups prior to hydrolysis of the bound DMA or even prior to removal of the excess of DMA from the solution resulted in a decrease in the activity of the antibodies immobilized on Eupergit C in comparison with untreated antibodies. This result indicates that additional bonds between the unblocked carrier and the newly exposed amines may take place. Thus, formation of multiple bonds between the native antibody molecule and the carrier, which are inhibited by pretreatment of the antibody with DMA, are at least partially responsible for the loss of the activity of the immobilized antibodies.

Treatment of the antibody with DMA would block mainly the most reactive amines of the antibody molecule. Hence the DMA-protected antibody would be immobilized via the less reactive amines, those which in the native protein would have less chance of interacting with the matrix. This reorientation of the protein–matrix site of interaction may cause an increase in the activity of the immobilized antibody if the most reactive amines are located near the active site while the less reactive amines are remote from it. On the other hand, it may happen that the protein–matrix interaction site would be relocated to an area close to the active site, in which case a decrease in the activity of the immobilized antibody should be observed.

As the number and distribution of amines in different immunoglobulin molecules is similar, the DMA treatment is expected to lower the number of antibody–matrix interactions, and consequently increase the activity, of most immobilized antibody molecules. On the other hand, the effect of reorientation of the antibody–matrix binding site is likely to be variable between different mAbs, as discussed above. Our experiments demonstrated a marked improvement in the activity of immobilized polyclonal antibodies and all five mAbs tested. Hence one might expect that this novel procedure could be suitable for preserving the activity of the majority of immobilized mAbs.

To the best of our knowledge there have been no previous reports on the use of reversible amino group blocking reagents for protecting the activity of immobilized proteins. Hence the DMA method for immobilization of antibodies, and probably also other proteins, on Eupergit C or other carriers should be a valuable adjunct to currently available immobilization methods.

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Eupergit C as a carrier for high-performance liquid chromatographic-based immunopurification of antigens and antibodies

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ABSTRACT

An immunoaffinity purification system using C30N and C1Z Eupergit C beads was developed and optimized. Poly- and monoclonal antibodies were purified using immobilized antigens and antigens were purified using immobilized antibodies. Antigens were used that possess enzymic activities and the efficiency of antigen binding was determined from the enzymic activity of the matrix-bound immunocomplexes. High-performance immunoaffinity purification using Eupergit C beads proved to be highly specific, reproducible, free from protein leakage and possessed a low degree of non-specific adsorption of tissue proteins. These characteristics of the system were illustrated by the isolation of immunoglobulin G from serum and of human decidua proteins from the decidua tissue and from seminal plasma. These proteins were obtained at high purity in a single purification step, as shown by sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

INTRODUCTION

The increasing availability of a variety of monoclonal antibodies and the rapid development of new water-insoluble matrices and covalent binding methods[1] during the last decade have led to a marked increase in the application of immunoaffinity methods to the purification of biologically active proteins. Of the various commercially available matrices, agarose and Sepharose are most widely used for the affinity purification of proteins on the laboratory and industrial scales. However, their application in high-performance immunoaffinity chromatography (HPIAC) is limited by their low mechanical stability[2] and by leakage of antibodies off the matrix, especially when coupled via cyanogen bromide activation[3]. In addition, agarose, as a natural polymer, may be susceptible to biological degradation which may limit the life span of antibody–agarose conjugates. Silica is mostly used as a support for HPIAC[2] and a variety of methods have been developed for covalent coupling of proteins onto this matrix. However, the sensitivity of silica to alkaline pH (unless

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especially capped) may limit its application in immunoaffinity purification processes since buffers of high pH (*e.g.*, ammonia, pH 10) are often used to elute the antigen from the matrix-conjugated antibody.

Recently, a number of synthetic polymeric matrices have been developed that bear active groups for covalent coupling of proteins[4]. We chose to study the applicability of one of these matrices, Eupergit C[5], to the high-performance liquid chromatographic (HPLC)-based immunoaffinity purification of antigens and antibodies. This matrix is a cross-linked copolymer of methylacrylamide, N-methylenebis-acrylamide and monomers containing reactive oxirane groups which react with amino, thio and hydroxyl groups of proteins. It is available as beads of 150 μm (standard, porous), 30 μm (C30N, porous) and 1 μm (C1Z, non-porous). The last two are excellent candidates for use as HPIAC supports.

In the process of the development of a carrier for immunoaffinity purification, several parameters have to be determined and optimized. Among these are the capacity of the matrix for protein binding, the activity and specificity of the matrix-bound antibodies, the elution of the antigens from the matrix, the absence of leakage of immobilized antibodies and the reproducibility and the stability of the immunoaffinity purification system. The results of these investigations are presented in this paper.

EXPERIMENTAL

Materials

Carboxypeptidase A (CPA) and horseradish peroxidase (HRP) were purchased from Sigma (St. Louis, MO, U.S.A.). Goat anti-rabbit immunoglobulin G (IgG) was purchased from Bio-Makor (Rehovot, Israel). Eupergit C was obtained from Rohm-Pharma (Darmstadt, F.R.G.).

The preparation of anti-CPA and anti-HRP monoclonal antibodies (mAbs), the determination of enzymic activities of CPA and HRP and the periodate oxidation of antibodies by the multi-step procedure were described previously[6].

Protein determination

Protein was determined according to the method described by Bradford[7] using the Bio-Rad Labs. (Richmond, CA, U.S.A.) protein assay reagent. The reagent was diluted 5-fold in water before use. Samples containing 0.2–8 μg of protein in 10–50 μl of phosphate-buffered saline (pH 7.4) (PBS) were mixed with 200 μl of the diluted reagent. The intensity of the colour developed was measured at 690 nm using an enzyme-linked immunosorbent assay (ELISA) reader (SLT, Grodig, Austria).

Alternatively, protein concentrations were determined spectrophotometrically by the absorbance at 280 nm using molar absorptivities of 1.8, 1.13 and 1.4 for 1 mg/ml solutions of CPA, HRP and IgG, respectively.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed with 10% gels according to the procedure of Laemmli[8].

High-performance immunoaffinity chromatography

The chromatographic system was obtained from Gilson (Villiers-le-Bel, France),

equipped with Model 303 pumps, a Model 111B UV detector and a Model 421 Autoinjector. Unless stated otherwise, all HPIAC separations where carried out with antigens or antibodies conjugated to Eupergit C C30N beads. The beads were packed into the columns in PBS using a high vacuum pump.

Preparation and selection of monoclonal antibodies

Anti-CPA and anti-HRP were prepared as described previously[6,9]. Antibodies which do not affect the activities of these enzymes were selected. The antibodies were assayed by ELISA as described previously[6,9].

Protein iodination

Iodination of proteins was performed by the chloramine T method[10]. Excess of free iodine were removed by gel filtration on a Sephadex G-25 column. ^{125}I-labelled CPA and rabbit IgG possessed specific activities of 4000–6000 cpm/μg.

Immobilization of proteins on Eupergit C

Eupergit C beads were thoroughly washed with PBS until no absorbance at 280 nm was detected in the supernatant and then the beads were washed once with 1 *M* potassium phosphate buffer (pH 7.4). Solutions of proteins to be immobilized (0.025–15 mg, 1–10 mg/ml of the same buffer) were incubated with washed Eupergit C beads (usually at 5–10 mg/g of matrix) for 16 h at 4°C or for 3–4 h at room temperature. The amount of enzyme antigens bound to the matrix was determibed by assays of the matrix-bound enzymic activities as described previously[6]. The amount of antibody bound was determined by adding a ^{125}I-labelled rabbit IgG as a tracer or by a protein assay of the reaction mixture supernatant at the end of the coupling reaction.

Excess oxirane groups on the matrix were blocked by incubation with 0.2 *M* β-mercaptoethanol for 4 h at 4°C. When used in HPIAC the Eupergit C conjugates were packed into stainless-steel HPLC columns (10 × 0.8 cm I.D.) and repeatedly washed with PBS and 0.2 *M* ammonium acetate buffer (pH 10) before application to immunopurification of the respective antibodies or antigens.

Formation of the immunocomplex by Eupergit C-conjugated antigens or antibodies

Antigens or antibodies were bound to the respective Eupergit C-conjugated antibodies or antigens by mixing the beads with a molar excess of the specific protein to be bound in PBS. The mixture was incubated for 3 h at room temperature or 16 h at 4°C. The amount of immunologically bound protein was determined after centrifugation of the reaction mixture at 12 000 *g* by enzymic assays of the matrix-bound CPA or HRP (when the respective antibodies were conjugated to the matrix) or by protein assays of the respective supernatants. In HPLC columns antigens or antibodies were loaded in PBS at a flow-rate of 0.5 ml/min. After the non-bound protein had been washed off the column, elution of the specific protein by washing with 0.2 *M* ammonium acetate buffer (pH 10.0) was monitored by UV adsorption at 280 nm.

RESULTS AND DISCUSSION

Protein-binding capacity of Eupergit C

Protein binding to the different forms of Eupergit C (standard, C30N and C1Z)

was determined by incubation of radioactively labelled IgG with the matrix and direct counting of the matrix-bound radioactive protein. Non-covalently adsorbed protein was excluded by washing the beads with a solution containing 1% SDS in 8 *M* urea. Both the standard Eupergit C and C30N exhibited a high degree of protein binding, which was more than 85% of the protein loaded onto the matrices even at a load of 100 mg protein per gram (dry weight) of matrix. With protein concentrations not exceeding 5 mg per gram of C30N binding was achieved within 15 min. With higher protein loads maximum binding was observed only after 4 h of incubation at room temperature (Fig. 1). Binding onto standard Eupergit C was similar to that onto C30N and is not shown. As might be expected, with the non-porous 1-μm beads C1Z the degree of protein binding was much lower, reaching a maximum at about 7 mg per gram of matrix. For all the bead forms the amount of protein non-covalently adsorbed to the matrix was relatively low (<2% of the input protein).

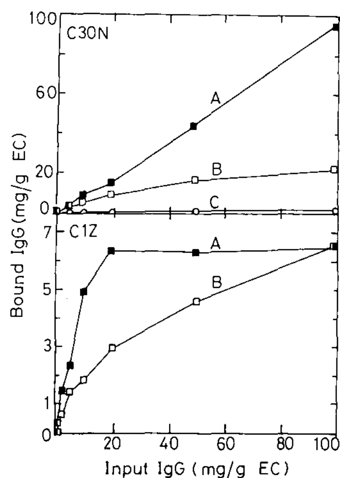

Fig. 1. Protein binding to Eupergit C (EC) C30N and C1Z beads. Aliquots of rabbit IgG (0–1000 μg) were mixed with 5 μg of ^{125}I-labelled rabbit IgG (36 600 cpm) and incubated in 1 *M* potassium phosphate buffer (pH 7.4) with 10 mg portions of Eupergit C C30N or C1Z for (□) 15 min or (■) 4 h at room temperature with slow agitation. At the end of the reaction, the beads were washed three times with PBS, transferred into counter tubes and counted. The amount of radioactive material adsorbed on the beads (○) was determined after washing the beads three times with 1% SDS in 8 *M* urea and counting the radioactivity released to the solution. (A) 4 h; (B) 15 min; (C) adsorption.

Blocking of residual oxirane groups on the matrix

Titration of the oxirane groups with thiosulphate showed that Eupergit C (standard and C30N) contains about 1.6 mmol of oxirane groups per gram of matrix[5,7]. About half of these groups are readily available to reaction with amino or thio groups whereas the other half are much less reactive and require a long time (hours to days) to react. Coupling of a protein usually uses only a tiny amount of these oxirane groups; the remainder have to be blocked to prevent undesirable covalent protein binding at a later stage. We found β-mercaptoethanol to be an efficient blocker. In

contrast to the commonly used ethanolamine, it may by applied to the matrix at almost neutral pH (pH 8.0) and, as it does not form charged groups when bound to the matrix, non-specific ionic adsorption of proteins is eliminated. Our investigations indicated that the immobilized antibodies were not affected by the presence of 0.2 *M* of β-mercaptoethanol used for blocking of the matrix (data not shown).

Immunoaffinity purification of antibodies using immobilized antigens

An efficient immunoaffinity purification is characterized by high selectivity, specificity, capacity and reproducibility. In order to study the applicability of Eupergit C to immunoaffinity purification various antigens (CPA, HRP and rabbit IgG) were coupled to C30N beads, packed into HPLC columns and used to isolate poly- and monoclonal antibodies from the respective antisera and ascites fluids. Purification of poly- and monoclonal anti-CPA antibodies is shown in Fig. 2. The purified antibodies were eluted as sharp peaks, in contrast to about 10-fold broader peaks which eluted using a comparable LC affinity purification system. Reproducibility of the purification system was demonstrated by reapplication of the purified antibodies (peak II), after dialysis against PBS, to the same column and re-elution with ammonium acetate buffer (pH 10). As shown by an ELISA test for anti-CPA antibodies (Fig. 3), most of the antibody loaded onto the column was eluted in peak II whereas peak I did not contain any active antibody. Assay for purity by SDS-PAGE showed that under the above conditions the antibody obtained by the single-step purification was about 90% pure. The impurities have been shown to result from non-specific adsorption of serum proteins by the matrix. Inclusion of polyethylene glycol (PEG) 400 in the loading buffer was found to reduce this type of adsorption, leading to the elution of almost pure antibody[11]. Purification of anti-HRP and anti-IgG mono- and polyclonal antibodies on the respective C30N-conjugated antigens yielded similar results to those described above (data not shown).

Immunoaffinity purification of antigens using immobilized antibodies

The different affinity-purified antibodies were conjugated to standard Eupergit C and their capacity to bind the antigen was tested. As shown in Table I, the specific antigen-binding activity of these antibodies depended markedly on the amount of antibody loaded onto the matrix. At a low antibody load (*ca.* 1 mg per gram of matrix) 0.60–0.80 mol of antigen were bound per mole of antibody whereas at a high antibody load (*ca.* 10 mg per gram of matrix) the specific activity decreased to 0.15–0.50 mol/mol. The polyclonal antibody immobilized on Eupergit C showed comparable binding activity. As shown in Table II, the activity of C30N-conjugated monoclonal antibodies was 10–20% higher than that observed for standard Eupergit C conjugates. C1Z-conjugated antibodies possessed higher activities (30–50% higher than the standard Eupergit C conjugates, but the efficiency decreased markedly at antibody concentrations higher than 2 mg per gram of C1Z.

In order to illustrate the applicability of the conjugated antibodies to HPIAC antigen isolation, C30N-conjugated anti-CPA mAb9 was packed into an HPLC column and CPA was applied, washed with PBS and eluted with ammonium acetate (pH 10) (Fig. 4). From the amount of CPA eluted a specific antigen-binding activity of 0.16 mol/mol was calculated, compared with 0.5 mol/mol observed under batch operation. The different efficiencies of antigen binding by immobilized antibodies in

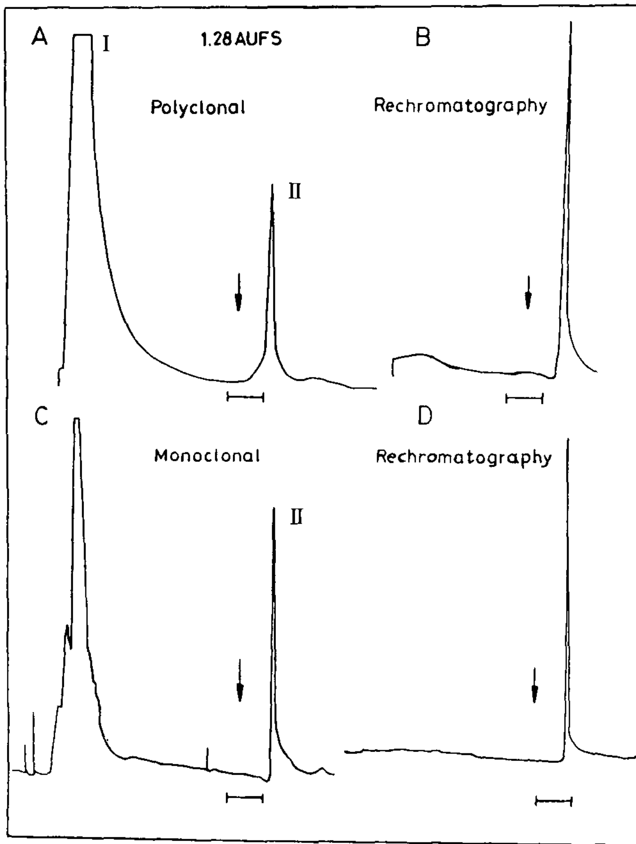

Fig. 2. Immunopurification of anti-CPA antibodies on a Eupergit C C30N–CPA column. (A) Purification of polyclonal antibodies from anti-serum: 0.5 ml of immunized rabbit serum was applied to a column (250 × 4 mm I.D.) containing Eupergit C-bound CPA (10 mg/g of matrix) in PBS at a flow-rate of 0.5 ml/min. Immunologically bound antibodies were eluted from the column with 0.2 *M* ammonium acetate buffer (pH 10.0) at a flow-rate of 1 ml/min. (B) Rechromatography of peak II in A. The material eluted in this peak was collected, dialysed against PBS (18 h, 4°C), concentrated and reapplied to the same column. (C) Purification of monoclonal antibody anti-CPA mAb14 from ascites fluid; 0.5 ml of ascites fluid was applied to the column and chromatographed under the same conditions as described for A. (D) Rechromatography of peak II in C as described for B. Arrows mark the application of ammonium acetate buffer (pH 10). The bars indicate 10 min.

column and batch operations may result from diffusion barriers which occur in the column. This phenomenon is still under investigation. Recently, we have developed binding methods that led to increased antigen-binding activity of the immobilized antibodies. These include coupling of oxidized antibodies (by sodium periodate or enzymatically) to adipic dihydrazide-modified Eupergit C[6,13,14] and reversible protection of the amino groups of the antibodies by dimethylmaleic anhydride prior to coupling to the matrix[15].

By immobilization of properly selected antibodies we were able to purify from decidua tissue two specific proteins named hDP55[16] and hDP71[17]. The last protein

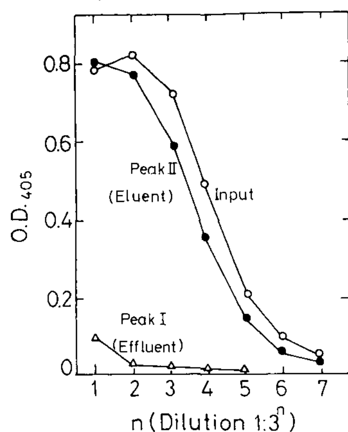

Fig. 3. Ag-Binding activity of antibodies purified by immunoaffinity chromatography. ELISA test for anti-CPA antibodies in the fraction loaded into the column (Input) (○), peak I (△) and peak II (●) (see Fig. 2 A). CPA was adsorbed on the wells of a polystyrene microtitre plate. Serial dilutions of the solutions to be tested were added and the amount of antibody bound was determined by reaction with alkaline phosphatase-labelled goat anti-rabbit IgG.

was found also to be present in large amounts in seminal plasma. Using immunoaffinity purification with C30N-immobilized monoclonal antibody (DEC21) we purified this protein to homogeneity from seminal plasma in one immunopurification step (Fig. 5).

