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JOURNAL OF

CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

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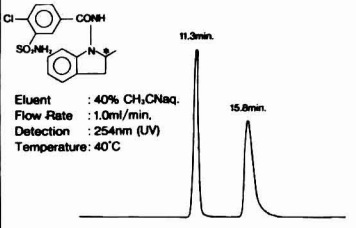

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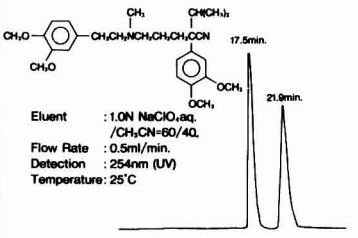

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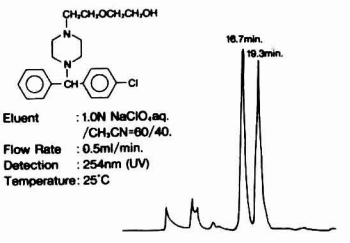

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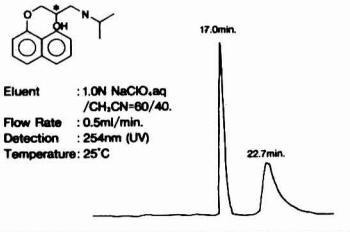

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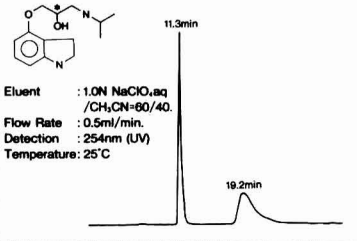

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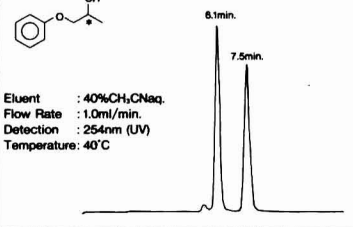

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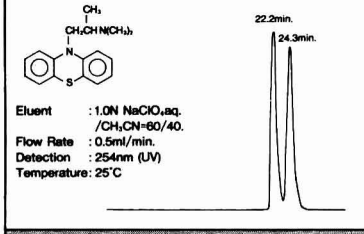

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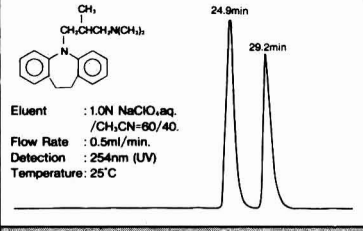

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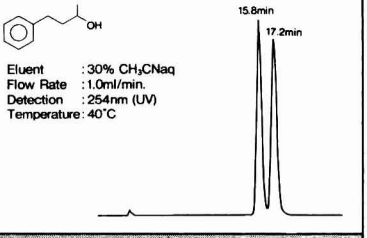

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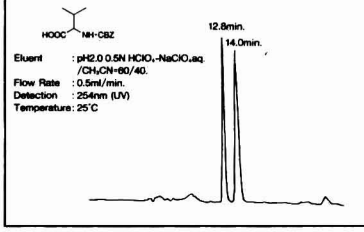

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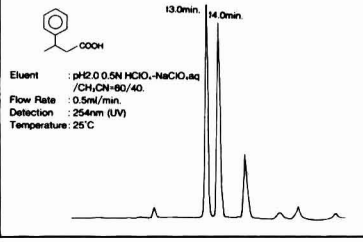
3-Phenylbutyric acid



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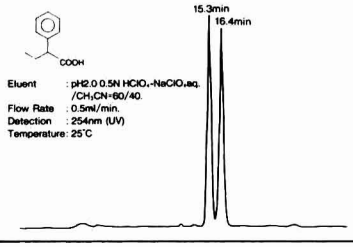
Detection : 254nm (UV)
Temperature: 25°C

2-Phenylbutyric acid



Eluent : pH2.0 0.5N $HClO_4$-$NaClO_4$aq. /CH_3CN=60/40.
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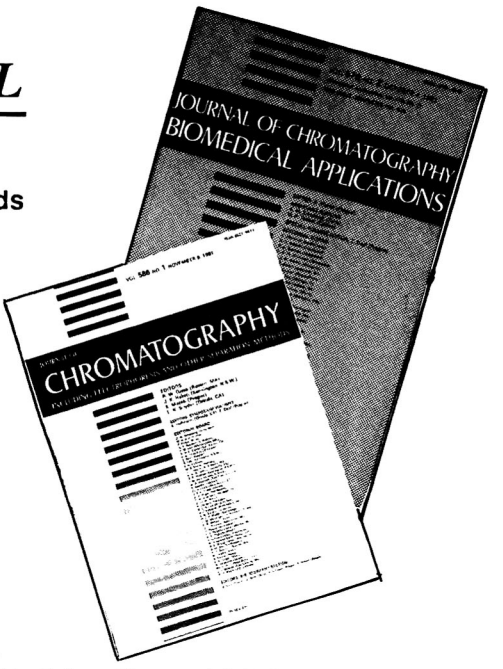