This antigen was also used to test the durability of our HPLC-based immunopurification system. Samples of cell-free seminal plasma (0.01–0.5 ml) were applied to

TABLE I

BINDING OF ANTIBODIES TO STANDARD EUPERGIT C

Antibody[a]	*Anti-CPA*[b]			*CPA*[c]			*[CPA]/[Ig] (mol/mol)*
	Input (mg/g)	*Bound (mg/g)*	*%*	*Input (mg/g)*	*Bound (mg/g)*	*%*	
mAb1	0.7	0.7	100	0.5	0.10	20	0.60
	7.7	5.0	65	2.5	0.15	6	0.15
mAb9	1.5	1.4	93	0.5	0.23	46	0.69
	15.7	8.0	51	2.5	0.80	32	0.50
mAb14	1.4	1.4	100	0.5	0.25	50	0.75
	14.2	9.3	65	2.5	0.45	18	0.24
Polyclonal	5.0	4.5	90	1.0	0.60	60	0.55

[a] Names of antibodies are abbreviated; mAb1 is anti-CPA mAb1, etc.

[b] Antibodies were immobilized onto 100-mg fractions of Eupergit C as described under Experimental. Input and bound protein were determined by the Bradford reaction.

[c] The amount of bound antigen was determined by enzymatic assay of the antigen bound to the matrix-conjugated antibody as described under Experimental.

TABLE II

CHEMICAL AND IMMUNOLOGICAL PROPERTIES OF VARIOUS EUPERGIT C BEADS AND MATRIX-CONJUGATED ANTIBODIES

Eupergit C	*Size (μm)*	*Porosity*	*Oxirane content (mmol/g)*	*Protein-binding capacity[a] (mg/g)*	*Antigen-binding capacity[b] (mol/mol)*
Standard	150	Porous	> 1000[c]	> 100	0.75 (1.4) 0.24 (14.2)
C30N	30	Porous	> 1000[c]	> 100	0.8 (1.2) 0.4 (11.5)
C1Z	1	Non-porous	20[d]	6.5	0.95 (2.0)

[a] See legend to Fig. 1 for details of analysis of protein binding.

[b] Anti-CPA mAb14 was immobilized onto 100-mg fractions of Eupergit C as described under Experimental. Bound protein (numbers in parentheses, mg/g matrix) was determined by a Bradford assay of the supernatants before and after the coupling reaction. The amount of bound antigen was determined by enzymic assay of CPA as described under Experimental.

[c] Determined by reaction with thiosulphate[12]. See also ref. 8.

[d] Determined by binding of ^{35}S-labelled cysteine.

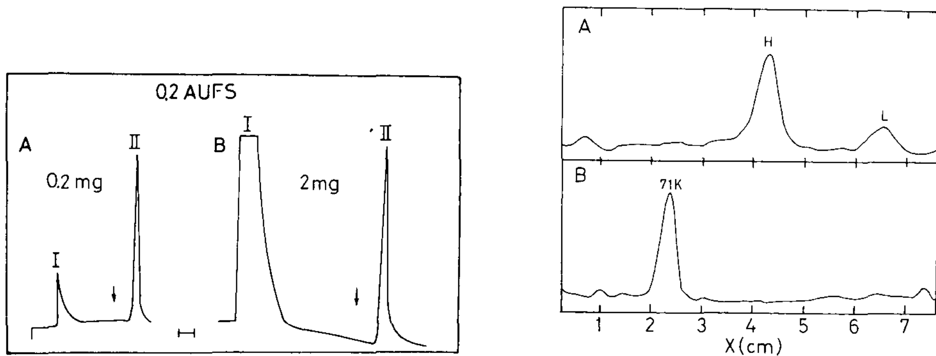

Fig. 4. Purification of CPA by Eupergit C C30N-conjugated anti-CPA mAb9. (A) 0.2 mg of enzyme was applied to a column (60 × 8 mm I.D.) packed with 0.8 g of matrix containing 4 mg of immobilized antibody. Protein eluted in peak I was found to be devoid of enzymatic activity. See text for experimental details. (B) Effect of overloading of the column by 2 mg of CPA. Peak II was used to calculate the column capacity for binding of CPA. Arrows mark the application of ammonium acetate buffer (pH 10). The bar indicates 5 min.

Fig. 5. SDS-PAGE densitometer patterns of IgG and hDP71 purified in the presence of 1% PEG 400. (A) Rabbit IgG (0.1 mg) was mixed with 0.5 ml of horse serum and purified by C30N-conjugated goat anti-rabbit IgG antibodies (0.5 mg Ab bound to 100 mg of matrix). (A) 100-mg amount of matrix, containing 0.5 mg of antibody, was packed into an HPLC column (60 × 6 mm I.D.) onto which 0.5 ml of rabbit IgG-containing horse serum was applied. After washing the column with PBS, IgG was eluted with ammonium acetate buffer (pH 10) as described in the legend to Fig. 2. (B) Human decidua protein (hDP71) was purified from cell-free seminal plasma by C30N-conjugated mAb DEC21. The beads were packed into an HPLC column as described in A. A 0.2-ml volume of seminal plasma was applied to the column. Elution conditions were as in A. H and L denote the peaks corresponding to the heavy and light chains of IgG. 71K denotes the peak corresponding to hDP71.

the column in PBS, hDP71 was eluted with ammonium acetate buffer (pH 10) and the column was re-equilibrated with PBS. This process was automatically repeated and the peak area of the eluted protein was recorded. A high efficiency of hDP71 purification was maintained for over 200 cycles of operation, lasting altogether over 2 months.

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Enzymic oxidation of monoclonal antibodies by soluble and immobilized bifunctional enzyme complexes

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ABSTRACT

Site-specific modification of monoclonal antibodies was achieved by oxidation of the carbohydrate moieties of antibodies which are located remote from the antigen binding sites. Sialic acid and galactose are terminal sugars of these carbohydrate chains. Concomitant treatment of the antibodies with neuraminidase and galactose oxidase generated aldehyde groups in the oligosaccharide moieties of immunoglobulins which reacted selectively with amino or hydrazide groups of the matrix. Subsequent immobilization of neuraminidase and galactose oxidase on Eupergit C–adipic dihydrazide proved to be an efficient and selective system for the enzymic oxidation of the monoclonal antibodies without impairing their immunological activity. Oriented immobilization of enzymically oxidized monoclonal antibodies on hydrazide or amino Eupergit C derivatives thus leads to the formation of antibody matrix conjugates which possess high antigen-binding activities.

INTRODUCTION

Site-specific modification of antibodies and possible oriented immobilization may be achieved by chemical or enzymic oxidation of the carbohydrate moieties of the antibodies prior to carrier attachment[1–3]. As the carbohydrate residues on the Fc fragment of the antibody molecule are located remote from the binding site of the antibody, immobilization via this region usually does not impair the immunological activity of the antibody. Previous attempts have been described that utilize enzymic oxidation of various glycoproteins for labelling or attachment to liposomal membrane[1,4–6].

The contents and compositions of carbohydrates in immunoglobulin G (IgG) from rabbit, horse, human and bovine serum are very similar. Each molecule of IgG contains 0–1 residues of fucose, 3 of mannose, 1–2 of galactose, 3–4 of N-acetylglucosamine and 0–3 of sialic acid (N-acetylneuraminic acid; NANA)[7]. The sialic acid was shown to be terminal by its release with neuraminidase and by identification as the N-acetyl derivative by gas chromatography. Incubation with β-galac-

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tosidase subsequent to treatment with neuraminidase (NA) liberated galactose, indicating the presence of a sequence of NANA–GAL. In principle, each of these carbohydrate residues may be oxidized by specific enzymes to yield the corresponding aldehyde or keto derivatives.

In this work we used the oxidation of C-6 of galactose by galactose oxidase to form a corresponding C-6 aldehyde on galactose. Galactose oxidase, in combination with neuraminidase, may be used for the modification of galactosyl residues penultimate to sialic acid. The newly formed aldehyde groups may then react with hydrazide groups of the carrier [*e.g.*, Eupergit C–adipic dihydrazide (ADH)] to form stable covalent bonds of the antibody with the matrix. The data presented in this paper show that the co-immobilization of neuraminidase and galactose oxidase on Eupergit C–ADH beads provides an economical, efficient and selective system for the enzymic oxidation of monoclonal antibodies without impairing the immunological activity of the antibodies.

EXPERIMENTAL

Enzymes

Carboxypeptidase A (CPA) and horseradish peroxidase (HRP) were purchased from Sigma (St. Louis, MO, U.S.A.). Determination of the enzymatic activity of CPA and HRP were described previously[3].

Galactose oxidase (GO), Cat. No. G-3385, purchased from Sigma, was assayed according to the manufacturer's instructions. One unit of enzyme activity is defined the amount which produces an increase of 1.0 unit of absorbance at 425 nm in 1 min under the experimental conditions specified. Neuraminidase from *Clostridium perfringens*, Cat. No. 107590 (free from protease) was purchased from Boehringer (Mannheim F.R.G.). The enzymic activity of NA was determined according to Potier[8].

Preparation and purification of monoclonal antibodies

The monoclonal antibodies (mAbs) anti-CPA mAb100 and anti-HRP mAb2 were purified and characterized as described previously[3,9]

Enzyme-linked immunosorbent assay (ELISA)

ELISA plates were coated with intact or oxidized mAbs (10 μg/ml, 100 μl, 1 h at 37°C) and blocked by 1% fat milk for a further 1 h. HRP was added at various concentrations to the wells and incubated for a further 1 h at 37°C. The enzymic activity of antibody-bound HRP was determined as described previously[3].

Preparation of Eupergit C–ADH

Eupergit C beads (100 mg) were treated with ADH (Sigma) for 16 h at room temperature in 0.2 *M* sodium carbonate buffer (pH 9.0). The amount of ADH added to the carrier varied between 0.01 *M* (Eupergit C–$ADH_{0.01}$) and 0.1 *M* (Eupergit C–$ADH_{0.1}$). For immobilization of minute amounts of neuraminidase and galactose oxidase, 0.01 *M* ADH proved to be sufficient. The modified carrier was thoroughly washed with phosphate-buffered saline (pH 7.4) (PBS) and excess of oxirane groups of the Eupergit C were blocked with 0.2 *M* β-mercaptoethanol for 2 days at

room temperature. After thorough washing with 0.1 *M* acetate buffer (pH 5.5), the modified beads were stored at 4°C for up to 60 days for further use. With longer storage periods new oxirane groups are exposed to the solution and/or blocked β-mercaptoethanol groups are hydrolysed and additional blocking is required. The amount of ADH groups introduced onto the matrix was determined by reaction with trinitrobenzenesulphonic acid (TNBS)[10,11].

Chemical oxidation of the monoclonal antibodies

The chemical oxidation and oriented immobilization of anti-CPA mAb100 and anti-HRP mAb2 were carried out with sodium periodate in a one-step oxidation procedure as described previously[12]. A similar reaction in solution was carried out at 0°C in the dark using 10 m*M* sodium periodate. The reaction was stopped after 1 h by addition of 10 m*M* sodium thiosulphate.

Antigen-binding activity of native and oxidized antibodies

In order to determine the antigen-binding activity of native and oxidized antibodies, they were conjugated to intact Eupergit C or Eupergit C–ADH, respectively, as described previously[3,13]. The matrix–antibody conjugates (5 mg containing 10 μg of immobilized antibody) were incubated with a 5-fold molar excess of the respective antigen (HRP or CPA). After thorough washing of the beads with PBS, the respective enzymic activity was determined as described previously[3].

The antigen-binding activity of anti-HRP antibodies was also determined using an ELISA test: anti-HRP mAb2, chemically or enzymatically oxidized (100 μl, 10 μg/ml) was coated on a ELISA plate for 1 h at 37°C and blocked with milk for a further 1 h. HRP was added to the wells at various concentrations and incubated for a further 1 h at 37°C. In control experiments, unmodified antibody was assayed under the same conditions. The enzymic activity of antibody-bound HRP was determined as described previously[3].

Determination of aldehyde groups on the antibody molecule

Aldehyde groups on oxidized antibodies were determined using the avidin–biocytin–hydrazide system followed by a modified dot blotting method. A 3-μl volume of oxidized antibody was applied to nitrocellulose discs, dried and incubated with 10 μg/ml of biocytin–hydrazide for 1 h. After repeated washings with PBS and blocking with bovine serum albumin (BSA) for 1 h, avidin–alkaline phosphatase conjugate was added and the mixture incubated for 30 min at 37°C After repeated washings the amount of alkaline phosphatase bound to the blot was determined by hydrolysis of the soluble substrate *p*-nitrophenyl phosphate. The amount of alkaline phosphatase bound was directly proportional to the amounts of aldehyde groups on the oxidized antibodies. A calibration curve for lactoperoxidase–biotin containing a known number of biotin molecules per molecule of protein was used.

Enzymic oxidation of monoclonal antibodies by soluble neuraminidase and galactose oxidase

Anti-CPA mAb100 (10 μg in 0.1 ml of 0.1 *M* acetate buffer, pH 5.0) was incubated with neuraminidase containing 0.012 units (0.1 ml) at 37°C for 20 h with gentle shaking. After dialysis for 16 h against 0.1 *M* phosphate buffer (pH 6.0), the

mAb was treated with galactose oxidase containing 2.5 units (25 μl) for 17 h under the same conditions as for the neuraminidase treatment. Aliquots (25 μl) were withdrawn at various time intervals and newly formed aldehyde groups on the antibody molecule were determined as described above. Other aliquots were withdrawn and conjugated to Eupergit C–ADH in order to analyse for antigen-binding activity as described above.

Immobilization of neuraminidase and galactose oxidase on Eupergit C–ADH

Neuraminidase and galactose oxidase in 0.1 *M* acetate buffer (pH 5.5) were oxidized with 10 m*M* sodium periodate and immobilized onto Eupergit C–$ADH_{0.01}$ using a one-step oxidation procedure[12]. Three different preparations of co-immobilized neuraminidase and galactose oxidase were prepared by the reaction of 25 mg of the matrix with a mixture of enzymes containing total protein of 0.05, 0.1 and 0.5 mg in a 1:4 ratio (in units of enzymic activity) of galactose oxidase to neuraminidase. After coupling of the enzymes to the carrier, the preparations were treated with 0.2 *M* acetaldehyde in 0.1 *M* acetate buffer (pH 5.5) for 2 days to block the residual reactive hydrazide groups. The amount of enzymes immobilized on the carrier was determined by measuring the respective enzymic activities in the reaction mixture supernatants before and after the reaction.

In parallel experiments, galactose oxidase was immobilized separately by binding directly to Eupergit C or after oxidation to Eupergit C–$ADH_{0.01}$ as described above. Soluble neuraminidase was added to the reaction mixture. The enzymes were tested for their ability to oxidize the antibodies anti-CPA mAb100 and anti-HRP mAb2.

Enzymic oxidation of antibodies by co-immobilized enzymes

In order to determine the capability of the three Eupergit C–$ADH_{0.01}$-conjugated enzyme preparations to oxidize antibiodies, anti-CPA mAb100 (20 μg) was incubated with each of these preparations for various periods of time. The degree of oxidation of the antibodies was estimated by their immobilization by binding onto Eupergit C–$ADH_{0.1}$ beads as described above. In addition, a method for a concomitant determination of aldehyde groups formed on the antibody molecules and the immunological activity of these antibodies was developed: 25 mg of Eupergit C–$ADH_{0.01}$ containing 0.5 mg of immobilized enzymes were incubated with 20 μg of mAb100 and 20 μg of biocytin hydrazide. The supernatant containing the hydrazide derivative of mAb100 was bound to Eupergit C-conjugated streptavidin via the biocytin moiety. The amount of bound mAb directly proportional to number of aldehyde groups induced in the antibody molecule by enzymic oxidation. The enzymic activity of CPA immunologically bound to the immobilized mAb was determined as described previously[3].

Operational stability

The above three preparations were incubated repeatedly with new amounts of mAb for various periods of time. The operational stability was determined during 60 days.

RESULTS

Enzymic oxidation of monoclonal antibodies by a soluble bifunctional enzyme system

The applicability of soluble GO and NA to the enzymic oxidation of antibodies was investigated using soluble enzymes. The formation of aldehyde groups on the antibody molecules, the binding of oxidized antibodies onto the Eupergit C–ADH matrix and the antigen-binding activity of the immobilized antibodies were studied and optimized. For this purpose we developed an assay based on the reaction of biocytin–hydrazide with the aldehyde groups formed on the antibody molecules. The antibody-bound biocytin was then reacted with streptavidin-conjugated alkaline phosphatase. The activity of alkaline phosphatase was directly proportional to the number of aldehyde groups formed on the antibody molecules. By using of a calibration curve for a lactoperoxidase–biotin complex possessing a predetermined number of biotin residues per lactoperoxidase molecule, the number of aldehyde groups on the antibody molecule was determined. By this assay it was found that the oxidation by soluble enzymes produced a maximum of two aldehyde groups per mole of antibody, compared with 3.2–4 aldehyde groups obtained after periodate oxidation.

The antigen-binding activity of anti-HRP mAb2 was determined by an ELISA test. Enzymically and chemically oxidized antibodies and intact antibodies were adsorbed on a polystyrene plate and their binding activities for HRP were assayed. As show in Fig. 1, a substantial increase in immunological activity of mAb HRP2 occurred after enzymic oxidation, corresponding to 1.9 mol of HRP bound per mole of antibody, compared with 0.8 and 1.4 mol/mol obtained with the chemically oxidized and intact antibody, respectively.

As the enzymically oxidized antibody fully retained its antigen-binding capacity, we used the enzymic activity of the antigen (HRP) immunologically bound to the antibody to monitor the kinetics of the coupling of oxidized antibody to Eupergit C–ADH$_{0.1}$. As shown in Fig. 2, the enzymic activity of HRP bound to oxidized antibodies immobilized on Eupergit C–ADH is dependent on oxidation time. Treatment with NA increased the amount of antibody bound to the matrix, apparently owing to the release of sialic acid leading to exposure of galactose to oxidation by GO.

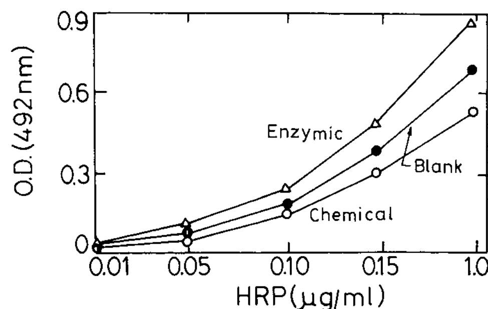

Fig. 1. Determination of immunological activity of anti-HRP mAb2 after (△) enzymic and (○) chemical oxidation and (●) of unmodified antibody measured by an ELISA assay.

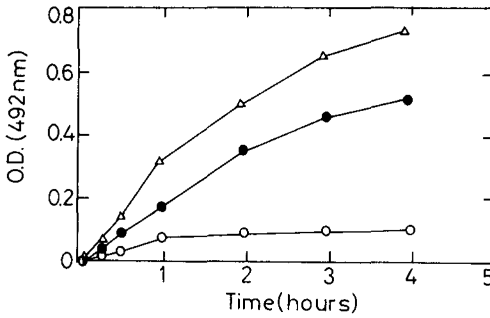

Fig. 2. Kinetics of enzymic oxidation of anti-HRP mAb2 with (△) neuraminidase–galactose oxidase or (●) galactose oxidase only. The oxidized antibodies and (○) unmodified antibody were immobilized on Eupergit C–$ADH_{0.1}$ and the enzymic of immunologically bound HRP was determined.

Enzymic oxidation of monoclonal antibodies by immobilized bifunctional enzyme complex

Neuraminidase and galactose oxidase (glycoproteins) were oxidized by reaction with sodium periodate using a one-step procedure[12]. Binding of the enzyme to Eupergit C–ADH was optimized by testing several preparations of Eupergit C–ADH, obtained by treatment of Eupergit C with various amounts of ADH and various concentrations of blocking reagent (β-mercaptoethanol). The enzymic activity of the co-immobilized NA and GO was measured by determination of newly formed aldehyde groups on the antibody molecule. As shown in Fig. 3, maximum activity of immobilized enzymes (2 mol of aldehyde per mole of anti-CPA mAb100) was obtained when 0.5 mg of the two enzymes (a ratio of 1:4 units of the enzymic activity of NA to GO) was co-immobilized onto Eupergit C–ADH prepared with 0.01 *M* ADH and blocked with 0.2 *M* β-mercaptoethanol for 16 h.

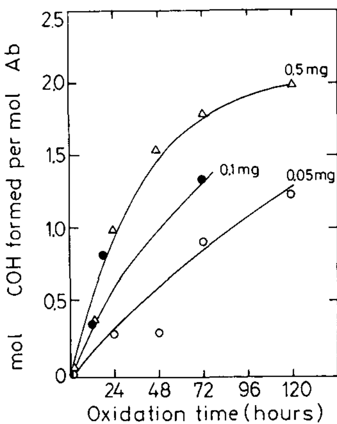

Fig. 3. Determination of aldehyde groups formed on anti-CPA mAb100 using the immobilized bifunctional enzymic system. Different amounts of immobilized enzymes (○, 0.05 mg; ●, 0.1 mg; △, 0.5 mg) were incubated with 10 μg of antibody. The enzymic activity of alkaline phosphatase–avidin measured as the increase in absorbance at 405 nm was proportional to the number of aldehyde groups formed.

TABLE I

ENZYMIC OXIDATION OF mAbs BY A HETEROGENEOUS COMPLEX OF NEURAMINIDASE AND GALACTOSE OXIDASE

Oxidation system	*Enzymic activity of AP–avidin*[a]	
	4 h[b]	*24 h*[b]
GO + NA	0.17	0.17
EC–GO + NA	0.02	0.07
EC–ADH–GO + NA	0.14	0.18
EC–ADH–GO–NA	ND[c]	0.15

[a] The values given indicate the absorbance at 405 nm as a measure of the enzymic activity of alkaline phosphatase (AP)

[b] Time of oxidation.

[c] Not determined.

The activity of a mixed preparation in which oxidized GO was immobilized onto Eupergit C–$ADH_{0.01}$ while native NA was added in solution was also determined. As shown in Table I, the mixed preparation was essentially as active as the two enzymes added in solution. In contrast, when native GO was immobilized directly onto Eupergit C its activity was lost. The co-immobilized enzyme preparation retained 80% of the original enzymic activity.

The antigen-binding capacity of oxidized anti-CPA mAb100 obtained using the co-immobilized bifunctional enzyme preparation was the same as the activity obtained with a mixture of the enzymes in solution. The amount of CPA bound to the immobilized antibody, as determined by the enzymic activity of the complex, corresponded to 6 μg of CPA per 20 μg of mAb or a molar ratio of 1.5 mol of antigen bound per mole of antibody.

DISCUSSION

Binding of antibodies on Eupergit C by reaction of their amino moieties with oxirane groups of the matrix may cause partial or complete loss of immunological activity. Site specific modification of antibodies by oxidation of their carbohydrate moieties and their oriented immobilization onto Eupergit C–ADH proved to be a highly efficient alternative procedure. As the sites of attachment of oligosaccharides to antibodies are specific and remote from the antibody-combining site, the selective coupling of newly formed aldehyde groups to hydrazide groups of the matrix often yields conjugates with unimpaired antigen-binding characteristics. Site-specific modification of antibodies by oxidation with sodium periodate as a means of their oriented immobilization onto insoluble matrices is widely used[1–3]. However, chemical modification by the periodate oxidation of vicinal hydroxyl groups of carbohydrate may also modify some amino acid residues, such as serine, threonine, proline and methionine, which, if located in the Fab region, may interfere with the immunological activity of the antibodies.

An enzymic procedure for the generation of aldehyde groups on the oligo-

saccharide moieties of antibodies utilizes the neuraminidase–galactose oxidase system[1,2]. In this procedure, terminal sialic acid residues are first removed by treatment with neuraminidase. Subsequent treatment with galactose oxidase results in the formation of aldehydes on the exposed galactose residues. This method has been used by Wilchek and Bayer[6] for the labelling of cell surface glycoproteins and by Chua *et al.*[5] to generate aldehyde on IgM immunoglobulins prior to coupling to liposomes. We have used soluble and immobilized bifunctional enzyme preparations of neuraminidase and galactose oxidase for the oxidation of affinity-purified anti-CPA mAb100 and anti-HRP mAb2. The course of oxidation was followed by determination of the aldehyde groups formed on the antibody molecules and by determination of the antigen-binding activities of the modified mAbs. Determination of the aldehyde groups in the oxidized antibodies by spectrophotometric methods, using TNBS[10,11] or 3-methyl-2-benzothiazolinone hydrazone hydrochloride[14], was not sensitive enough. Determination of aldehyde groups using the avidin–biocytin–hydrazide system[15,16] was adopted with a modification of the dot blotting method (see Experimental). This method proved to be highly sensitive, allowing the determination of 1–2 aldehyde groups formed on the antibodies after enzymic oxidation.

Enzymic oxidation of monoclonal antibodies is expected to be more specific than chemical oxidation. We indeed found the formation of a smaller number of aldehyde groups on the enzymically than on the chemically oxidized antibodies. Nevertheless, the 2 mol of aldehyde formed per mole of enzymically oxidized antibodies were sufficient to achieve efficient binding of the antibody to the matrix. These antibodies possessed a higher antigen-binding activity than the corresponding chemically oxidized antibodies immobilized on the same matrix. The amount of antigen bound to the corresponding antibody–matrix conjugate was close to the theoretical value of 2 mol of antigen (CPA or HRP) bound per mole of immobilized antibody. The co-immobilization of galactose oxidase and neuraminidase exhibits the well known advantages of immobilized enzymes such as repetitive use of the same enzymes, a good recovery of the antibodies and the possibility of continuous oxidation of mAb.

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Purification of soluble cytokine receptors from normal human urine by ligand-affinity and immunoaffinity chromatography

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ABSTRACT

Affinity chromatography of crude human urinary proteins on either human recombinant interleukin-6 (rIL-6) or human recombinant interferon-γ (rIFN-γ) or anti IFN-γ receptor (IFN-γ-R) monoclonal antibodies (McAb) yielded the two respective soluble receptors in significant amounts. A single sequence of 30 amino acid residues was obtained by N-terminal microsequencing of the protein peak purified in tandem by affinity chromatography on an IL-6 column and reveresed-phase high-performance liquid chromatography. This sequence was identical with the predicted N-terminal sequence of IL-6-R as previously reported. The purified IL-6-R retained its biological activity. It was used for the preparation of specific anti IL-6-R monoclonal antibodies. Analysis of the eluted proteins from both IFN-γ and anti IFN-γ-R columns by inhibition of solid-phase radioimmunoassay, enzyme-linked immunosorbent assay, sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blotting proved the existence of soluble IFN-γ-R in normal urine. This finding together with the already known presence of soluble TNF receptors and a soluble IL-2 receptor found both in plasma and in urine indicates that release of soluble cytokine receptors into body fluids is a general phenomenon which occurs under normal physiological conditions.

INTRODUCTION

Soluble extracellular fragments of receptors may serve as natural blockers or enhancers of their respective hormones or cytokines. Only a limited number of such

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soluble receptor fragments have been reported, including receptors for red blood cells[1] and for the Fc portion of immunoglobulins[2]. Among the cytokine receptors, interleukin-2 receptor was the only one reported to be released in a soluble form from activated human lymphoid cells[3]. Moreover, it was detected in body fluids of normal individuals and its level was increased in disease states[4]. To address the question of whether release of soluble cytokine receptors into body fluids is a general phenomenon, we analysed urine from normal donors for the presence of additional cytokine receptors. Affinity chromatography of crude human urinary proteins using either the corresponding ligand [human recombinant interleukin-6 (rIL-6) or recombinant interferon-γ (rIFN-γ)] or the corresponding monoclonal antibodies [anti IFN-γ receptor (IFN-γ-R)] yielded the respective two soluble receptors in significant amounts. It is therefore sugested that shedding of various cytokine receptors is a general phenomenon which occurs under normal physiological conditions.

EXPERIMENTAL

Cytokines

rIL-6 and rIFN-γ (InterPharm Labs., Ness-Ziona, Israel) were purified to homogeneity on an anti IL-6 monoclonal antibody (McAb) column[5] and an IFN-γ McAb column[6], respectively. Iodination of cytokines or receptors was performed by the chloramine-T method to a specific activity of $2.2 \cdot 10^7$ cpm/μg for rIL-6, $2.5 \cdot 10^6$ cpm/μg for rIFN-γ and $5 \cdot 10^7$ cpm/μg for IL-6-R.

Antibodies

McAb No. 34-1[5] was used for affinity purification of IL-6; McAb No. 3-3[7] was used both for affinity purification of rIFN-γ and for coating microtitre plates in a solid-phase radioimmunoassay (sRIA); McAb No. 177-1[8] was used both for immunoaffinity purification of the IFN-γ-R from crude urine and for coating microtitre plates in a double antibody enzyme-linked immunosorbent assay (ELISA). Rabbit anti IFN-γ-R polyclonal antibodies were obtained by immunization with a ligand affinity-purified IFN-γ-R from human placenta.

Immunoaffinity chromatography

Agarose–polyacrylhydrazide beads[9] (BioMakor, Ness-Ziona, Israel) were used in order to couple all the monoclonal antibodies used in immunoaffinity chromatography.

Ligand affinity chromatography of urine

Each of the cytokines (2.5 mg) was coupled to Affigel-10 (1 ml) (Bio-Rad Labs. Richmond, CA, U.S.A.). Concentrated crude or partially purified urinary proteins[10] were placed on each of the columns. Following washing with phosphate-buffered saline (PBS), bound proteins were eluted with citric acid (25 mM, pH 2.5) and immediately neutralized.

Reversed-phase high-performance liquid chromatography (RP-HPLC)

Eluted fractions from the immobilized IL-6 column were further resolved by RP-HPLC on an Aquapore RP-300, column (30 × 4.6 mm I.D.) (Brownlee Labs.,

Santa Clara, CA, U.S.A.) using an acetonitrile gradient in 0.3% aqueous trifluoroacetic acid (TFA). Fractions of 0.5 ml were collected.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting

Proteins resolved by SDS-PAGE (12%) under non-reducing conditions[11] were rendered visible either by silver staining[12] or electroblotting on nitrocellulose sheets as described previously[8]. Electroblotted proteins were reacted either with [^{125}I]rIL-6 ($1 \cdot 10^6$ cpm/ml) or with McAb No. 177-1 (1:500), followed by [^{125}I]goat anti mouse antibodies ($0.7 \cdot 10^6$ cpm/ml). The nitrocellulose sheets were then washed, dried and autoradiographed.

Hybridoma growth factor (HGF) activity assay[13]

The bioactivity of the IL-6 was measured by stimulation of [^{3}H]thymidine. The assay was performed in the presence or absence of a soluble preparation of IL-6-R. One unit of IL-6 is defined as the amount of protein that gives 50% of the maximum effect in the assay.

Protein determination

Protein concentrations were determined by the fluorescamine method[14].

N-Terminal sequence analysis

The HPLC-purified protein was subjected to N-terminal sequence analysis on a pulsed liquid–gas-phase protein microsequencer (Model 475A; Applied Biosystems; Foster City, CA, U.S.A.).

RESULTS

Affinity chromatography on an immobilized rIL-6 column was used as the main step of IL-6 receptor (IL-6-R) purification. A concentrate of crude urinary proteins (1.5 g of protein in 100 ml obtained from 40 l of urine) was placed on the IL-6 column and bound proteins (50 μg) were eluted by low pH. Further purification was achieved by RP-HPLC which yielded a major protein peak eluting at 39% acetonitrile (14 μg)[15]. On scale-up of these procedures, 110 μg of pure protein were obtained from CM-Sepharose-purified urinary proteins[10], starting with 900 l of urine. Analysis by SDS-PAGE of the protein peak from the RP-HPLC column gave a single broad band with an apparent molecular weight of 50 000 under non-reducing conditions. Following electroblotting of the partially purified protein sample from the affinity column eluate, only the mol.wt. 50 000 band reacted specifically with [^{125}I]IL-6 (Fig. 1). The protein peak from the RP-HPLC was further characterized by N-terminal microsequencing and a single sequence of 30 amino acid residues was obtained (single-letter code): LAPRR(C)PAQEVARGVLTSLPGDSVTLT(C)PG. This sequence was identical with the predicted N-terminal sequence of IL-6-R reported previously[16]. The apparent molecular weight of this fragment may correspond to the extracellular portion of mature IL-6-R.

The ligand affinity-purified IL-6-R retained its biological activity: it enhanced the HGF activity of IL-6 on mouse plasmacytoma cells in a dose-dependent manner

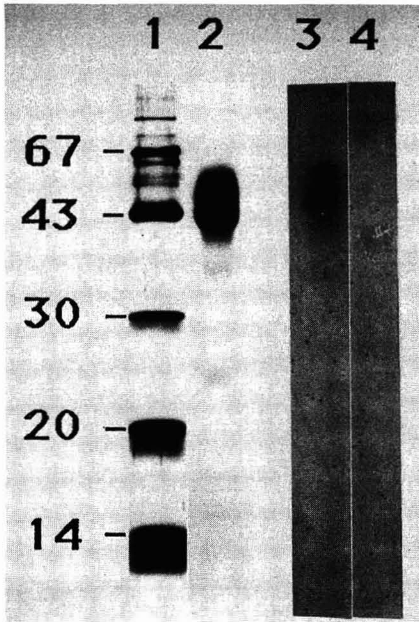

Fig. 1. Analysis of purified IL-6-R by SDS-PAGE and by [^{125}I]rIL-6 binding. Silver-stained SDS-PAGE: lane 1 = molecular weight (kilodalton) markers; lane 2 = aliquot (850 ng) of HPLC peak fraction (39% acetonitrile)[15]. Autoradiograph of [^{125}I]rIL-6 bound to electroblotted proteins: lane 3 = ligand (IL-6)–affinity-purified urinary proteins (LAPUP, 1.8 μg); lane 4 = L(IFN-γ)–APUP (1.5 μg, negative control).

(Fig. 2) while the IL-6 alone at the dose used (0.5 unit/ml) did not stimulate HGF activity at all. Taga *et al.* [17] recently reported that the soluble part of the rIL-6-R increased the growth-inhibitory effect of human IL-6 on mouse M1 cells. Moreover, our soluble IL-6-R strongly increased the anti-growth effect of IL-6 on human breast ductal carcinoma cells T47[18,19].

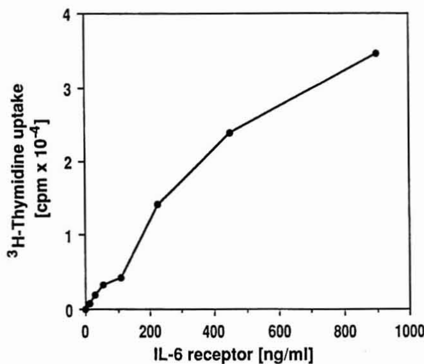

Fig. 2. Enhancement of IL-6 HGF activity by L(IL-6)–APUP on T1165 mouse plasmacytoma cells. Background [^{3}H]thymidine uptake (5000 cpm) of the cells was subtracted from all the readings.

TABLE I

SCREENING OF HYBRIDOMAS BY AN INVERTED sRIA

Hybridoma No.	*cpm*	*Ig class*	*Hybridoma No.*	*cpm*	*Ig class*
4.4	20455	IgGl	36	1455	IgM
5	1085	IgM	37	9640	IgG1
17.1	36565	IgG2a	38.4	35975	IgG1
20.2	31450	IgG1	39.1	5195	IgG2
22	11465	IgG2	40	1415	IgG1
24.2	8850	IgG1	41	1870	IgG1
25	2000	IgG2a	42.5	33565	IgG1
28.7	1645	IgG1	43	1255	IgG1
29	4165	Not done	46	6090	Not done
30.8	1755	IgM	48	18000	IgG1
31	3060	Not done	49	8000	IgM
32.5	31465	IgG1	50.3	28440	IgG1
33.2	14875	IgG1	51	1075	IgG1
34.1	33480	IgG1	52	3945	IgM
35.2	35495	IgG3	53.4	3440	IgG1

The pure soluble IL-6-R preparation was used in order to prepare monoclonal antibodies. Fusion of NSO cells with spleen cells from a Balb/c mouse that received five injections (2.5 μg per injection) was performed. An inverted solid-phase RIA was used for screening. Briefly, microtitre plates were coated with pure goat anti-mouse antibodies and washed and incubated with the hybridoma supernatants. The specific antibodies were detected by a pure preparation of an iodinated soluble IL-6-R. Thirty anti IL-6-R antibodies were obtained out of 800 hybridomas screened (Table I). Some of the antibodies were found to be suitable for detection of IL-6-R in Western blots. They are now being characterized for neutralization of the biological activity of IL-6 and for immunoaffinity purification of IL-6-R from different sources.

In parallel we searched for the possible existence of soluble IFN-γ-R or other IFN-γ binding proteins. Two approaches were attempted. In one ligand affinity chromatography and in the other immunoaffinity chromatography was used. Application of crude proteins from 125 l of urine on the rIFN-γ column[20] and elution at low pH yielded 37 μg of IFN-γ binding proteins. Application of crude proteins from 100 l of urine on an immobilized anti IFN-γ-R McAb No. 177-1[8] and elution at low pH yielded 70 μg of proteins. Analysis of the eluted proteins from both columns by SDS-PAGE, under non-reducing conditions followed by silver staining revealed a similar pattern of protein bands (Fig. 3). Following electroblotting onto nitrocellulose membrane, only the mol. wt. 40000 protein in both preparations reacted specifically with the anti IFN-γ-R McAb (Fig. 3). The same band also reacted specifically with [^{125}I]IFN-γ but the signal was faint (data not shown). From the results obtained it was concluded that the protein showing a molecular weight of 40000 is the ligand-binding domain of the IFN-γ receptor. Further confirmation of the identity of the IFN-γ binding protein as IFN-γ receptor was obtained by two additonal tests. The eluate from the rIFN-γ column inhibited in a dose-dependent manner the binding of [^{125}I]IFN-γ to anti IFN-γ McAb in an sRIA[15], and the same eluate gave a specific signal in a double antibody ELISA based on monoclonal and polyclonal anti IFN-γ-R antibodies[15].