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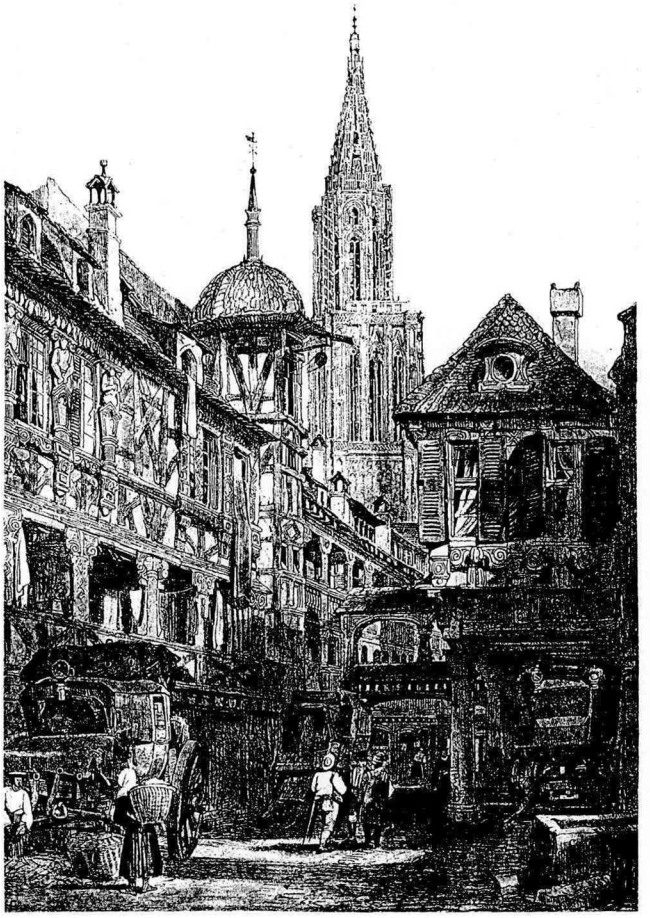

FIRST EUROPEAN SYMPOSIUM ON FAST PROTEIN LIQUID CHROMATOGRAPHY OF BIOMOLECULES

Strasbourg (France), October 19–20, 1992

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1ST EUROPEAN CONFERENCE ON FAST PROTEIN LIQUID CHROMATOGRAPHY OF BIOMOLECULES, STRASBOURG, OCTOBER 19–20, 1992

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CHROM. 25 263

Preface

The first European Symposium on FPLC of Biomolecules was held at the Palais de Congrés in Strasbourg, France, October 18–20, 1992. FPLC meetings have been held before in Germany and The Netherlands. In a year of European integration, their success suggested that it was the right time to organize an European meeting offering a wider platform for exchange of experience between researchers in the many fields in which FPLC is now used. In the 10 years following the introduction of FPLC, it has become an important tool in many biochemical and biomedical research areas, changing protein purification from a tedious and troublesome task to a quick and simple technique where results are obtained in minutes rather than hours. The topics for the meeting also included recent extensions of the technology to both larger scales (BioPilot) and micropurification (SMART System).

The symposium was attended by 150 registered delegates from 16 countries and included participants from outside Europe. The contributions covered a range of topics from summaries of purification and analysis strategies and schematic overviews of specific applications of FPLC in a wide range of research fields to fascinating presentations of innovative instrumentation for protein analysis.

Biomolecules need to interact under optimal circumstances with a well chosen matrix before they can be separated. The Strasbourg meeting was indeed a well chosen matrix for scientific interactions and a fitting celebration of 10 years of productive work with FPLC.

The present issue of the *Journal of Chromatography* contains a number of papers presented at this first European FPLC symposium. Hopefully it will be the beginning of a series, and from the positive response my guess is, there will be a next European symposium in . .