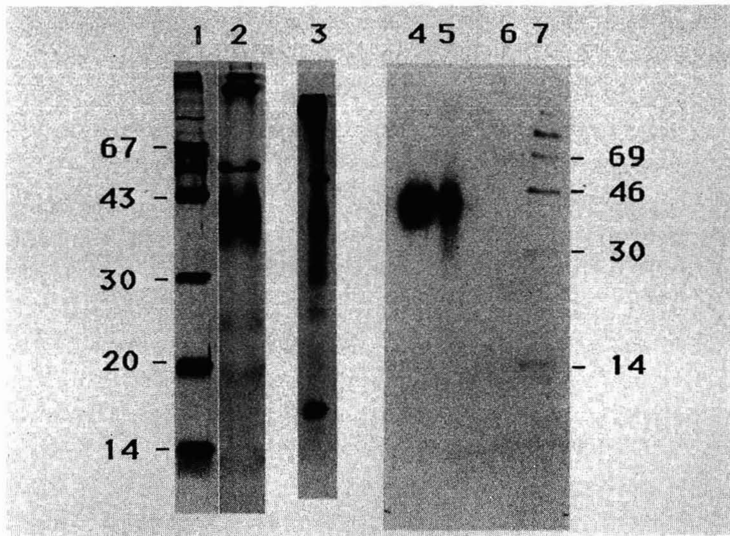

Fig. 3. Analysis of IFN-γ binding proteins by SDS-PAGE and by Western blotting. Silver-stained SDS-PAGE: lane 1 = molecular weight (kilodalton) markers; lane 2 = L(IFN-γ)–APUP (300 ng); lane 3 = immunoaffinity (anti IFN-γ-R McAb)-purified urinary proteins (IAPUP, 250 ng). Autoradiograph of a Western blot performed with anti IFN-γ-R McAb: lane 4 = L(IFN-γ)–APUP (600 ng); lane 5 = IAPUP (900 ng); lane 6 = L(IL-6)–APUP (1200 ng, negative control); lane 7 = ^{14}C molecular weight (kilodalton) markers.

DISCUSSION

This study has demonstrated the convenience and success of ligand and immunoaffinity chromatography in the purification of proteins that are present in trace amounts in very crude protein mixtures. It also proves the existence of specific receptors for the IL-6 and IFN-γ cytokines in normal human urine. Moreover, the purified IL-6-R retained its biological activity; it enhanced the growth-stimulatory action of subminimal doses of IL-6 on mouse cells and also increased the growth-inhibitory effect of IL-6 on human breast cancer cells. This finding, together with the already known presence of soluble IL-2-R in both plasma and urine, indicates that release of soluble cytokine receptors into body fluids is a general phenomenon that occurs under normal physiological conditions, and might have significant consequences *in vivo*. We recently found that a urinary protein that inhibits TNF activity[21], shown by us and others to function by binding TNF specifically[10,22], also represents a soluble version of a cell surface TNF receptor[23]. Soluble receptors might be derived either by shedding of cell surface receptors or by a separate biosynthetic pathway starting from alternatively spliced mRNA or even from a distinct gene. However, the protein sequence identity of the soluble part of the IL-6-R described by us and the reported IL-6-R cDNA indicates that both proteins are derived from the same gene. The observed prevalence of soluble cytokine receptors suggests that they may have an immunoregulatory role, either by participation in the process of

eliminating cytokines via the kidney or, if present in the plasma as shown for IL-2-R, by modulating the availability of their corresponding cytokines.

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Immobilization of α-human atrial natriuretic peptide on insoluble supports and affinity purification of specific antibodies from a polyclonal goat anti-α-human atrial natriuretic peptide serum

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ABSTRACT

α-Human atrial natriuretic peptide (α-hANP) was covalently coupled via single attachment onto two different insoluble matrices. Controlled-pore glass–α-hANP matrices were well suited for the purification of monospecific antibodies, whereas Enzacryl AA–α-hANP did not withstand the inevitable chemical and physical stresses during affinity purification.

INTRODUCTION

Human atrial natriuretic peptide (hANP) is one of the recently discovered heat-stable vasoactive peptides. It is produced and processed in the cardiac atrium. The main target organs of the peptide hormone are the kidneys, the adrenal cortex, smooth vascular musculature and several regions of the central nervous system. The fragment of interest is α-hANP (28 amino acids), which is derived from the prohormone γ-hANP (126 amino acids) by carboxyterminal proteolytic cleavage. Several other circulating forms have been described[1–3]. The half-life of α-hANP in circulating blood is about 3–5 min[4]; most of the peptide is removed from the bloodstream by the kidneys and the liver[5]. The standard procedure for the determination of α-hANP in plasma or urine is a radioimmunoassay of an extract of urine or EDTA–plasma obtained by use of Sep-Pak C_{18} cartridges[6–9]. Various cross-reactions with its metabolites or degradation products cannot be excluded[9–14].

The use of affinity chromatography or an adapted version thereof should yield

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antibodies of known specificity, to be tested for their applicability in radioimmunoassays[15].

EXPERIMENTAL

Materials

The following were used: α-hANP (CB-PP051041) and [^{125}I]-α-hANP tracer (AW-A3103) (Biomedica, Vienna, Austria); Enzacryl AA 70871 (00-12506/8) (Koch Light, Hatfield, U.K.); controlled-pore glass (CPG) (44741) (Serva, Heidelberg, F.R.G.); RIAzid radioimmunoassay (Henning, Berlin, F.R.G.); EDTA disodium salt, 99% pure (Sigma, Deisenhofen, F.R.G.); BSA (Sigma, St. Louis, MO, U.S.A.); and powdered charcoal, highest purity (2184), Triton X-100, for scintillation measurement, citric acid monohydrate and disodium hydrogenphosphate (Merck, Darmstadt, F.R.G.).

Coupling procedure

Enzacryl AA. Enzacryl AA was activated with 2 *M* hydrochloric acid for 15 min at 4°C, followed by treatment with a 2% sodium nitrite solution for 20 min at 4°C. After washing with 10 m*M* phosphate buffer (pH 7.0) the activated Enzacryl AA was incubated with α-hANP for 48 h. The immobilizate was washed with 10 m*M* sodium acetate buffer (pH 4) to remove unreacted peptide, treated with 3 *M* sodium acetate buffer (pH 4) in order to destroy any unreacted spacers, washed repeatedly with 10 m*M* sodium acetate buffer (pH 4) and stored at 4°C for use (Fig. 1). This matrix is referred to as Enzacryl AA–α-hANP.

Controlled-pore glass (CPG) beads. The CPG surface[16] was treated as follows: 10 g of CPG were incubated with 3% nitric acid for 12 days at room temperature (after degassing) in order to produce free silanol groups. After two washing steps with distilled water, 250 ml of 10% aqueous 3-aminopropyltriethoxysilane were added to

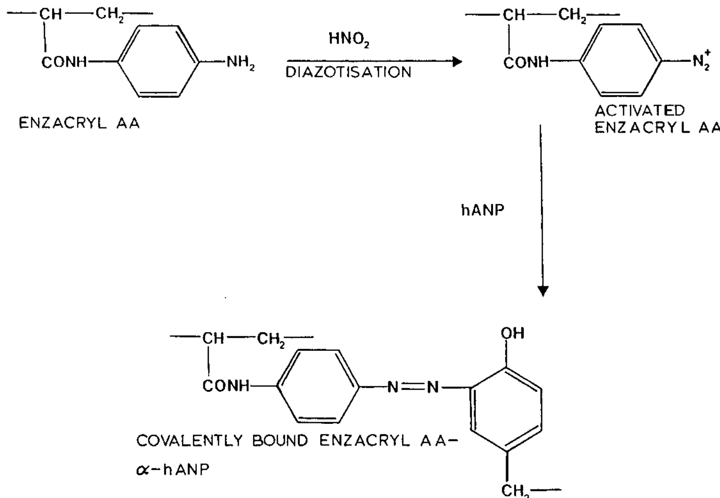

Fig. 1. Diazo coupling of the C-terminal tyrosine of α-hANP to Enzacryl AA.

the support while shaking vigorously. The pH was adjusted to 3.5 using 6 *M* hydrochloric acid and the suspension was evacuated in a water-bath at 75°C for 2 h. The glass beads were filtered off, thoroughly washed with distilled water and dried overnight at 115°C.

The free amino groups introduced by this reaction were used to link glutardialdehyde spacers to the glass beads (Fig. 2). After degassing, the CPG suspension was treated with a 10% glutardialdehyde solution under a fume-hood, rotated at room temperature for 4 h, filtered and washed extensively with ice-cold water. The structure was reduced using aqueous sodium tetrahydroborate (the support is now stable under water at 4°C) prior to diazotisation (Fig. 3) and the peptide hormone covalently bound to the activated support employing reaction conditions analogous to those used with Enzacryl AA. Unreacted sites were saturated by addition of 0.01 *M* hydrochloric acid or a 1:1 mixture of normal goat serum and phosphate-buffered saline (PBS) [without bovine serum albumin (BSA)]. Subsequent treatment was analogous to that for Enzacryl AA.

In addition, a control matrix without α-hANP was prepared. The reaction conditions and post-coupling treatment were identical with those described above with the exception that the buffer solution used for the coupling procedure contained no α-hANP. The control matrix is referred to as CPG and the affinity purification matrix as CPG–α-hANP.

Affinity purification of anti-α-hANP antibodies

Either polyclonal goat anti-α-hANP serum or the antibody fraction thereof

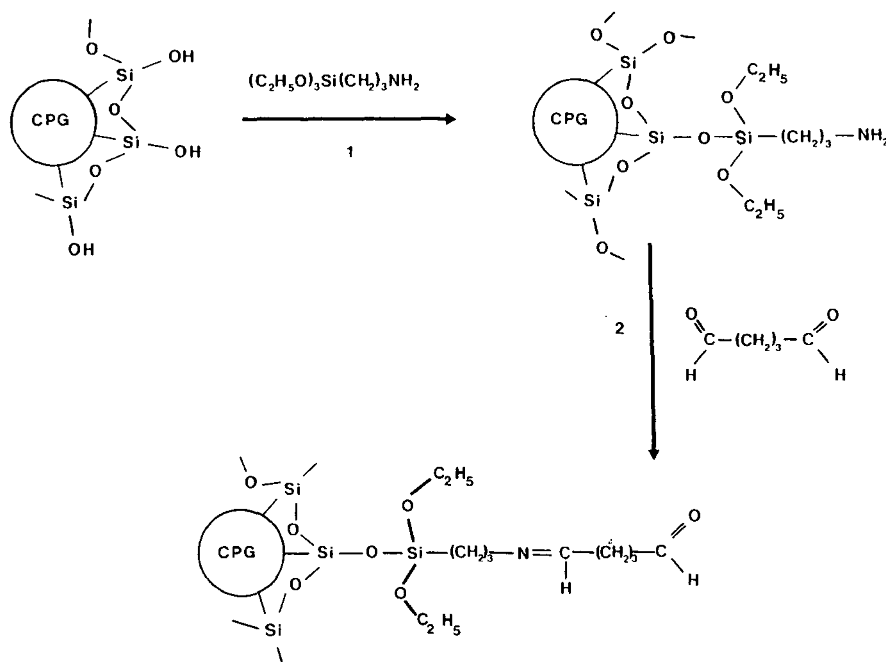

Fig. 2. Derivatization of the active surface silanol groups of CPG using 3-aminopropyltriethoxysilane (1) and the addition of glutardialdehyde to provide a spacer group (2).

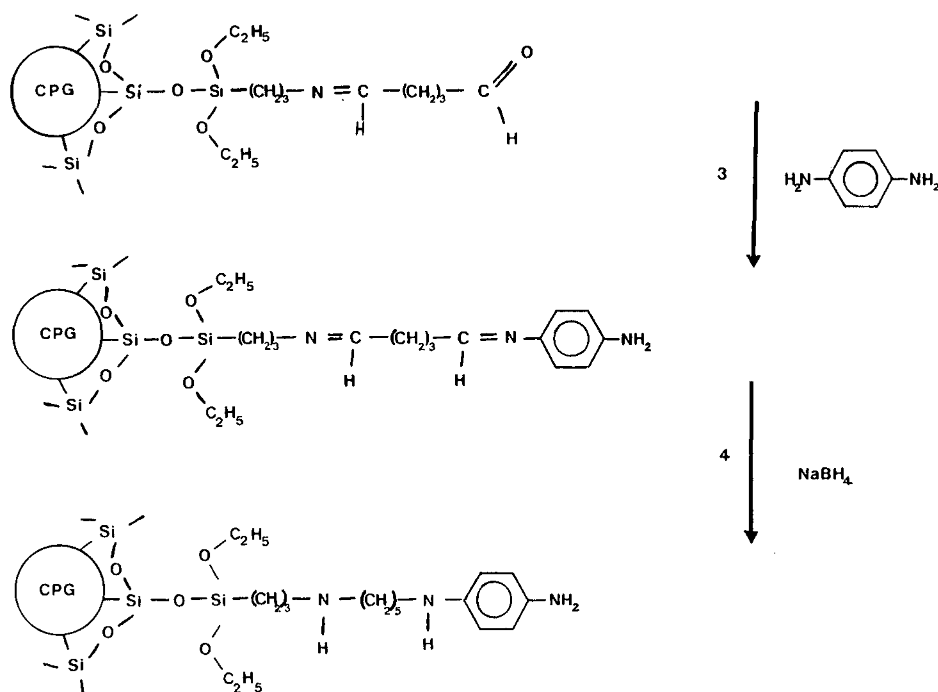

Fig. 3. Processing of the spacer's terminal aldehyde by reaction with 1,4-diaminobenzene (3) and reduction of the carbon–nitrogen double bonds (4), in order to provide a suitably reactive site for the subsequent coupling procedure (Fig. 2).

(obtained by ammonium sulphate precipitation) was used. An adapted version of affinity chromatography was used in this study.

Enzacryl AA–α-hANP. A batch technique was used at 4°C throughout. The antibodies (or antiserum) were applied at pH 7 in Tris–HCl or McIlvaine buffer solutions (McIlvaine-buffer, pH 2.6, 7.0, 7.6: 0.1 *M* citric acid monohydrate–0.2 *M* disodium hydrogenphosphate) and allowed to bind to the matrix. The excess antibodies and other serum components were removed by thorough washing with the loading buffer. Following this, the antibody–immobilized antigen complex was dissociated by use of McIlvaine buffer (pH 2.6). The supernatant was collected in this buffer at pH 7.6; the same buffer was used to neutralize the matrix (Fig. 4).

CPG–α-hANP. Three types of matrices were used: CPG–α-hANP pretreated with dilute hydrochloric acid and the control matrix CPG (see *Coupling procedure*).

The batch technique was carried out at room temperature. Antibodies in PBS (pH 7.4) and also the antiserum were allowed to thaw at 4°C before being applied to the matrix at pH 7. McIlvaine buffer (pH 7) was used to wash out excess antibodies and serum components after the matrix had been allowed to interact with the sample for 1 h. The affinity chromatographic matrix was then washed with PBS (without BSA), in order to remove any unspecifically adsorbed material. McIlvaine buffer (pH 2.6) was used to elute the matrix-bound antibody, which was collected in the same buffer at pH 7.6 (Figs. 5 and 6)[15].

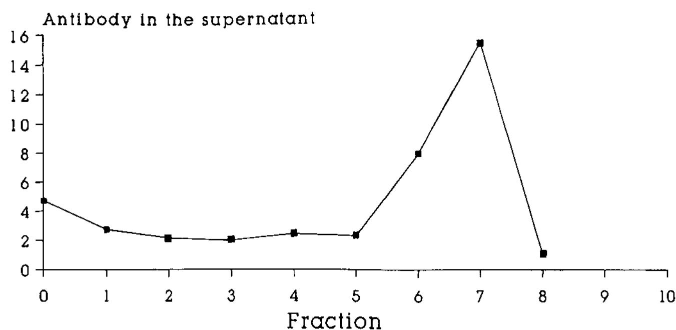

Fig. 4. Batch affinity purification steps employed when using the Enzacryl AA–α-hANP matrices: 0, application of the antibody fraction or antiserum; 1–5, washing with buffer to remove excess of antibodies or other serum components; 6,7, elution of the bound specific antibodies; 8, neutralization of the matrix used.

Treatment of the supernatants obtained by use of the batch technique. The batch technique results in a number of supernatants being collected. An aliquot of each was treated with a constant amount of tracer and subsequently processed as below. A plot of cpm in the supernatant against the fraction number provides a discontinuous elution profile for the affinity purification procedure (see Figs. 4–6).

Determination of antibody titre. A constant amount of [^{125}I]-α-hANP tracer (antigen) was added to a dilution series of the antibody in PBS, mixed well and kept at 4°C overnight (the AB + AG $\rightleftharpoons$ ABAG reaction will have reached equilibrium by then). A 2% (w/v) suspension of charcoal in radioimmunoassay (RIA) buffer [RIA buffer: sodium phosphate buffer (pH 7.4) containing 1 m*M* EDTA (disodium salt), 0.3% BSA and 0.1% Triton X-100] was agitated at 4°C overnight in order to block the macromolecule complex adsorption sites of the charcoal particles. The suspension was centrifuged at 5000 rpm for 15 min, the supernatant discarded and the pellet resuspended in RIA buffer to make a 2% (w/v) suspension. A constant volume of this suspension was used to adsorb the excess of unbound tracer and was centrifuged off as

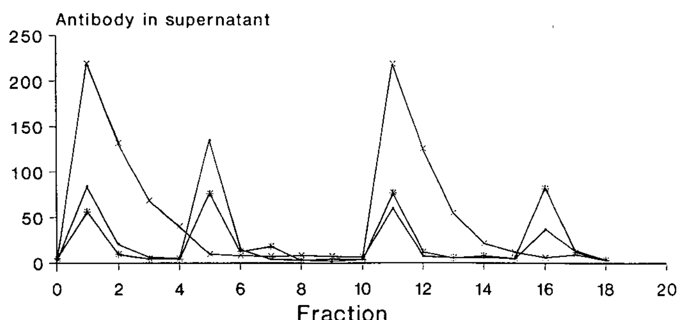

Fig. 5. Batch affinity purification steps employed when using the CPG–α-hANP matrices pretreated with normal goat serum: 1,11, application of the antibody fraction or antiserum; 2–4, 12–15, washing with two buffers to remove excess antibodies or other serum components and any adsorbed material; 5,6, 16,17, elution of the specific anti-α-hANP antibodies bound to the matrix; 7–10,18, neutralization and additional washing of the matrix used. ● = Antibody/CPG–α-hANP; × = antiserum/CPG; ∗ = antiserum/CPG–α-hANP.

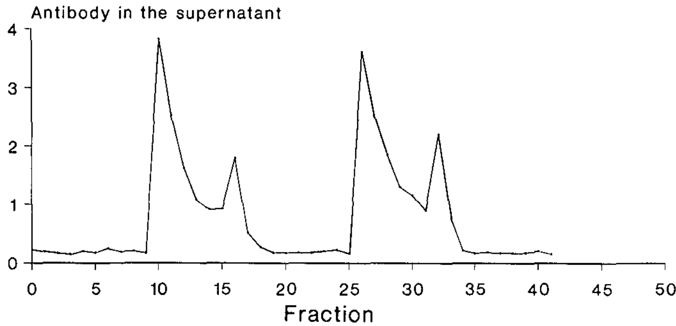

Fig. 6. Batch affinity purification steps employed when using the CPG–α-hANP matrices pretreated with 0.01 *M* HCl: 0–9, 18–25, 34–41, conditioning and neutralization of the matrix before sample application; 10,26, application of the antibody fraction or the antiserum; 11–15, 27–31, washing with buffer in order to remove excess antibodies or other serum components; 16,17, 32,33, elution of the specific anti-α-hANP antibodies bound to the affinity matrix.

above after incubation at 4°C for 30 min. The charcoal-bound excess of tracer in the pellet and the antibody-bound tracer in the supernatant were counted separately in a gamma-counter (Canberra Packard, Model A 5410). The data were plotted as cpm in the supernatant (or pellet) against the dilution (not shown).

RESULTS AND DISCUSSION

An adapted version of affinity chromatography was used throughout this study. Owing to the nature of Enzacryl AA, its use in column form would not have suited our purposes. Chemically modified CPGs exhibit a large available surface area onto which synthetic α-hANP may be immobilized by a stable covalent bond, thus specifically oriented to allow optimum antibody recognition. By use of the batch technique, the small amounts of the prepared affinity chromatographic matrix needed to isolate large amounts of antibodies are more easily handled, with optimum recovery.

The spacer group (aromatic amine) which is already present as an integral part of the Enzacryl AA polymer may be diazotized and coupled to the peptide hormone directly, whereas the CPG beads require treatment in order to be of use. Dilute nitric acid provides the free silanol groups required for the subsequent even coating of the CPG surface by use of 3-aminopropyltetraethoxysilane. This reaction in turn provides the aliphatic amine necessary for the introduction of the spacer, glutardialdehyde. The free aldehyde group obtained reacts with 1,4-diaminobenzene, followed by reduction with sodium tetrahydroborate, thus completing the spacer, while making available the aromatic amine for the peptide coupling procedure (as for Enzacryl AA). The adapted CPGs may be stored indefinitely under water at 4°C before coupling to α-hANP. After removing excess of unreacted peptide, the CPG beads were treated with dilute hydrochloric acid or a 1:1 mixture or normal goat serum and PBS (without BSA) in order to block the residual adsorption sites (mainly unreacted spacers).

To show that the antibodies eluted from the matrix are not simply due to reversible adsorption, a control matrix was prepared by simply neglecting to add any α-hANP to the coupling buffer; all other steps were identical with those for the

production of the CPG–α-hANP matrices. The control matrix was subjected to the same batch procedure as the affinity chromatographic matrices.

As can be seen in Figs. 5 and 6, the serum or antibody fraction applied to the control matrix was washed out quantitatively, with no reversibly adsorbed antibodies being eluted. This implies that the eluate peaks obtained during the batch procedure are due only to specific anti-α-hANP antibodies.

The influence of pH on the loading of the matrix was found to be minimal; the affinity of the antibodies to Enzacryl AA–α-hANP suffices to resist several (up to seven) washes with buffers of different ionic strength at pH 7. Elution of the antibodies was performed at pH 2.6. It is necessary to neutralize the eluted antibodies immediately in order to avoid their denaturation (Fig. 4).

Enzacryl AA–α-hANP was found to be suitable for the storage of the specific anti-α-hANP antibodies at 4°C over a long period of time. This was achieved by simply interrupting the batch procedure before the elution step. These highly purified antibodies would normally be very unstable at such temperatures without a stabilizer, but their biological activity proved to be intact on elution after a 4-month storage period; the antibodies may be eluted as required.

Unfortunately, the Enzacryl AA matrix tended to produce large amounts of fines during the washing and elution procedure, rendering its re-use impossible. Therefore, CPG beads were tested. This immobilizate showed no problems during repeated adsorption and desorption. No decrease in capacity was observed after re-use (Figs. 5 and 6). The antibody titres[15] found were dilutions of 1:4096, 1:8192 and 1:600 for goat anti-α-hANP, antibody fraction of goat anti-α-hANP and antibody eluate sources, respectively.

The prepared CPG–α-hANP matrix was found to be ideal for the isolation of biologically active anti-α-hANP-antibodies. The batch purification was easy to handle, clean and efficient. The matrix should prove suitable for use in a column, *i.e.*, for classical affinity chromatography.

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Review

Biosensors

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1. INTRODUCTION

The last few years have seen unprecedented interest in the development of a newly emerging area of pleuridisciplinary technology, biosensors, lying at the confluence of biotechnology, materials science and electronics[1–4]. Biosensors are analytical devices that respond selectively to analytes in an appropriate sample and convert their concentration into an electrical signal via a combination of a biological recognition system and a physico-chemical transducer. Biosensors promise to provide a powerful and inexpensive alternative to conventional analytical strategies for assaying chemical species in complex matrices; they do this by being able to discriminate the target analyte from a host of inert and potentially interfering species without the requirement for separating and, subsequently, identifying all the constituents of the sample.

The requirement for accurate chemical intelligence is particularly conspicuous in human health care but is becoming increasingly important in veterinary medicine, the agri-food, horticultural, pharmaceutical and petrochemical industries, environmental surveillance, defence and security[3]. For example, it is now generally recognised that inexpensive and reliable sensors for monitoring key metabolites, hormones, drugs, gases or ions in the ward, surgery, home, work place, outpatients department and central laboratory are essential for the delivery of effective patient care. Biosensor technology is eminently suitable for satisfying the needs of "alternate" site diagnosis and is particularly apposite in circumstances where there are advantages in obtaining immediate analytical results; for example, in assessing cancer markers in tissue proximal to an excised tumour within an operating theatre or in assessing the nature of

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the drug in patients suspected of an overdose. In circumstances such as these, the sensor output could be qualitative, whereas for therapeutic drug monitoring, where the "window" between therapeutic and toxic effects could be relatively narrow, the sensor must display high precision even at the expense of longer assay times. These differences in performance criteria for biosensors also extend to other parameters such as price; for example, biosensors designed for monitoring glucose in the home by diabetics will be extremely price-sensitive whilst similar devices for critical care units and industrial bioreactors could be quite price-insensitive. Thus, the features required for particular sensors will depend on the individual application, although in all cases the device should be sufficiently specific, sensitive and reliable to permit analysis of the target species.

2. BIOSENSOR ARCHITECTURES

All biosensors exploit a close harmony between a selective biorecognition system and a transducer which translates a physico-chemical perturbation associated with the biorecognition process into a usable signal[1,2]. Generally speaking, the biorecognition system is typically an enzyme, sequence of enzymes, lectin, antibody, membrane receptor protein, organelle, bacterial, plant or animal cell or whole slice of plant or mammalian tissue. This component of the sensor is responsible for the selective recognition of the analyte, the generation of the physico-chemical signal monitored on the transducer and, ultimately, the sensitivity of the final device[5,6]. Discrimination ratios of 10^7–10^8 or greater may be required for the biology to recognise the target molecule in the presence of a complex matrix of other substances. The action of these "bioreceptors" can be categorised into three principal types: firstly, biocatalytic systems such as enzymes, organelles, whole cells or tissue slices where the selective binding sites "turn over". Such systems are more appropriate for monitoring analytes such as metabolites in the mM–μM concentration range, are reusable and, thus, display a capability for continuous sensing in real time. Secondly, "irreversible" binding systems which exploit antibody, binding protein or receptor systems where interactive sites can become saturated and where such devices are more applicable to "single use" disposable devices. These devices tend to be more applicable to analytes such as hormones, steroids, drugs, microbial toxins, cancer markers and viruses where concentrations lie in the μM–pM range. Finally, amplified systems represent a hybrid configuration between biocatalytic and "irreversible" systems and exploit an antibody, DNA/RNA probe or other appropriate high-affinity binding systems as the initial biorecognition event followed by a suitable amplification, cycling or cascade system linked to an appropriate transducer. Such systems are capable of monitoring analytes in the pM–aM concentration range and lower. Selective biosensors have now been developed in all three categories where recent advances in immobilisation technology have provided improved stabilisation, localisation and activity of the sensing surfaces[7].

3. BIOCATALYTIC SYSTEMS

The majority of successful biosensors exploit enzymes as the biological recognition/response system linkd to transducers capable of responding to protons,

ions, gases, heat, light, mass or electrons generated during the catalytic cycle. Conceptually, the simplest systems catalyse the generation or uptake of protons or other ions that can be coupled to an appropriate potentiometric sensor. In this type of device, local equilibrium is establishd at the sensor surface and leads to the generation of a potential proportional to the logarithm of the analyte activity. The most universal potentiometric biosensors are enzyme electrodes, where an appropriate enzyme is immobilised over an ion-selective electrode[8]. Enzyme electrodes for the estimation of glucose, urea, antibiotics, L-amino acids and a plethora of other substances have now been realised and commercial devices have been in the market place for over a decade. However, more recently, considerable effort has been directed towards the miniaturisation of enzyme electrodes. This has been achieved by exploiting monolithic silicon fabrication technology coupled with appropriate enzyme immobilisation techniques to produce highly selective microsensors[9,10]. Enzyme-sensitised field-effect transistors (ENFETs) for urea, penicillin, glucose, acetylcholine and ATP have all been fabricated from ion-selective field-effect transistors (ISFETs) by combining an enzyme-loaded gel with the ion-selective membrane over the gate region of the field-effect transistor (FET). Considerable interest has been shown in this device technology because of their small size and potentially low production costs. Furthermore, the approach is also amenable to producing monolithic multi-analyte biosensors with photolithographically patterned enzyme-loaded gels for the simultaneous monitoring of K^+, urea and glucose[11–13]. However, despite exciting advances in technology, attempts to commercialise enzyme-modified FET biosensors have been plagued by poor device sensitivity and response times, difficulties in assaying analytes in "real" samples and prohibitively high encapsulation and fabrication costs. Thus, despite some elegant solutions to these problems, many researchers are sceptical as to whether potentiometric ENFETs could ever be exploited without dramatic improvements in the technology.

Current measuring or amperometric devices exploit electron exchange between biocatalytic systems and electrodes and offer a wider scope of applications than potentiometric techniques[14]. They give a current response which is directly proportional to analyte concentration, a normal dynamic range and a normal response to errors in the measurement of current[15]. First-generation amperometric devices monitored oxygen consumption or hydrogen peroxide production associated with the oxidation of substrates by a number of oxidases. Unfortunately, such devices suffer from a dependence on ambient oxygen concentrations and interference by contaminating electroactive species found in crude samples at the high electrode potentials required for electron exchange. Second-generation devices have largely circumvented these problems by substituting an artificial electron mediator for oxygen in order to facilitate electron shuttling between the enzyme and electrode[16]. Ideally, such mediators should participate in enzymatic redox reactions, exhibit rapid electron-exchange rates, be stable and non-toxic, be amenable to immobilisation alongside the enzyme system and display redox potentials sufficiently removed from other electroactive species present in samples to avoid interference. Mediators such as quinones[17], hexacyanoferrate[18], phenazine methosulphate[19], ferrocene[16], tetrathiafulvalene[20] and tetracyanoquinodimethane[21] have all been used to couple the redox enzyme glucose oxidase to suitable electrodes. However, despite the commercialisation of mediated-enzyme sensors, biosensor technology is rapidly moving into the realms of

third-generation devices in which reduced enzymes react "directly" with the electrode itself. For example, conducting organic salts such as N-methylphenazinium tetracyanoquinodimethane (NMP^+TCNQ^-) appear to promote electron exchange with reduced enzymes[15,22]. However, the precise mechanism of electron transfer between the active site of the enzyme and the conducting organic salts is still a matter for conjecture.

More recently, the entrapment of redox enzymes in electrically conducting organic polymers has been suggested as a means of promoting close liaison between the enzyme and the electrode surface[23]. Initial studies have shown that glucose oxidase may be incorporated into polypyrrole[23,24], poly-N-methylpyrrole[25] and polyaniline[26]. This technique of incorporating enzymes into electrodepositable-conducting polymer films also permits the localisation of biologically active molecules on electrodes of any size or geometry[27] and is particularly appropriate for the fabrication of multi-analyte microamperometric biosensors[28]. Recent work in our laboratory with monolithic silicon devices showed the feasibility of constructing miniature multi-analyte enzyme sensors. The microelectronic devices comprised five pairs of serpentined and interdigitated gold electrodes (1000–3500 nm thick) deposited over Ti and Pt metal layers (*ca.* 100 nm thick) on a thermally oxidised silicon "chip" mounted on a ceramic carrier[28]. The microelectrodes were bonded to pads located at the periphery of the wafer and comprised two large electrodes (*ca.* 500 × 500 μm each) bonded in series and used as a counter electrode and three smaller electrodes, each of dimensions 200 × 500 μm. The middle pair of electrodes were converted to an Ag/AgCl reference electrode whilst the outer two electrodes were exploited to deposit electrochemically in polypyrrole, glucose oxidase and galactose oxidase respectively. Glucose and galactose were assayed by monitoring the oxidation of hydrogen peroxide at the respective enzyme microelectrodes held at a fixed potential of +0.7 V (*versus* Ag/AgCl). Fig. 1 shows the calibration curves for glucose and galactose of the microfabricated dual-enzyme biosensor.

A recent extension of this approach has included the co-entrapment of mediators into the polypyrrole films by electrodeposition of polypyrrole copolymers bearing redox mediators with glucose oxidase[29]. This technique of electrodepositing enzymes

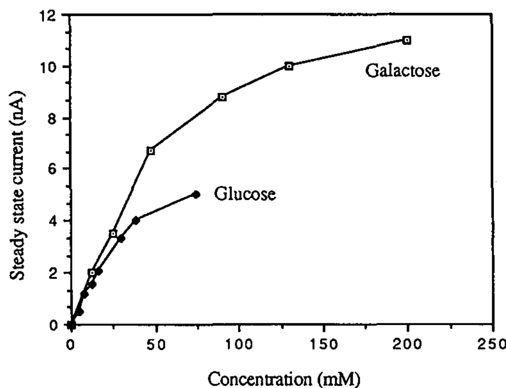

Fig. 1. Steady-state current responses of a polypyrrole-entrapped glucose oxidase–galactose oxidase dual-analyte microamperometric sensor to glucose and galatose.

within polymers modified with redox mediators provides an elegant means of generating reagentless enzyme systems at amperometric electrodes. Other developments include the covalent electro-immobilisation of gluose oxidase in conducting polymers[30] and the claimed "direct" electron transfer between a reduced oxidase and the conducting polymer[31]. Furthermore, covalent attachment of redox mediators such as ferrocene analogues[32] or ruthenium pentammine complexes to glucose oxidase itself has provided electron relays between the active site and the electrode and thus provided a route for direct electron exchange. Finally, covalent attachment of the flavin cofactor of an oxidase to an electronically conducting support via a conducting link has provided an alternative route for electronic communication between the enzyme and the electrode[34].

The technique of enzyme entrapment in conducting polymers has also been exploited for the construction of microconductimetric devices[28]. The development and operation of an inexpensive, rapid and accurate microconductimetric biosensor that exploits the change in conductance by the catalytic action of enzymes immobilised proximal to a planar microelectronoic conductance cell has been described previously[35]. More recently, it has been possible to construct a five-electrode microelectronic device dual-measurement principle device which is capable of detecting glucose amperometrically and urea conductimetrically with both enzymes entrapped in polypyrrole[28].

4. "IRREVERSIBLE" SENSORS

Biocatalytic systems based on enzymes can display poor stability, limited selectivity towards some key analytes and insufficient sensitivity when the analyte is present at very low concentrations. Nevertheless, highly selective and sensitive devices based on immunological recognition systems can be devised to circumvent these shortcomings. The development of "direct" immunosensors, which require only the addition of the analyte to elicit a response, has proved a worthwhile, but challenging, objective. Early attempts at constructing an immunologically sensitised field-effect transistor (IMMUNOFET) have not proven entirely promising despite the use of a number of innovative approaches to membranes[36]. Indeed, it is now generally considered highly unlikely that an immunologically sensitive potentiometric device will ever be constructed in view of the unlikelihood of realising an ideally polarised interface at which measurements could be made.

In principle, direct sensing of antigens by antibodies could be achieved by exploiting sensitive mass to frequency transducers based on piezoelectric materials[37]. For example, a piezoelectric immunosensor has been developed based on ST-cut surface acoustic wave (SAW) quartz crystals comprising interdigitated transducers between which was deposited a goat antibody by covalent immobilisation to the silanized surface. However, despite the elegance of the approach, difficulties associated with damping on immersion in aqueous solutions and with non-specific adsorption and sensitivity were experienced. Fortunately, it is possible to use other acoustic wave modes than the Rayleigh mode operation at 10 MHz used in these early studies which may resolve some of these deficiencies. Thus, exploitation of more sophisticated approaches to SAW technology may breathe a new lease of life into biosensors based on piezoelectric technology for application in aqueous media.

Research into optical techniques for direct immunosensing probably holds the most promise for the future, since refractive index is one of the few physical parameters which varies on formation of immune complexes. Thus, optical techniques such as ellipsometry[38,39], evanescent wave immunoassay[40], dynamic light scattering[41] and surface plasmon resonance[42–44] have all been applied to the detection of immunological reactions. In the latter approach, antibodies are immobilised on thin metal films, usually gold or silver, deposited on the surface of glass prisms[42,43] or diffraction gratings[44]. If light of an appropriate wavelength is directed on the metal–glass interface at an incident angle within certain narrow limits, the delocalised electrons of the metal at the metal–external medium interface are excited into a collective motion, termed a "plasmon". The transfer of energy from the light beam to the surface electrons results in decrease in the intensity of the reflected beam. The angle at which the incident light excites the surface plasmon is extremely sensitive to the refractive index of the medium immediately adjacent to the metal surface and is thus influenced by immune reactions occurring at the surface[44]. Fig. 2 shows a typical plot of normalised reflectivity *versus* the angle of incidence of a helium–neon laser on sequentially binding protein A, anti-lysozyme antibody and hens egg lysozyme to a silver (150 nm) on chromium (3 nm) diffraction grating. At a fixed angle of incidence, immune interactions may be followed in real time directly at the device surface[44]. However, despite the obvious attractions of this relatively simple optical technique, non-specific adsorption of serum components to the sensor surface was found to be significant and would be expected to limit the sensitivity for estimating specific analytes in serum[45]. Nevertheless, new approaches aimed at investigating the nature of the adsorbed serum components, orientated antibody immobilisation procedures and engineering both the sensor surface and the antibody may reduce non-specific adsorption to an acceptable level[45].