For the Scientific Committee

(Rotterdam, Netherlands) Felix W.M. de Rooij

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Review

Preparative chromatography of biomolecules

Alois Jungbauer

Institut für angewandte Mikrobiologie, Universität für Bodenkultur, Nussdorfer Lände 11, A-1190 Vienna (Austria)

ABSTRACT

An overview of preparative chromatography is given with a description of both theoretical and practical aspects. Owing to the large-scale production of recombinant proteins in various hosts, the requirements for speed, recovery and purity are ever increasing. The introduction of new gels with higher stability, a better understanding of the adsorption process and significant improvements in equipment such as injectors, pumps, fractionation devices and valves have transformed chromatography from an art to science and technology. The rules for scale-up are well understood (constant height, constant height-to-diameter ratio, dynamic similarity) and theoretical solutions including computer programs are available to minimize experimental work.

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1. INTRODUCTION

The requirements for amount, purity and final concentration of particular biomolecules may influence the purification strategy, the technique of scale-up of column liquid chromatography and the selection criteria for packings, etc. The requirements for scale-up also depend on the

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intended use of the material, *i.e.*, is it only for research purposes, for clinical trials or for marketing as a fine chemical? In this paper, an attempt is made to give an overview of these requirements (Table 1). Detailed instructions depend on the particular case, but general guidelines for preparative chromatography can be applied for all of the purposes listed in Table 1.

The degree of purity, composition of starting material, stability of product and amount are the most important parameters in the design of a preparative chromatography protocol. These parameters influence the complexity and the selection of unit operations. For optimization of a total process, the cloning of the protein product with the appropriate expression system and host, the fermentation strategy and the purification must integrate well. For an understanding of these aspects, this paper will focus on the influence of the preparation of starting material on chromatography at a later time.

Conventional laboratory purification procedures for protein consist of a sequence starting with clarification by centrifugation (often in connection with flocculation), precipitation, fol-

TABLE 1

REQUIREMENTS FOR PURITY, HOMOGENEITY, AND RELATED PROPERTIES, OF PROTEINS FOR RESEARCH AND DEVELOPMENT

Intended use	Amount required	Purity criteria
Immunization	Depends on immunogenicity, less than 100 mg per animal, even for large animals such as goats	>95% for conventional immunization; crude material for sensitizing spleen cells used for hybridoma production
Animal experiments	Depends on experiment, number of animals, etc.	Avoid denaturation for long-term studies; avoid bacterial endotoxins
Clinical trials with humans[a]	Depends on experiment, potency of substance, etc.	>99.9% purity; less than 10 (100) pg DNA per dose; avoid adventitious agents, *e.g.*, viruses, incomplete virus particles, bacterial endotoxins
Protein characterization		
Crystallization	10–100 mg	99%
NMR studies	10–100 mg	High concentration necessary, >1 mg/ml
Spectorscopic studies, *e.g.*, CDR studies	1–10 mg	Avoid denaturation, may influence result
Amino acid sequencing, amino acid composition	<1 mg	Avoid amino acid buffers in final purification steps; partial denaturation is acceptable; purity is not as critical as with amino acid sequencing
Fine chemicals		
Growth factors, inhibitors, nutrition factors, etc., especially in cell culture	Depends on situation	Purity is not most important criterion; avoid bacterial endotoxins and contaminants with mitogenic, inhibitory and growth-interfering properties
Preparation of reference materials	Depends on situation	Purity is often not most important criterion; high stability; avoid proteases

[a] Very detailed instructions are available, *e.g.*, for recombinant DNA-derived products and monoclonal antibodies.

lowed by a series of chromatographic steps [1,2]. Desalting, if necessary, is performed by dialysis. Modern procedures often use microfiltration and ultrafiltration combined with a series of chromatographic steps [3–5]. Desalting is carried out by gel filtration on small columns packed with gels of low exclusion limits such as Sephadex G-25 (Pharmacia, Uppsala, Sweden) Trisacryl (Sepracor/IBF, Villeneuve la Garrene, France) or Bio-Gel P media (Bio-Rad, Richmond, CA, USA). With a few exceptions, chromatography is not used in the initial step of protein purification procedures [6,7].

The development of affinity chromatography [8,9] in the 1960s brought a new dimension to biochemistry and related fields. Proteins could be purified to homogeneity easily and purification sequences could be greatly simplified.