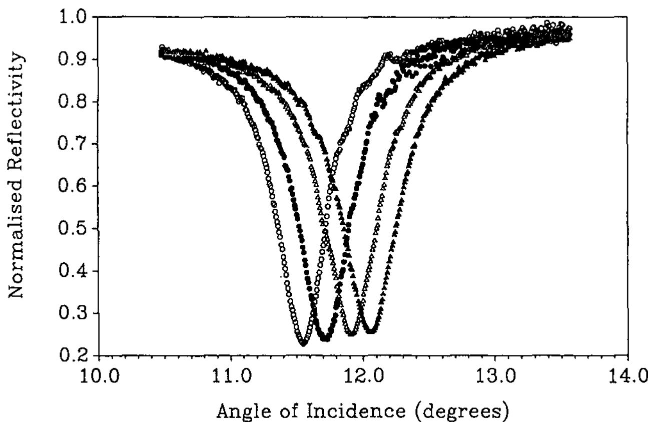

Fig. 2. Resonance curves obtained by sequential addition of protein A (333 μg ml^{-1}), anti-hens egg lysozyme antibody (333 μg ml^{-1}) and hens egg lysozyme (33 μg ml^{-1}) to a silver–chromium diffraction grating in 10 m*M* sodium phosphate buffer, pH 7.0. All measurements were made after washing the grating with buffer following incubation with the respective proteins. ○ = Buffer; ● = protein A; △ = anti-lysozyme; ▲ = lysozyme.

5. PERSPECTIVES AND CONCLUSIONS

It is now universally recognised that biosensors are likely to form a vital and pivotal feature of any future chemical surveillance and control system. Work over the past decade has now identified the problems facing the development of biosensors and future trends in the technology. There is a detectable trend to scrutinise the biological component in more detail than hitherto. For example, novel biorecognition systems isolated from newly discovered microbial, plant or microbial sources, from "extremophiles", from specifically engineered organisms by recombinant DNA techniques or exploiting the opportunities offered by artificial enzymes, catalytic antibodies (abzymes) or chemically imprinted polymers could lead to more selective and durable sensing elements. An alternative approach to biorecognition which could hold considerable promise in the longer term is the use of more complex chemoreceptor systems found in living organisms[46,47].

Novel biorecognition systems may still be sufficiently stable to permit reliable measurements over extended time regimes and thus require the development of intelligent interfaces to offset some of these limitations[4]. The interface could perform data acquisition and conrol, implement intelligent algorithms and communicate to the central controller. This system could recognise the performance characteristics of individual sensors, correlate, reject faulty signals, compensate for interferences and perform all necessary calibration steps. A multi-function chip comprising an array of biologically sensitive electrodes on a monolithic silicon device a few square millimetres in size could, at least in principle, encompass sufficient signal processing circuitry to address each sensor in turn and output the concentration of the analyte by comparing it with a calibration standard. Unfortunately, modern silicon microelectronics is considerably in advance of the biorecognition, protein and surface chemistry required to realise these concepts.

6. ABSTRACT

This review introduces biosensors as analytical devices that respond selectively to analytes in appropriate samples and convert their concentrations into electrical signals via a combination of a biological recognition system and a suitable transducer. The last decade has seen dramatic advances in the design of sensor configurations, the marriage of biological systems with modern monolithic silicon and optical technologies, the development of effective electron-exchange systems and the introduction of direct immunosensors.

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Electrochemical biosensors on thin-film metals and conducting polymers

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ABSTRACT

Electrochemical biosensors using advanced thin-film technology employing 1,4-arenequinones substituted with at least two halogens in *para* positions as new agents for the immobilization of enzymes are described. For special applications, thin-film electrodes were combined with permeation-selective polypyrrole layers as sensory modifying devices.

INTRODUCTION

The interest in miniaturized biosensors in medicine, food and process technology has increased significantly in the last few years[1–7] and a wide variety of oxidase-based biosensors have been developed. However, various problems have to be solved in order to allow the construction of satisfactory sensors for practical use in large numbers. The stability of the immobilized biosystem is very important, *e.g.*, enzyme (even under dry storage conditions) and can be increased by stable and efficient coupling procedures. The selectivity of the electrochemical transducer and the reproducibility with respect to response and temperature dependence must not be neglected[8–12]. Thin-film technology is able to provide the high purity and reproducibility required of the electrode surface and the high spatial resolution of the electrode structure. Our aim was to obtain devices for the rapid and highly reproducible measurement of small samples.

For microdevices, stepwise derivatization starting from the metal electrode surface turned out to be the most practical procedure. To achieve this aim two different techniques were studied and compared with each other: covalent coupling procedures with halogeno-1,4-arenequinones[13–15] on flat ultra-thin metal films, and covalent

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immobilization of enzyme on electrochemically polymerized, porous, substituted polypyrroles as top layers on vapour-deposited metal films.

EXPERIMENTAL

Materials

Platinum, palladium, rhodium and titanium were purchased from Balzers (Vaduz, Liechtenstein). Silicon nitride was prepared by plasma-enhanced chemical vapour deposition from silane, nitrogen and ammonia, all from Matheson (Oevel, Belgium). The negative lift-off photoresist AZ 1350 was from Hoechst (Wiesbaden, F.R.G.). Buffer substances, iron(III) chloride, potassium dichromate, nitric acid, bromine, sodium sulphate, calcium chloride, tin(II) chloride, titanium(III) chloride, hexamethyldisilazane (10% solution in xylene), N-cyclohexyl-N′-[2-(N-methylmorpholino)ethyl]carbodiimide 4-toluenesulphonate, *p*-chloranil, tetrabutyl tetrafluoroborate, organic solvents (acetone, toluene, ethanol, acetonitrile, acetic acid, etc.) and triethylamine were from Merck (Darmstadt, F.R.G.). *p*-Fluoroanil and *p*-bromoanil were purchased from Lancaster Synthesis (White Lund, U.K.). Glucose oxidase (GOD) (E.C. 1.1.3.4), galactosidase (E.C. 3.2.1.23), naringinase (E.C. 3.2.1.21 and 3.2.1.40), alkaline phosphatase (E.C. 3.1.3.1) and glucose-6-phosphate were supplied by Sigma (Deisenhofen, F.R.G.). Nafion (10% solution in water) and aliphatic alcohols was purchased from Aldrich (Steinheim, F.R.G.). 3-Aminopropyltriethoxysilane, lithium perchloride and tetrabutyl hexafluorophosphate were from Fluka (Buchs, Switzerland). Pyrrole, obtained from Merck, was distilled twice and stored at $-20°C$ in the dark.

Preparation of the sensor electrode

Mechanically stable sodium silicate glass sheets of thickness 100–300 μm were used as electrode carriers. After standard cleaning procedures with detergents, ultrasonication and organic solvents, metals were evaporated by an electron gun in a high-vacuum instrument (Balzers) and used to coat glass sheets with thin titanium layers up to a thickness of 50–100 nm as an adhesion layer. Platinum, rhodium or palladium layers up to a thickness of 100 nm, evaporated on top of the titanium film, act as electrochemical electrodes. Structuring of these thin films was performed by a lift-off technique with AZ 1350 photoresist. These layers were isulated by a 3-μm silicon nitride layer and structurized by plasma etching[16]. The platinum surface was cleaned by etching with an oxygen plasma (30 W) for 3 min and if necessary the surface could now be hydrophobized by dipping into a 10% solution of hexamethyldisilazane in xylene for 15 min.

Using these procedures, three different electrode types were constructed. (i) A single 0.64 mm^2 Pt/Ti sandwich working electrode (Fig. 1a) was made in order to test chemical processes and instrumentation. (ii) A three-electrode miniaturized electrochemical cell (Fig. 1b) with an outer diameter of 200 μm was produced for further studies. The Ag/AgCl reference electrode was produced by evaporating and structuring a 1-μm thick silver film, which was subsequently chlorinated with 10 m*M* iron(III) chloride to obtain a thickness of 2 μm. (iii) A four-electrode electrochemical cell (Fig. 1c) with an outer diameter of 2500 μm and possessing two identical working electrodes was produced for differential measurements.

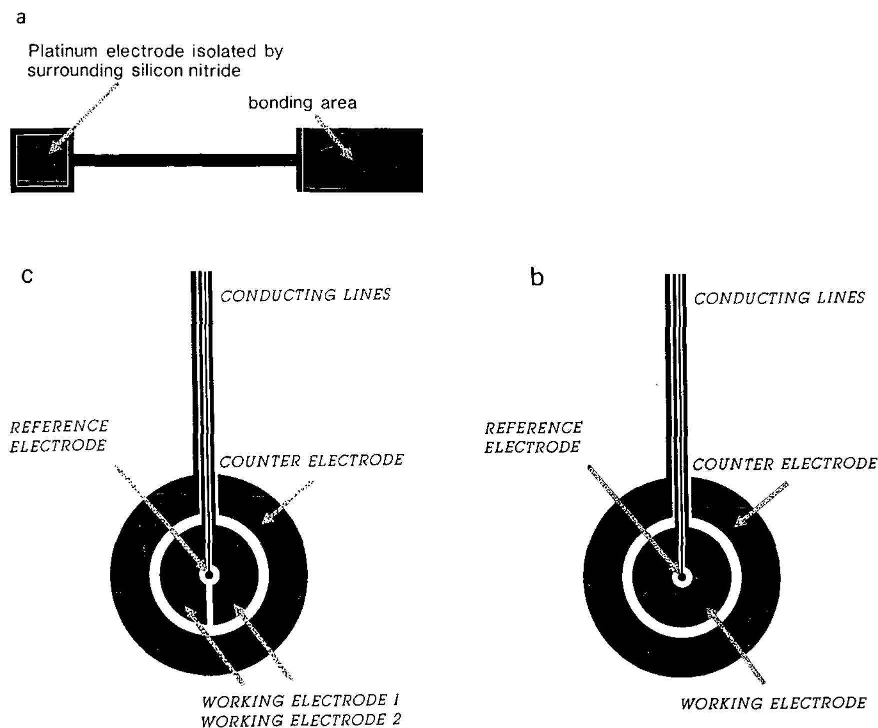


Fig. 1. Electrode types: (a) test electrode; (b) three-electrode type; (c) four-electrode type.

Preparation of the biosensors using 1,4-arenequinones[13–15,17] *(Fig. 2)*

Activation of the metal surfaces. Platinum electrodes were oxidized either in a 2.5% solution of potassium dichromate in 15% nitric acid at a potential of 2.5 V *vs.* Ag/AgCl for 2 min or by a coupled oxygen plasma. Palladium and rhodium electrodes were oxidized in a solution containing sodium nitrate instead of nitric acid. The oxidized electrodes were rinsed with water, followed by acetone, dried and imemdiately derivatized with silane.

Coupling with 3-aminopropyltriethoxysilane. The oxidized electrodes were dipped into a 10% solution of silane in toluene and kept at 60°C for 30 min. The silylated surface was cleaned with toluene and ethanol. An alternative procedure was spinning of a 1% solution of the silane in propanol–water (95:5, v/v) on the electrodes at 3000 rpm for 30 s and drying in an oven at 90°C for 3 min.

Reaction with p-chloranil (Fig. 3). The silylated electrodes were kept in a 1% solution of *p*-choranil in toluene at 40°C for 30 min. The electrodes were rinsed several times with toluene followed by acetone. The derivatized electrodes were stored dry in the dark.

Reaction with modified p-quinones (Fig. 4). In addition to *p*-chloranil, other derivatives such as *p*-fluoroanil, *p*-bromoanil and related quinones were used to activate the electrode surface for coupling of the enzyme.

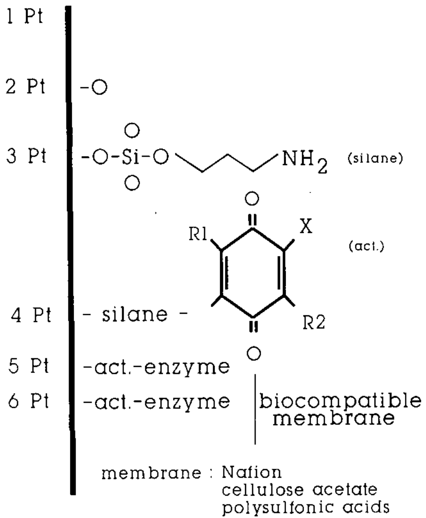


Fig. 2. Immobilization steps: 1 = untreated electrode; 2 = oxidized surface; 3 = derivatization with silane; 4 = activation; 5 = enzyme coupling; 6 = membrane coating.

Coupling of the enzymes. To couple glucose oxidase to the activated surface, the electrodes were immersed in a solution of 5 mg/ml glucose oxidase in 0.1 *M* phosphate buffer (pH 7.0) for 2 h. The electrodes were rinsed several times with 4 *M* phosphate-buffered saline to eliminate adsorbed protein. Immobilized apo-glucose oxidase (GOD freed from its FAD coenzyme)[18] was used for an apoenzyme reactivation assay and, likwise, β-galactosidase, naringinase and alkaline phosphatase (using glucose-6-phosphate as a substrate) in a GOD coupled assay.

Coating of the electrodes[19]. A polymeric top layer may be applied to prevent unwanted adsorption of most proteins and to minimize clotting effects in biological

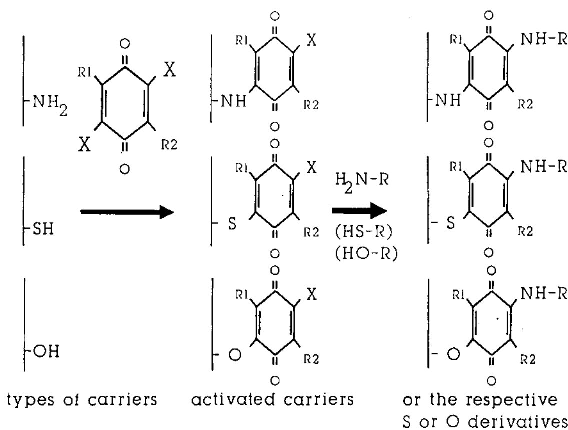
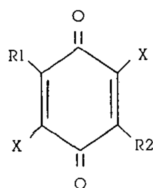


Fig. 3. Reaction scheme of immobilization with halogeno-1,4-arenequinones.



X = halogen
R1, R2 = halogen, R, COR, COOR,
C_1 - C_8 alkyl, COOH, CN, CNS, NO_2, N_3

Fig. 4. Halogeno-1,4-arenequinone reagents for enzyme coupling.

samples. Using Nafion (a perfluorinated and sulphonated polymer) or other polysulphonic acids a further ion sieving effect can suppress unwanted inferences of anionic compounds. A 1-ml volume of Nafion solution was buffered by the addition of 20 μl of triethylamine and 25 μl of acetic acid. The electrodes were either dipped into the solution or the solution was sprayed or spun onto the surface. The solvents were evaporated with water-saturated nitrogen at room temperature for 10–30 min.

Preparation of 3- and N-substituted pyrroles

The following pyrroles were studied: (1) 1-(Ω-carboxymethyl)pyrrole, (2) 1-(Ω-carboxyethyl)pyrrole, (3) 1-(Ω-carboxypropyl)pyrrole, (4) 1-(Ω-carboxypentyl)pyrrole, (5) 1-(Ω-carboxydecyl)pyrrole, (6) 1-(1,3-dicarboxypropyl)pyrrole, (7) 1-[(1-carboxy-3-methyl)butyl]pyrrole, (8) 2-(1-pyrrolo)acetylglycine, (9) 1-dodecylpyrrole, (10) 1-(4-carboxybenzyl)pyrrole, (11) 1-(4-nitrophenyl)pyrrole, (12) 1-(4-carboxyphenyl)-pyrrole, (13) 3-carboxymethyl-4-methylpyrrole, (14) 1-*o*-tosylpyrrole, (15) 1-*p*-tosyl-pyrrole, (16) 1-benzenesulphonylpyrrole, (17) 4-(3-pyrrolo)-4-hydroxybutyric acid, (18) 4-(3-pyrrolo)-4-ketobutyric acid, (19) 3-[(hydroxy-4-nitrophenyl)methyl]pyrrole and (20) 3-[(keto-4-nitrophenyl)methyl]pyrrole.

Pyrroles 1–12 were prepared according to the method of Giuliano *et al.*[20], 13 according to the method of van Leusen *et al.*[21], 14–16 by a new synthesis based on the procedure of Giuliano *et al.*[20] and 17–20 by a new synthesis based on the procedure of Rokach *et al.*[22] followed by 3-alkylation.

Preparation of biosensors using substituted polypyrroles

Cleaning of the electrode. Platinum thin-film electrodes were cleaned by ultrasonication in distilled water, rinsed with acetone and dried carefully under dust-free conditions. The electrodes were cycled five times in acetonitrile–2.5% lithium perchlorate between -500 and 1800 mV (100 mV/s) *versus* Ag/AgCl using a three-electrode configuration of an electrochemical cell. A potentiostat interfaced by a 14-bit analog-to-digital, digital-to-analog converter to an AT personal computer was employed for generating the required voltages.

Coating with polymerized substituted homopolypyrroles[23]. Pyrroles 1–20 were used as monomers for the coating of platinum electrodes with thin polymeric layers. A 0.5% solution of these compounds in acetonitrile containing 2.5% of $LiClO_4$, NR_4BF_4 or NR_4PF_6 ($R = CH_3, C_2H_5, C_4H_9$) was dried over sodium sulphate or

calcium chloride for several hours. The clear solution was transferred to a glass cell and bubbled with argon for 10 min. The electrode immersed in the deaerated solution was then cycled 10–30 times between −300 and +1600 mV (100 mV/s). If the substituted pyrrole was polymerizable the formation of a thin, brown polymeric layer was observed.

Coating with polymerized heteropolypyrroles. It is necessary to make a copolymer having a high content of unsubstituted polypyrrole in order to obtain thick and stable substituted polypurrole films. As the polymerization speed of the substituted pyrroles is significantly lower than that of unsubstituted pyrrole it is nevertheless necessary to have a 10:1 excess of the substituted monomer. The electrolyte of the above-mentioned composition was complemented with an additional 0.05% of pyrrole. The electrode was cycled up to ten times between −300 and +1600 mV (100 mV/s). A black polymer layer of varying thickness was obtained.

Coupling of the enzymes. A variety of methods were employed to activate the terminal carboxylic acid groups. The use of water-soluble carbodiimides may be recommended for the best results. The polypyrrole-coated electrodes were incubated with a saturated solution of N-cyclohexyl-N′-[2-(N-methylmorpholino)ethyl]carbodiimide 4-toluenesulphonate for 60 min at 25°C without shaking. The electrodes were rinsed several times with water and immediately reacted with a 5 mg/ml enzyme solution in 0.1 *M* phosphate buffer (pH 7.0) for 2 h. Substituted polypyrrole films having nitro groups may be reduced using a solution of 15% titanium(III) chloride or 1% tin(II) chloride in 10% hydrochloric acid for 30 min forming a film with pendant amino groups. After thorough rinsing with dilute hydrochloric acid, distilled water and ethanol, these electrodes can be dried. Now 1,4-arenequinones can be employed to couple the enzyme using the same activating and immobilizing procedure as mentioned above.

The sensors thus obtained were rinsed with phosphate buffer and water and stored at 4°C. In addition to the enzymes mentioned above, ADH was used to study immobilized dehydrogenases.