In the early 1980s, when the first foundations of genetic engineering companies created a boom in biotechnology, the introduction of rigid gels and equipment such as fast protein liquid chromatography (FPLC) into the field of preparative chromatography initiated a new era in the purification of biomolecules. Fast and reproducible methods could be established by taking advantage of the high precision in flow, the simple packing and the construction of inert materials for use with buffers employed for protein chromatography. Later, the on-line monitoring of pH and conductivity converted preparative chromatography from art to science and technology. The development of a scaled-up version with the same features of the analytical process was a logical consequence as FPLC became popular in the protein chemist's world.

2. ANALYTICAL *VERSUS* PREPARATIVE CHROMATOGRAPHY

The common characteristic of both types of chromatography is the interaction and distribution of a solute between a mobile phase and a stationary phase while the mobile phase is passing through the stationary phase, hence the different solutes are separated according to their distribution coefficients and some other physical parameters with minor influences.

Protein purification is accomplished primarily by liquid chromatography (LC), therefore further considerations deal only with LC where the stationary phase is packed into a column. The different types of chromatographic procedures and the principles of action are listed in Table 2. Packings and stationary phases in preparative LC have been reviewed comprehensively several times [11–17]. Features of the material did not change, but a few packings disappeared from the market as new materials were developed.

In analytical chromatography, a small sample

TABLE 2

TYPES OF CHROMATOGRAPHY DISTINGUISHED ACCORDING TO THE NATURE OF RETARDATION FORCES AND THE PRINCIPLE OF SEPARATION

Modified from Jungbauer and Jansen [10]. All listed types are used for purification of biomolecules.

Name	Action principle	Separation according to
Adsorption chromatography	Surface binding	Molecular structure
Ion-exchange chromatography	Ion binding	Surface charge
Molecular sieve chromatography (gel filtration)	Steric exclusion	Molecular size and shape
Affinity chromatography	Biospecific adsorption/desorption	Molecular structure
Hydrophobic (interaction) chromatography	Hydrophobic complex formation	Hydrophobicity and hydrophobic patches
Covalent chromatography	Covalent binding	Functional groups
(Metal) chelate chromatography	Coordination complex formation	Complex formation with transition metals
Reversed-phase liquid chromatography	Hydrophobic complex formation	Hydrophobicity

is applied to the column and maximum resolution is sought by exploiting the characteristics of the different phases of the system. In preparative chromatography, maximum throughput at a defined purity is the principal consideration. Therefore, an adsorption/desorption mode is preferred. The process can be divided into several steps:

equilibration	→ adjusting mobile and stationary phase to binding conditions
sample application (loading)	→ loading of protein solution on to column
washing	→ removal of unbounded material, often several steps
elution	→ desorption of desired compound by stepwise or continuous change of mobile phase composition
regeneration/ cleaning	→ desorption of very sticky impurities, *e.g.*, colours, endotoxins, lipids
preservation/ sanitation	→ change of mobile phase to avoid microbial contamination during storage

Elution can be carried out be instantaneously either by changing the mobile phase (stepwise elution of step gradient) or by continuously changing the mobile phase (linear gradient).

The use of buffers and materials for different steps cannot be generalized, as they depend greatly on the separation method chosen. With a few exceptions [hydrophobic interaction chromatography (HIC), reversed-phase LC (RPLC)] aqueous buffers are used as mobile phases. One should use caution in using oxidizing agents for cleaning columns as most packing materials are very sensitive to these agents. Sodium hydroxide solutions combined with detergents are compatible with most gels and sufficient cleaning may be achieved. Hydroxyapatites and silicas are very sensitive to alkaline solutions [18–20]. Storage of columns in 20% ethanol or 2-propanol avoids microbial growth in most instances. Some columns also withstand practical mistakes such as pumping air into into them [21].

2.1. Theory of adsorption/desorption mechanism

Velayuahan and Horvath [22] generalized the adsorption/desorption phenomena for all types of chromatography by introducing the term mobile phase modifier (MPM), which is responsible for desorption, *e.g.*, salts in ion-exchange chromatography, chaotropic agents in affinity chromatography, acetonitrile or methanol in RPLC.

The interaction of a solute with the stationary phase expressed as the retention factor or distribution coefficient (K) is plotted against the MPM concentration in the mobile phase (Fig. 1). The biomolecule is adsorbed at low MPM concentrations and desorbed at high concentrations, as the retention factor becomes very high at low salt and very low at high salt concentrations. Several types of interactions are observed, *e.g.*, a strong dependence of the retention factor which coincides with the K value of MPM and a weak dependence of MPM. The relationship between

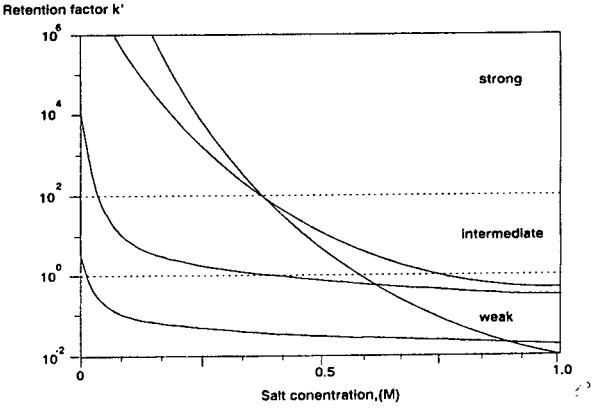

Fig. 1. Distribution coefficient (K) or capacity factor (k') *versus* salt concentration generalized as mobile phase modifier (MPM) concentration according to Velayudhan and Horváth [22]. A protein located in the strong binding domain has a tendency to be retained in the column. Changing the MPM retention will continuously alter the retention. For adsorption/desorption an on–off mechanism is desirable

the available K value and the retention factor k' is expressed by the equation

$$k' = (v_e - v_0)/v_0 \quad (1)$$

and

$$K = (v_e - v_0)/(v_t - v_0) \quad (2)$$

where v_e is the elution volume, v_t is the total column volume and v_0 is the void volume. The term $(v_t - v_0)$ is the dimensionless fraction of stationary phase, which can also be expressed by $1 - \varepsilon$, where ε is the void fraction. Inserting eqn. 2 in eqn. 1 leads to

$$k' = K(1 - \varepsilon) \quad (3)$$

The relationship between k' and salt for an isocratic retention model in ion-exchange chromatography (IEX) can be approximated by the equation

$$\log k' = m \log(1/c) + \log a \quad (4)$$

proposed by Kopaciewicz *et al.* [23], where c is the MPM concentration and m and a are constants characteristic for a given separation system describing the involvement of charged groups in the adsorption/desorption process. The curves shown in Fig. 1 can be fitted well by this equation.

As a consequence of the MPM concentration-dependent change of k' or K, several types of elution are observed. IEX is used as an example for the explanation of this behaviour.

Using a stepwise elution protocol, a sharp peak is often eluted together with the wave of the salt. This type of elution is also called fronting or type I [24]. When the salt concentration in the elution buffer is decreased, a broader peak will be observed. The substance is travelling down the column behind the salt front (Fig. 2). The elution profiles are called type II elution [24]. Of course, type I elution is preferred, as the protein is highly concentrated in contrast to that observed with type II and profiles generated by linear gradients (Fig. 3). The sample volume (loading volume) does not influence the peak width (Table 3). In a purification experiment for human monoclonal antibodies we obtained a 40-ml peak fraction from a

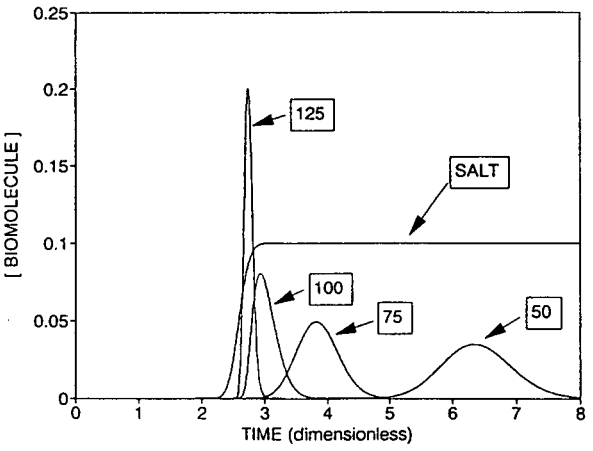

Fig. 2. Types of elution profiles in stepwise elution. Type I or fronting is characterized by elution of the protein together with the salt front and type II by elution of the protein behind the salt front. Calculated values of an IEX system described in ref. 64. Calculation was carried out with different salt concentrations.