RESULTS

Our aim was to construct biosensors that can be produced in large numbers with high reproducibility, stability and selectivity for commercial use. Given the well defined structure of the microelectrodes, we developed and studied two different strategies: covalent coupling on thin-film metals with a new immobilization technique and covalent coupling on modified polypyrrole layers.

Covalent immobilization with 1,4-arenequinones (Figs. 3 and 4)

For optimum results a defined covalent coupled monolayer should be achieved avoiding enzyme membranes or cross-linked gels using bifunctional reagents, both having characteristic disadvantages due to the varying thickness of the enzyme layer or mechanical and chemical instability.

Covalent coupling of glucose oxidase, naringinase, β-galactosidase and alkaline phosphatase was carried out employing an improved technique according to our recently desorbed method[13,14] adapted from the chemistry of quinones. 1,4-Arenequinones substituted with at least two halogens or pseudohalogens in *para* positions

are highly reactive as regards nucleophilic substitution. Amino and thiol groups react with the quinone compound, replacing two *para*-halo groups of the reagent. A related method with a different reaction mechanism is the immobilization of proteins by a redox coupling reaction with *p*-benzoquinone[24,25].

When using immobilized amines or thiol groups on the electrode surface, the quinone reagent reacts at one halogen position, eliminating hydrohalogenic acid. Thus the halogen atom in a *para* position is highly activated by mesomeric and inductive effects. Electrode material activated in this way may be stored dry and in the dark for at least 6 months.

To couple the enzyme, the activated electrodes should be dipped in a buffered solution of the enzyme for 1–12 h followed by a washing procedure (see Experimental) to eliminate adsorbed protein. The activated *para*-halogen atom is now capable of reacting with the amino and mercapto groups of proteins, peptides and coenzymes.

The influence of the X substituents (reactive halogen) (see Fig. 4) on the coupling efficiency of the immobilization of enzymes had been characterized: on varying the reactive halogen from F to Cl and Br no alteration in the surface enzyme coating of the electrodes could be observed. Changing the reactive halogen group from F to Cl and Br altered the reactivity, resulting in an increased coupling time. Using tetrahalogen compounds (*p*-fluoroanil, *p*-chloroanil and *p*-bromoanil) highly reproducible results were obtained[13,14].

As reactive groups of proteins, peptides and ligands being able to couple to the activated support, ε-amino groups of lysine or α-amino groups of amino acids as well as thiol groups of cysteine were characterized by the use of model compounds. Very stable bonds were formed, immobilizing proteins and ligands, by this technique. This immobilization technique was also adapted to porous glass and silica supports, polyacrylhydrazide, Enzacryl AA and SH, partially hydrolysed nylon membranes and aminolysed polyesters for the construction of multi-enzyme sensors.

The same method permitted the consecutive coupling of glucose oxidase as defined multi-layers to the same electrode, causing an increase in the electrode response.

Characterization of a glucose sensor based on this immobilization technique

The change in response as a function of voltage, substrate concentration and

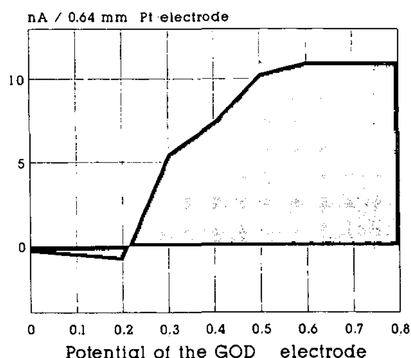


Fig. 5. Voltage (V) dependence of the electrode response (*vs.* Ag/AgCl). Shaded area: response 0.1 *M* phosphate buffer (pH 7.0); glucose = 820 mg/l (physiological concentration).

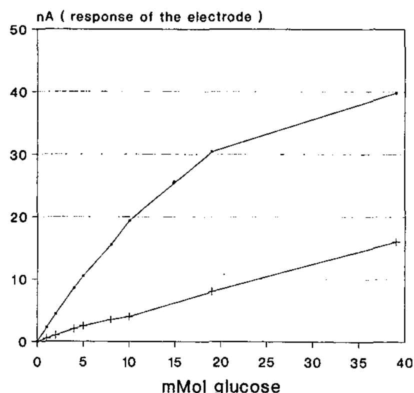


Fig. 6. Calibration graph for the Pt thin-film metal glucose oxidase electrode (0.64 mm^2). Immobilized with tetrachloro-1,4-benzoquinone. 0.1 *M* phosphate buffer (pH 7.0). • = Without Nafion; + = with Nafion.

time was studied in order to characterize the electrode (Figs. 5–7). Under optimum conditions the electrodes working at a potential of 500 mV *vs.* Ag/AgCl gave a linear response up to 12 m*M* without any further diffusion-limiting membrane. By covering the electrode with a dip-coated Nafion membrane, the linear range for glucose was extended to 50 m*M*.

Immobilization on substituted polypyrrole layers

A number of glucose sensors have already been constructed involving the immobilization of glucose oxidase by copolymerization with pyrrole and adsorption on polypyrrole surfaces. Free and immobilized redox-mediating compounds such as ferrocene or dimethylferrocene have been employed to enhance the redox transfer from FAD to the polypyrrole layer[26–28]. However, senors based on this principle have many disadvantages: low operational and storage stability; stability and linearity of response only in deaerated solutions; and low selectivity of the polypyrrole electrochemistry. By comparing the properties and limits of the above-mentioned electrodes, two fundamental advantages of electrodes covered by substituted polypyrrole layers having no redox activity were established: about a 20-fold increase in response per unit area due to the porous surface and significant permeation control by the polymeric

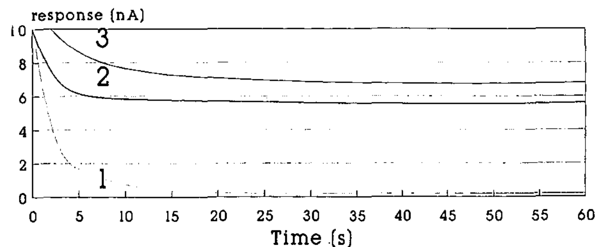


Fig. 7. Time dependence of electrode response. 1 = 0.1 *M* phosphate buffer (pH 7.0); 2 = 5 m*M* glucose buffer, stirred; 3 = 5 m*M* glucose buffer, unstirred.

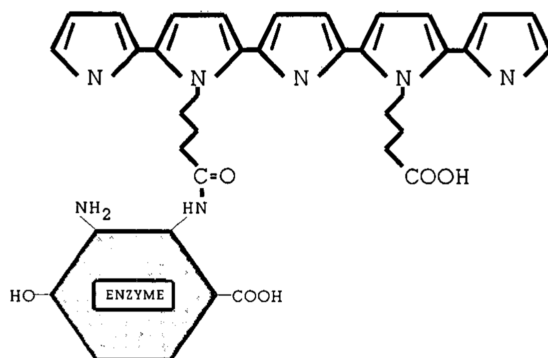


Fig. 8. Immobilization of enzymes to polypyrrole heteropolymers.

layer for interfering electroactive substances resulting in a distinct increase in selectivity (*e.g.*, 50–200-fold supression of ascorbic acid in glucose monitoring).

Optimum results for polypyrrole sensors described in this paper could only be obtained by synthesizing modified polypyrrole layers without significant conductivity and redox activity. For these electrodes, the electrocatalytic reaction should take place at the metal surface, otherwise high background currents and low selectivity will be obtained. Thus, the substituted polypyrrole layer acts as a highly effective barrier for interfering redox active compounds such as ascorbic acid, bioactive amines, sulphydryl-containing peptides and proteins, etc., but having a high permeability for, *e.g.*, hydrogen peroxide.

Various N- and 3-substituted pyrrole monomers were synthesized (see Experimental) in order to prepare layers posessing these properties. The synthesized pyrrole

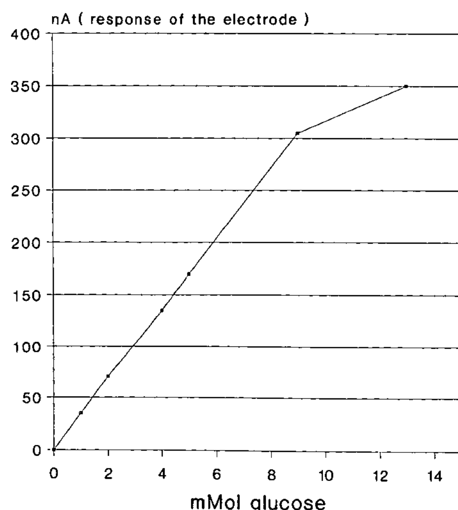


Fig. 9. Calibration graph for an N-alkylpolypryrrole glucose oxidase electrode (0.64 mm^2). 0.1 *M* phosphate buffer (pH 7.0).

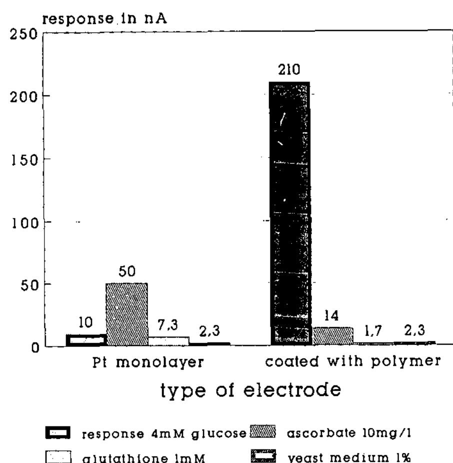


Fig. 10. Selectivity of the electrodes (0.64 mm^2). Comparison of a Pt–GOD monolayer and a Pt–polymer–GOD monolayer electrode. 0.1 *M* phosphate buffer (pH 7.0). Temperature, 25°C; potential, 500 mV.

derivatives were tested for their ability to form polymers. Polymeric films could be obtained with pyrroles 1–5, 8–11, 13 and 17–20. Homopolymers, heteropolymers and sandwich types of these polymer layers were prepared. The various layer types led to different properties of the resulting enzyme sensors. Satisfactory homopolymer layers could only be formed with pyrrole derivatives having no bulky side-groups directly attached to the pyrrole ring (*e.g.*, arenes). In synthesizing heteropolymers it was vital to consider that the compound with the more positive polymerization potential will be incorporated into the polymer to a much lower extent than its concentration in the polymerizing solution would normally allow.

It is necessary to introduce functional groups which are stable against oxidation under polymerizing conditions in order to obtain a modified polypyrrole layer which can be used for covalent coupling of enzymes. COOH and NO_2 groups have proved to be most suitable for this purpose. Not too hydrophobic polypyrrole layers containing carboxy groups turned out to give the best results (Fig. 8).

Characterization of electrodes

Typical properties of this type of sensor can be exemplified with glucose oxidase. The change in response as a function of substrate concentration and selectivity was studied in order to characterize the electrode (Figs. 9 and 10). The change in response as a function of voltage has the same shape as shown in Fig. 5, but the data must be multiplied by a factor of 20. Under optimum conditions the electrodes working at a potential of 500 mV *vs.* Ag/AgCl gave a linear response up to 9 m*M* without any further diffusion-limiting membrane. By covering the electrode with a polymer membrane, the linear range for glucose was extended to 50 m*M*.

CONCLUSION

The comparison of the two electrode types using immobilized glucose oxidase as

an example shows the advantages and limitations of the two sensor types, as follows.

A 0.64 mm^2 Pt–halogenoarenequinone immobilized glucose oxidase sensor has a response of 8–12 nA with 5 m*M* glucose–phosphate-buffered saline (pH 7.0) at 25°C. Porous polypyrrole immobilized glucose oxidase sensors have a 10–50-fold greater response per unit sensor surface area owing to a significant increase in the inner surface area.

The temperature dependence of the signal is 5%/°C and independent of the electrode type.

The base current is 0.2 nA per 0.64 mm^2 for the monolayer type and 1.5–3 nA/mm^2 for the polymer-coated type.

The storage stability is independent of the electrode type, showing a decrease in response per year of *ca.* 38% if stored in aqueous 4 *M* sodium chloride at 4°C.

With both electrode types the response time is in the range of a few seconds, increasing if the electrode is covered by a diffusion-limiting membrane.

The response of polymer-coated electrodes is nearly independent of fluctuations in the test solution in the vicinity of the sensors owing to the coverage of the enzyme layer by the polymer. Monolayer electrodes, being sensitive to these fluctuations, can be protected by a diffusion-limiting membrane (*e.g.*, Nafion), which supresses this effect.

The reproducibility of the immobilizing surface is optimum for thin-film metal electrodes; on the other hand, reproducibility is difficult to achieve when immobilizing on the internal surface of a polymer layer. Hence the reproducibility of the sensor response is significantly better on coated thin-film metal electrodes than on polymer-coated types.

To avoid unwanted adsorption layers on top of the enzyme film reversibly decreasing the electrode response in the range 10–40%, both electrode types should be covered with a hydrophilic membrane (*e.g.*, Nafion).

The non-specific response of interfering redox-active substances (*e.g.*, ascorbic acid, reduced glutathione, phenacetin) can be supressed by either using the four-electrode structure and employing differential measurement or using the polypyrrole type of electrodes exhibiting permeation-selective properties.

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Note

Adsorption chromatography on cellulose

VI. Further studies on the separation of D-and L-tryptophan on cellulose with aqueous solvents

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In a previous paper[1] we described some of the interesting aspects of the separation of the optical isomers of tryptophan on cellulose using aqueous solvents. The separation factors were almost independent of temperature and of the salt concentration. Further, the addition of a methyl group to various ring positions did not produce great changes in the separation of the optical isomers.

In this paper a number of further aspects of this separation are reported: the effect of various cations and anions in the eluent, the effect of changing the polarity of the eluent by addition of methanol and the effect of the kind of cellulose used as the stationary phase.

EXPERIMENTAL

All developments were performed in small, well stoppered glass jars [for thin-layer chromatography (TLC)] or in 40-cm tall cylinders (for paper chromatography) at room temperature (18–20°C). The development of thin layers took between 10 and 25 min, depending on the length of development. The developed chromatograms were dried for a few minutes in an air oven at 90°C, dipped in a solution of ninhydrin in acetone and heated again for several minutes. Full colour was usually produced within 24 h.

RESULTS AND DISCUSSION

Effect of cations and anions in the aqueous developing solvent

Previously we had observed[1] that the separation of optical isomers of tryptophan and substituted tryptophans is essentially the same in aqueous LiCl and aqueous $(NH_4)_2SO_4$ on Merck Art. 5577 DC Plastikfolien cellulose, and also that the temperature and the concentration of the salt had little influence on the separation.

We have now extended the work to the following salts:

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(a) 0.1 *M* NaCl, 1 *M* NaCl, 0.1 *M* KBr and 1 *M* KI. The separations obtained were identical, hence the size of the anion has no measurable effect.

(b) We also developed chromatograms with 1 *M* NaCl, 0.25 *M* $MgSO_4$, 0.25 *M* $CaCl_2$, 0.25 *M* $BaCl_2$ and 0.033 *M* $Al_2(SO_4)_3$. As shown in Fig. 1, there is no visible difference between the various chromatograms.

(c) When various salts of transition metals were used as eluents, only Cu(II) changed the chromatogram. Fig. 2 shows that 0.25 *M* $CdSO_4$, 0.05 *M* $MnSO_4$, 0.05 *M* $NiSO_4$, 0.05 *M* $Co(NO_3)_2$ and 0.05 *M* $ZnSO_4$ all yielded a separation of the two optical isomers of 5-methyltryptophan. With 0.05 *M* $CuSO_4$, on the other hand, only one spot was observed. Hence it seems complexation with Cu(II) interferes with the chiral distinction between the two optical isomers. We consider it remarkable that none of the other transition elements has an influence here, as they are well known to form complexes.

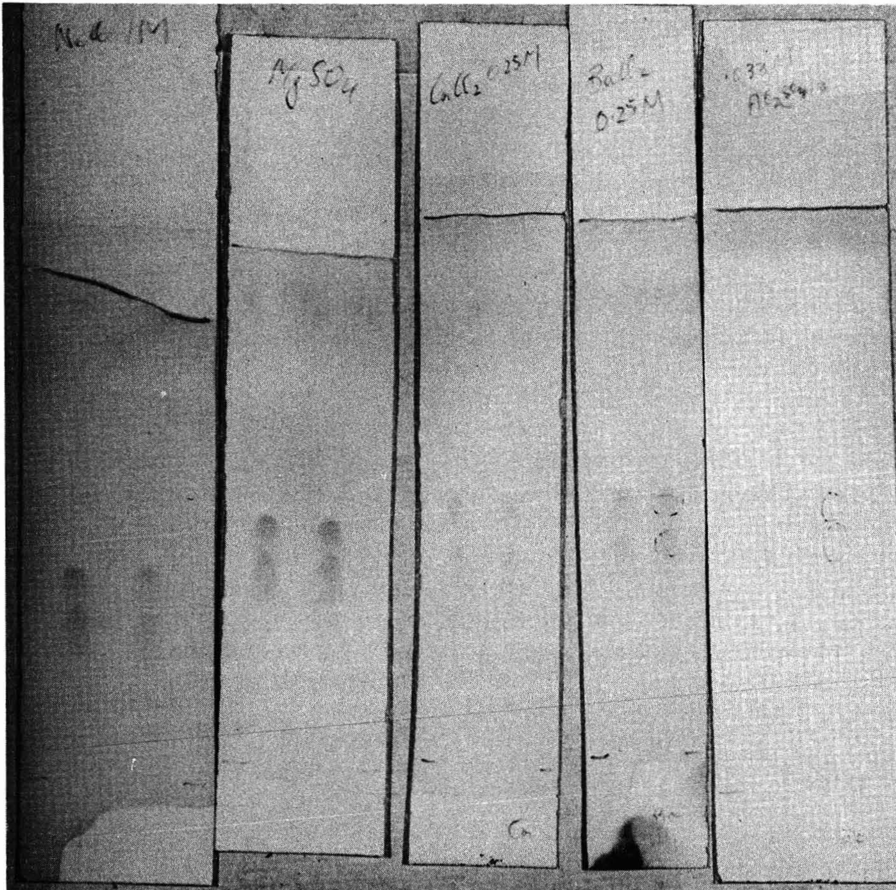

Fig. 1. Thin-layer chromatograms of DL-5-methyltryptophan on Merck Art. 5577 DC Plastikfolien cellulose. Eluents (from left to right): 1 *M* NaCl, 0.25 *M* $MgSO_4$, 0.25 *M* $CaCl_2$, 0.25 *M* $BaCl_2$ and 0.033 *M* $Al_2(SO_4)_3$.