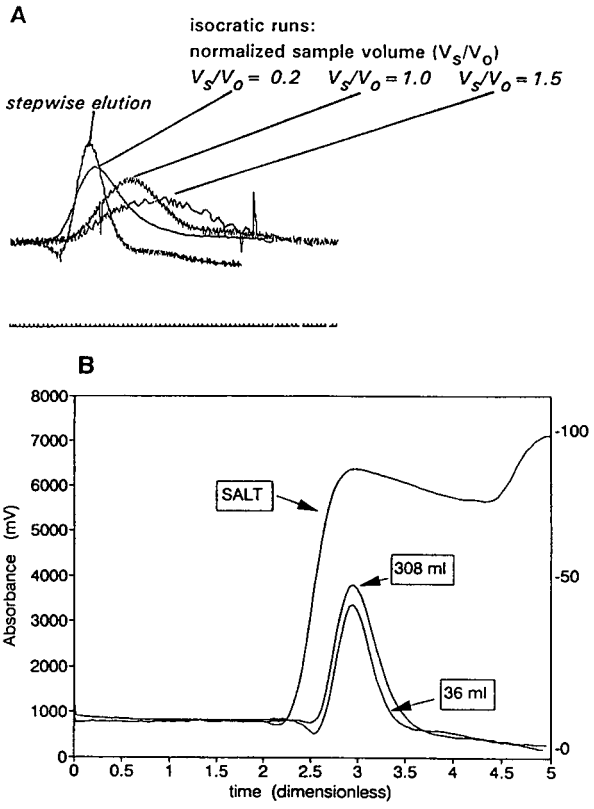

Fig. 3. Influence of loading volume on peak volume. (A) Experimental results of an ion-exchange purification of SOD on DEAE-Sepharose fast flow; (B) elution profile of the same purification system with stepwise elution.

TABLE 3

DEPENDENCE OF PEAK VOLUME ON LOADING VOLUME IN STEPWISE ELUTION GENERATING TYPE I ELUTION PROFILES

The model was purification of SOD with a DEAE-Sepharose fast flow using 100 m*M* NaCl for desorption. The detailed experimental conditions for loading, washing and desorption are described elsewhere [27]. v_s = Sample volume, v_p = peak volume and v_0 = void volume of the column.

Dimensionless sample volume, v_s/v_0	Dimensionless peak volume, v_p/v_0
0.2	1.5
1.1	0.65
10.7	0.6
11.3	0.6

10-ml CM-Sepharose fast flow column loading with 2150 ml of desalted culture supernatant [25]. In the scale version, 100 l were percolated over a 1000-ml column and the protein was recovered in less than 1 l, indicating a concentration factor higher than 100. Malm [26] reported a monoclonal antibody purification, in which concentration factors up to 200 were achieved. These examples indicate that optimization should be directed to type I elution profiles, when a salt gradient is defined. A simple approach is to load the sample under optimum binding conditions and conduct the elution procedure with a series of consecutive elution steps [25,27].

The different types of elution profiles should not be mixed with the concentration-dependent behaviour of adsorption isotherms, which are normally described as Langmuir adsorption isotherms (Fig. 4). As a consequence of this physical behaviour, the bulk of the solute is eluted earlier than traces. Therefore, one cannot transfer analytical resolution data to preparative chromatography under conditions of overloading biomolecules. Analytical chromatography should be carried out in the linear range, whereas preparative chromatography should be carried out in the saturated range. Divergences of Langmuir plots in the shape of sigmoid curves have also been reported [28], but may have minor

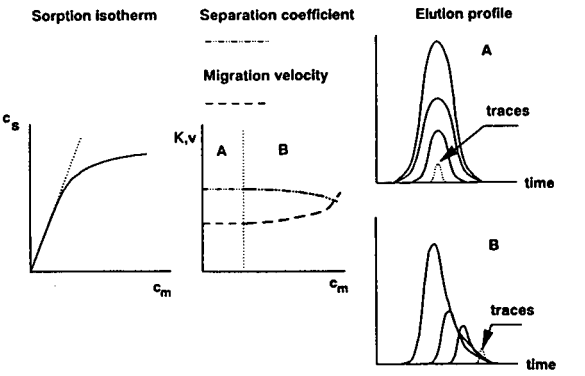

Fig. 4. Influence of sorption isotherm on the elution profile and migration velocity. K = distribution coefficient described by the slope of the sorption isotherm; v = migration velocity, c_m and c_s = mass concentration of solute in mobile and stationary phase, respectively.

consequences for the elution profile than chromatography under non-equilibrium conditions.

A preparative chromatographic system is sufficient characterized by a three-dimensional plot of *K versus* MPM concentration and concentration of the solute (Fig. 5). For practical work it is not necessary to know the exact relationship between *K*, MPM concentration and solute concentration, but one has to keep in mind the type (weak, moderate or strong) of elution behaviour when the gradient is designed.