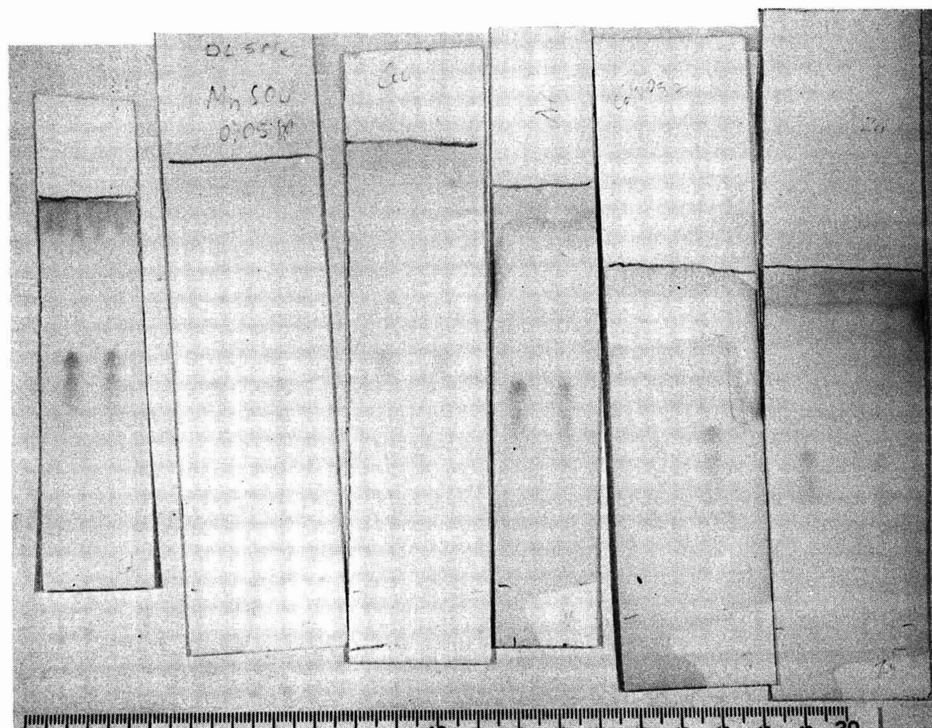

Fig. 2. Thin-layer chromatograms of DL-5-methyltryptophan on Merck Art. 5577 DC Plastikfolien celullose. Eluents (from left to right): 0.25 *M* $CdSO_4$, 0.05 *M* $MnSO_4$, 0.05 *M* $CuSO_4$, 0.05 *M* $NiSO_4$, 0.05 *M* $Co(NO_3)_2$, 0.05 *M* $ZnSO_4$.

Effect of methanol in the eluent

Our previous work on adsorption on cellulose from aqueous solvents showed that the main adsorption mechanism is "hydrophobic" and the only amino acid that is strongly adsorbed is the heterocyclic tryptophan[2]. Yuasa *et al.*[3] separated optical isomers on cellulose with solvent mixtures where a liquid–liquid partition mechanism would be expected. We therefore wanted to investigate the effect that a change in polarity would have on our kind of separation. This is shown in Fig. 3.

Mixtures of 1 *M* NaCl and methanol yield essentially the same separation of the isomers of 5-methyltryptophan between 0 and 75% methanol on Merck Art. 5577 DC Plastikfolien cellulose. Only with 80% methanol is the separation altered with lower R_F values and worse, or no, resolution.

We conclude that the chiral distinction mechanism is not disturbed by minor changes in polarity.

Effect of the structure of the cellulose

All experiments reported above, and also those in the previous study[1] were carried out with Merck cellulose thin layers. Essentially the same separations were also obtained on Merck Art. 5787 Fertigplatten cellulose HPTLC plates (10 cm × 10 cm), as is shown in Fig. 4.

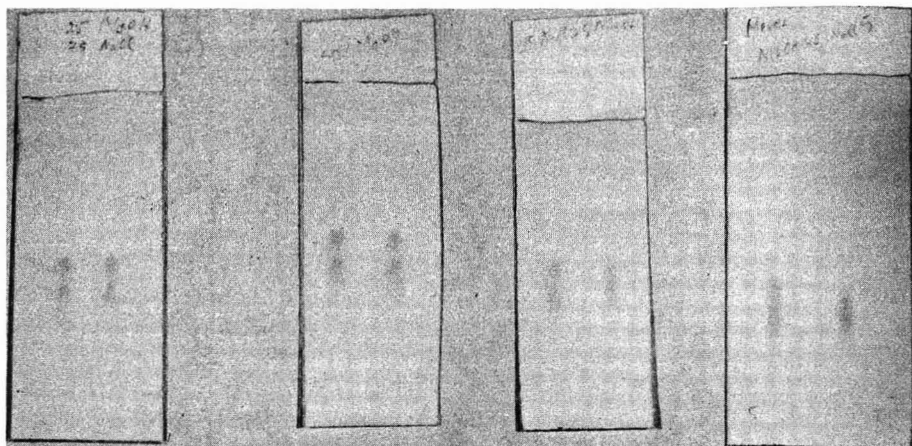

Fig. 3. Thin-layer chromatograms of DL-5-methyltryptophan Merck Art. 5577 DC Plastikfolien cellulose. Eluents (from left to right): 1 *M* NaCl mixed with methanol in the proportions 75:25, 50:50, 25:75, 10:80. On the last chromatogram the spot on the right is DL-tryptophan.

Several types of Macherey–Nagel cellulose layers, MN Polygram Cel 300 UV_{254}, MN Polygram Cell 300 (40 mm × 80 mm) and MN Polygram Cel 300 (20 cm × 20 cm), were tried but yielded no separations of the optical isomers. Typical results are shown in Fig. 5. The lack of separation is certainly not due to the short development (*cf.*, Fig. 4), as longer thin layers also did not yield separations.

On Whatman 1 and 3MM filter-papers, no distinct separation of D- and L-5-methyltryptophan could be obtained, but the DL-mixture usually gave an

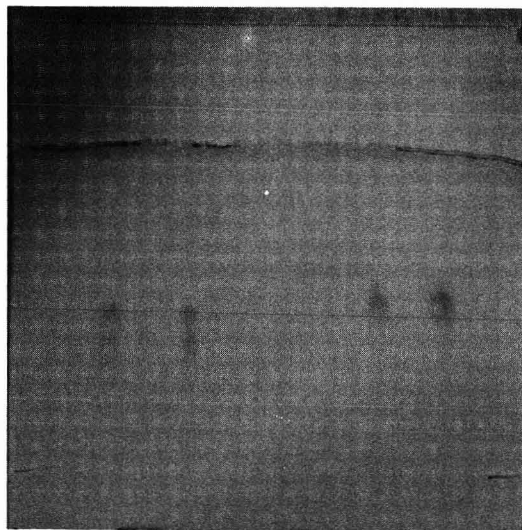

Fig. 4. Thin-layer chromatogram on a Merck Art. 5787 Fertigplatten cellulose HPTLC plate (10 cm × 10 cm) developed with 0.5 *M* NaCl. Spots (from left to right): DL-5-methyltryptophan (two spots); DL-tryptophan (two spots).

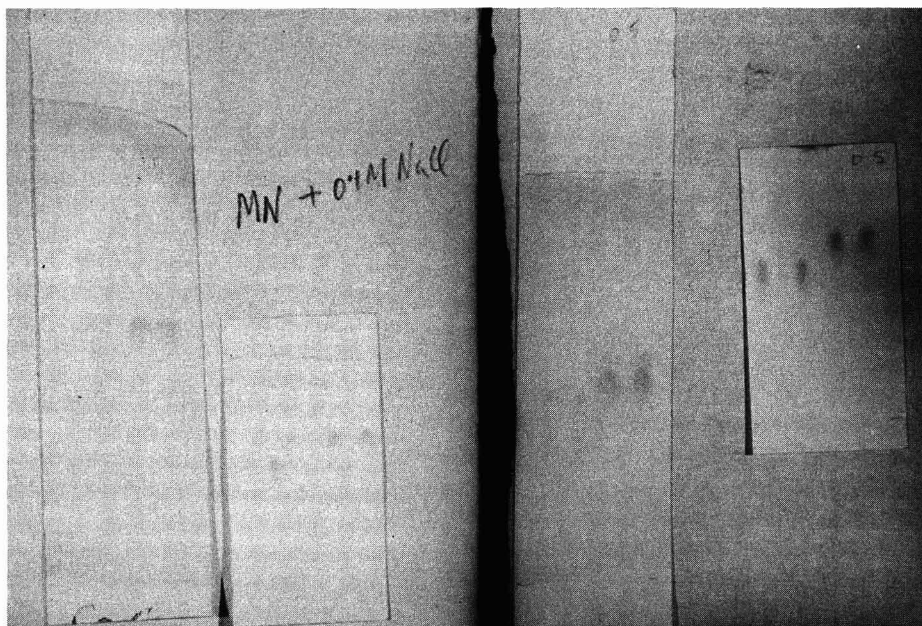

Fig. 5. Thin-layer chromatograms of DL-5-methyltryptophan (two spots on the right) and DL-tryptophan (two spots on the left) developed with 0.1 *M* NaCl (left) and 0.5 *M* NaCl (right) on Merck Art. 5577 DC Plastikfolien cellulose (right) and MN Polygram Cel 300 (40 mm × 80 mm) (left). The latter layers have the same dimensions as the chromatograms shown in Fig. 4.

elongated spot, as previously observed by Weichert[4], indicating a partial separation.

The structure of cellulose was discussed in a recent review by Kremer and Tubb[5], and their account suggests that the differences between the various cellulose supports are due to the content of "amorphous regions", a characteristic that may be enhanced by the mechanical "beating" action during the pulping process. They described these regions as a "two-dimensional colloidal system in which the surface fibrillae have two dimensions in the colloidal range but are anchored to the fibre in the third dimension".

It would be interesting to establish whether this chiral effect of cellulose can be altered at will by a suitable manufacturing process.

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SHORT CONFERENCE REPORT

8TH INTERNATIONAL SYMPOSIUM ON AFFINITY CHROMATOGRAPHY AND BIOLOGICAL RECOGNITION, JERUSALEM, ISRAEL, OCTOBER 29-NOVEMBER 3, 1989

Fig. 1. The audience during a lecture session. Front row from left to right: Sergio D. Rose, Irwin Chaiken, Daniel J. O'Shannessy, Ephraim Katzir, Jaroslava Turkova, Meir Wilchek, Edward A. Bayer.

Fig. 2. Group photograph of most of the participants next to the statue of the matriarch Rachel.

Fig. 3. Presentation of the Pierce Award. From left to right: Meir Wilchek, Chris R. Lowe and A. Krishna Mallia.

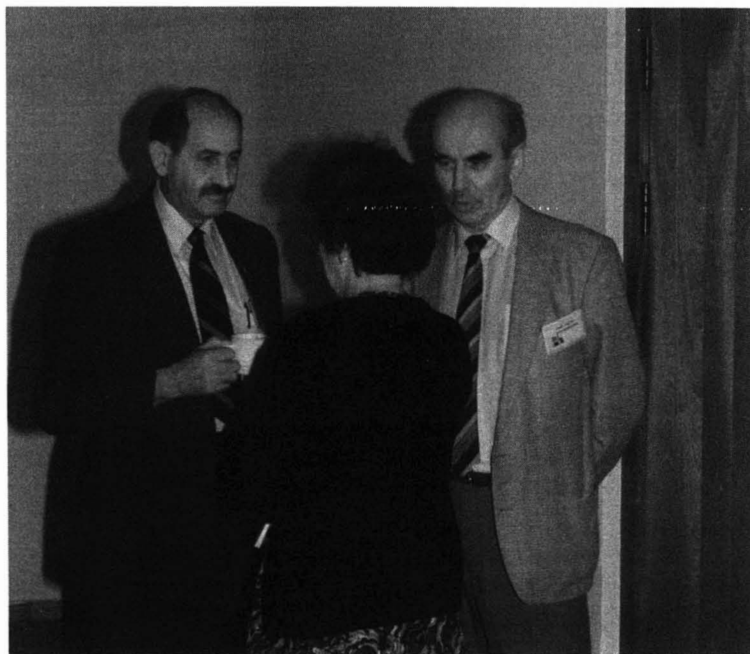

Fig. 4. Conversation during coffee break. From left to right: Meir Wilchek, Jaroslava Turkova and Karel Macek (Editor, *Journal of Chromatography*).

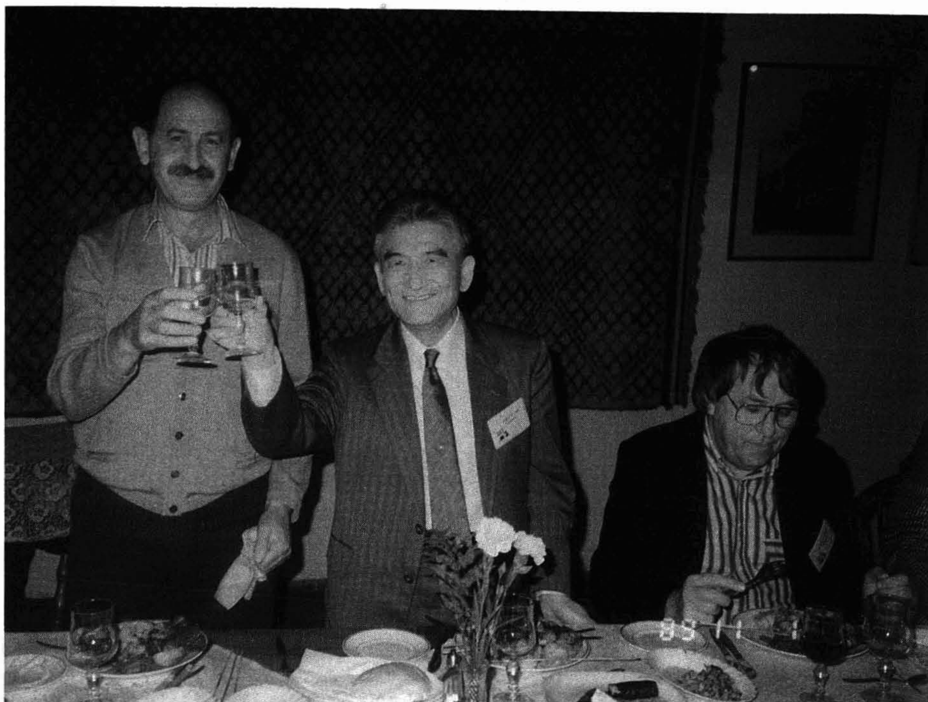

Fig. 5. A toast during dinner. From left to right: Meir Wilchek, Shin-ichi Ishii (chairman of the 1991 Symposium to be held in Yokohama, Japan), and Klaus Mosbach (recipient of the first Pierce Award).

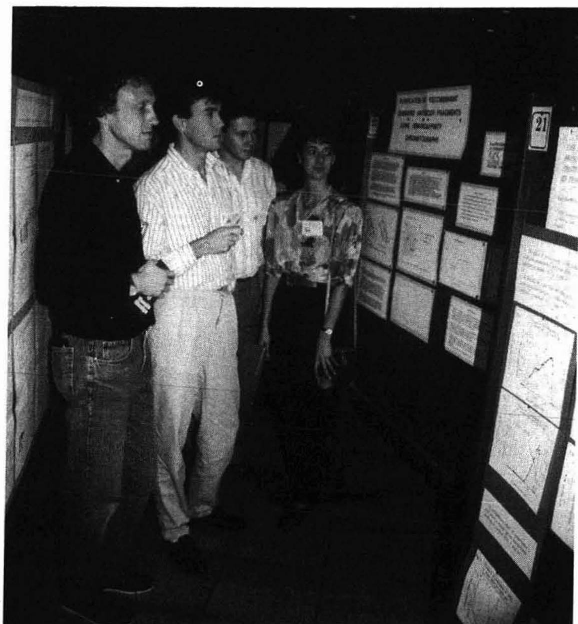

Fig. 6. A view of the poster session.

Polyimides: Materials, Chemistry and Characterization

Proceedings of the Third International Conference on Polyimides, Ellenville, New York, November 2-4, 1988

edited by **C. Feger,** *IBM, Yorktown Heights, NY,*
M.M. Khojasteh, *IBM, Hopewell Jct, NY, and* **J.E. McGrath,** *Virginia Polytechnic Institute and State University, Blacksburg, VA, USA*

The scope of this book is to give an up-to-date overview of the field of polyimide research - a field which has seen rapid growth and diversification in the last few years as well as a stronger involvement of university laboratories. Although synthesis of new materials still plays a major role, a number of groups work now on understanding the details of polyimide chemistry, structure and properties. This is reflected in the papers presented in this book which examine several major aspects of polyimides: new materials, synthesis, cure studies, structure-property studies, studies of mechanical and electrical properties, analysis of polyimides, interface studies, and applications.

This conference was hailed as the best yet in the series, and these articles, the most current in this fast-growing field, will ensure that the book will be a landmark in the field for years to come.

The papers, too numerous to list individually, are grouped under the following headings:

1. **New Materials** (18 papers): includes polyimide blends (a new field), photosensitive polyimides, silicon-containing polyimides, copolymers, bismaleimide resins, semi-interpenetrating networks.
2. **Synthesis** (6 papers): includes new synthetic methods, polymerization mechanisms and properties of precursor solutions.
3. **Curing Studies** (7 papers): includes studies on imidization and on degradative reactions at elevated temperatures.
4. **Structure-Property Relationships** (5 papers): includes the influence of fluoro-, carbonyl-, ether- and aliphatic groups on polyimide properties.
5. **Properties** (9 papers): includes mechanical and electrical properties.
6. **Characterization** (5 papers): includes spectroscopies (UV, LMFT-MS, RBS, IR) and determination of molecular weights and orientation.
7. **Interfaces and Adhesion** (4 papers): includes adhesion, interface reactions and surface modification.
8. **Application** (4 papers): includes electronic applications.

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Books for the Practising Chromatographer

SELECTIVE SAMPLE HANDLING AND DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, PART A

edited by **R.W. Frei** ***and*** **K. Zech**

(Journal of Chromatography Library, 39A)

This is the first of a 2-part work which attempts to treat the sample handling and detection processes in a liquid chromatographic system in an integrated fashion. The need for more selective and sensitive chromatographic methods to help solve the numerous trace analysis problems in complex samples is undisputed, but few workers realize the strong interdependence of the various steps - sample handling, separation and detection - which must be considered if one wants to arrive at an optimal solution. Since the sample handling side, although recognized as a major bottleneck, has been neglected in the chromatographic literature, special emphasis has here been placed on this aspect. Priority is given to the treatment of chemical principles as applied to the topics in this book, rather than to instrumentation.

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(Journal of Chromatography Library, 42)

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(Journal of Chromatography Library, 43)

This book describes in great detail the recent progress made in the isolation and separation of natural products. Written by experts in their respective fields, it covers antibiotics, marine and plant-derived substances, enzyme inhibitors and interferons. It offers the reader: up-to-date reviews of specific topics in the natural products field not to be found elsewhere; information on new chromatographic methods and techniques - described in sufficient detail to be utilized by investigators in this area of research; and extensive references to enable the serious researcher to pursue particular information. It will appeal to pharmaceutical and natural products researchers and is a valuable acquisition for university chemistry and biochemistry departments.

1989 634 pages US$ 150.00 / Dfl. 285.00 0-444-87147-0

ANALYTICAL ARTIFACTS: GC, MS, HPLC, TLC and PC

by **B.S. Middleditch**

(Journal of Chromatography Library, 44)

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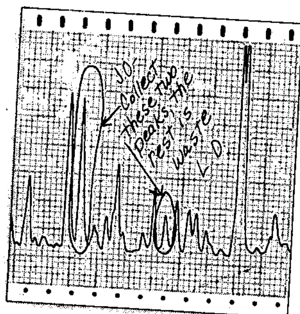

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