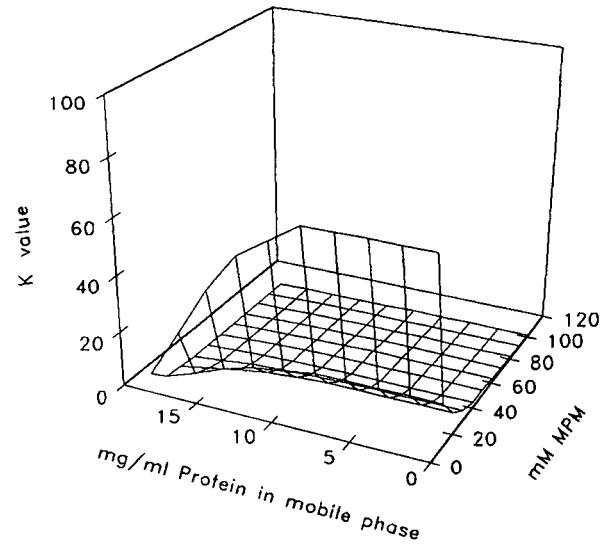

Fig. 5. Three-dimensional plot of *K versus* MPM concentration and protein concentration in the mobile phase under equilibrium conditions.

The adsorption/desorption conditions are also influenced by the ligand density [29]. Therefore, one cannot transfer the conditions from one matrix to another. Also, it should be noted that the ligand density influences the peak profile; the lower the density, the more pronounced is the band broading.

2.2. Adsorption/desorption in hydrophobic interaction chromatography

HIC has received increasing attention as it is an effective step that can be directly linked to IEX, affinity chromatographic (AC), gel filtration (gel permeation) chromatographic (GPC) or non-chromatographic purification steps. The effect of salt at a significantly high ionic strength (m) can be described as

$$\log(k'/k'_0) = \lambda m \quad (5)$$

where λ is a parameter that measures the retentive strength and is similar to the salting-out constant [30]. An early report from Pahlman *et al.* [31] noted that the protein may undergo a conformational change at the high salt concentrations used (up to 3 M) in HIC. Additionally, HIC is very sensitive to the temperature of the loading step and to the residence time in the column. Detailed instructions for the adjustment of retention behaviour in HIC were given by Melander *et al.* [30]. By modifying the hydrophobic ligand, the selectivity of the salt-promoted interaction (as named by Porath [32]) can be increased.

2.3. Adsorption/desorption in affinity chromatography

As the adsorption in AC is biospecific and driven by the interaction of a protein or a biomolecule with a ligand with a certain molecular structure [33], adsorption cannot be generalized. Adsorption also can occur at both low and high salt concentrations and in a broad range of pH values. The effects of ligand density on retention behaviour and capacity are comparable to those seen with other types of chromatography [34]. Desorption can be performed by addition of free ligand or of a mimic of the ligand to the mobile phase (affinity elution [35]) by changing the pH or ionic strength of the mobile phase. Electrophoretic elution and high-pressure elution have also been described, but are not broadly applied [36–38]. Chaotropic ions are very well suited for desorption. They disrupt the structure of the binding between the ligand and the biomolecule; often the recovery is low owing to denaturation during desorption. Group-specific adsorbents such as protein A [39], protein G, lectins [40,41] and triazine dyes [42] with a broad binding spectrum are very popular. This interaction is based on a particular protein domain which is conserved during evolution or is a random event as with lysine-Sepharose used for the isolation of plasminogen, plasminogen activators, rRNA and dsDNA.

Immunoaffinity chromatography is the most universal technique especially considering the monoclonal antibodies as ligands. Immunoaffinity chromatography has industrial applications for purifying materials such as interferon, FIX, FVIII and EPO and for vaccine purification. Ligand leakage is still an unsolved problem in AC. Binding chemistry and molecular orientation of the ligand are largely responsible for the leakage and loss in performance. The immobilization chemistry (reviewed by Narayanan and Crane [43]) is directed to immobilization of high-molecular-mass biomolecules with a single-point attachment, whereas small molecules are immobilized with a spacer with an optimum length.

The purification power of AC in the field of biomolecules is ranked the highest among all types of chromatography for its effectiveness in retaining biological activity.

3. SCALE-UP OF COLUMN LIQUID CHROMATOGRAPHY

Examples are discussed where the same particles are used for the separation of proteins on a different scale. Owing to the incompressibility of modern gel particles, the different packing density of various column heights also can be neglected. If particle types are changed during scale-up, the situation becomes complicated.

3.1. Starting material

Although it seems trivial, altering the starting material during processing can greatly change the characteristics of the system. Fermentation conditions may change for several pragmatic reasons (cheaper medium or ingredients) or the sampling of tissues or biological fluids is changed. To illustrate this problem, disintegrated *Escherichia coli* produced by different methods used in the experiment are described in Fig. 6. The composition changed dramatically.

3.2. Repeated cycles

Repeating cycles also may appear trivial unless one considers the details. When a purification procedure consists of several steps, one has to calculate carefully whether offset cycling or staggered cycling should be used (for definition, see Fig. 7). Fulton *et al.* [45] studied several cases and concluded that staggered cyclings were superior to offset cyclings. Offset cycling is simpler than staggered cycling and therefore applied more often on the laboratory scale.

One also has to take into account fouling of columns [46] and ligand leakage [47,48]. Fouling at the top of the column and ligand leakage change the peak profile. The peak becomes broader and tailing increases. For safety reasons and for obtaining reproducibly high yields, preparative chromatography is not carried out at the capacity limit in practice. Therefore, ageing of

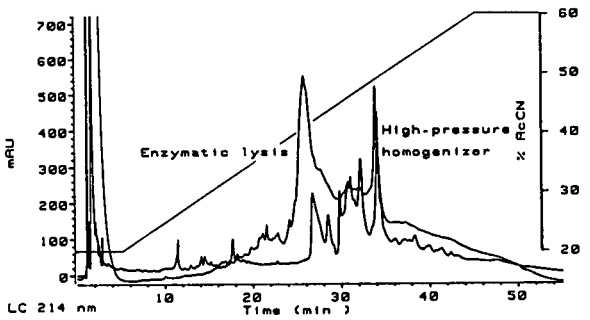

Fig. 6. Analytical reversed-phase liquid chromatography of an *E. coli* homogenate produced by enzymatic lysis with lysozyme (laboratory-scale method) and with a high-pressure homogenizer (pilot- and technical-scale method). The experimental conditions are described in ref. 44.

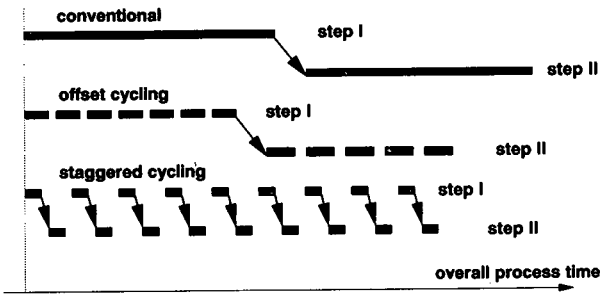

Fig. 7. Time profile of two subsequent chromatographic steps using conventional scale-up, offset cycling and staggered cycling, according to Fulton *et al.* [45].

columns caused by loss of ligands cannot be detected from decreased yields. Ageing only affects the purity of the eluted biomolecule; especially non-proteinaceous contaminants will increase.

As long as the column is not clogged, backflushing after several steps is advisable to increase the column lifetime. Lifetimes of more than 1000 cycles have been reported for preparative gel filtration columns [49,50] and HIC columns [51]. The earlier a column is used in the process scheme the shorter the lifetime will be.

3.3. Column overload and multiple injections

In practice, column overload with solute molecules is the first attempt to purify more material. Loss in resolution caused by column overloading was described in detail by Gareil *et al.* [52]. In this particular situation, the shape of the isotherm plays an important role. Under isocratic conditions the throughput can be optimized [53,54] more easily than under gradient conditions [55–58]. Recently an analytical solution for volume-overloaded gradient elution chromatography was described [59]. A detailed treatment of the problem would exceed the scope of this paper, but one should bear in mind that overloading can be achieved by application of a more concentrated sample (concentration overload) or by a larger volume (volume overload).

Especially in RPLC and HIC, multiple injections are in common use. Proteins are not soluble or stable in the mobile phases such as

50% saturated ammonium sulphate or 0.1 *M* trifluoracetic acid. Multiple injections of proteins dissolved in a suitable buffer followed by mobile phase circumvent this problem. In Fig. 8A, the pilot-scale purification of recombinant single-chain antibody by means of HIC is shown using the technique of multiple injections.

3.4. Increase column size by diameter

The conventional way to scale up preparative chromatography is to increase the column diameter using the adsorption/desorption mode, if the bead size or bead size distribution is not changed from the laboratory to the preparative

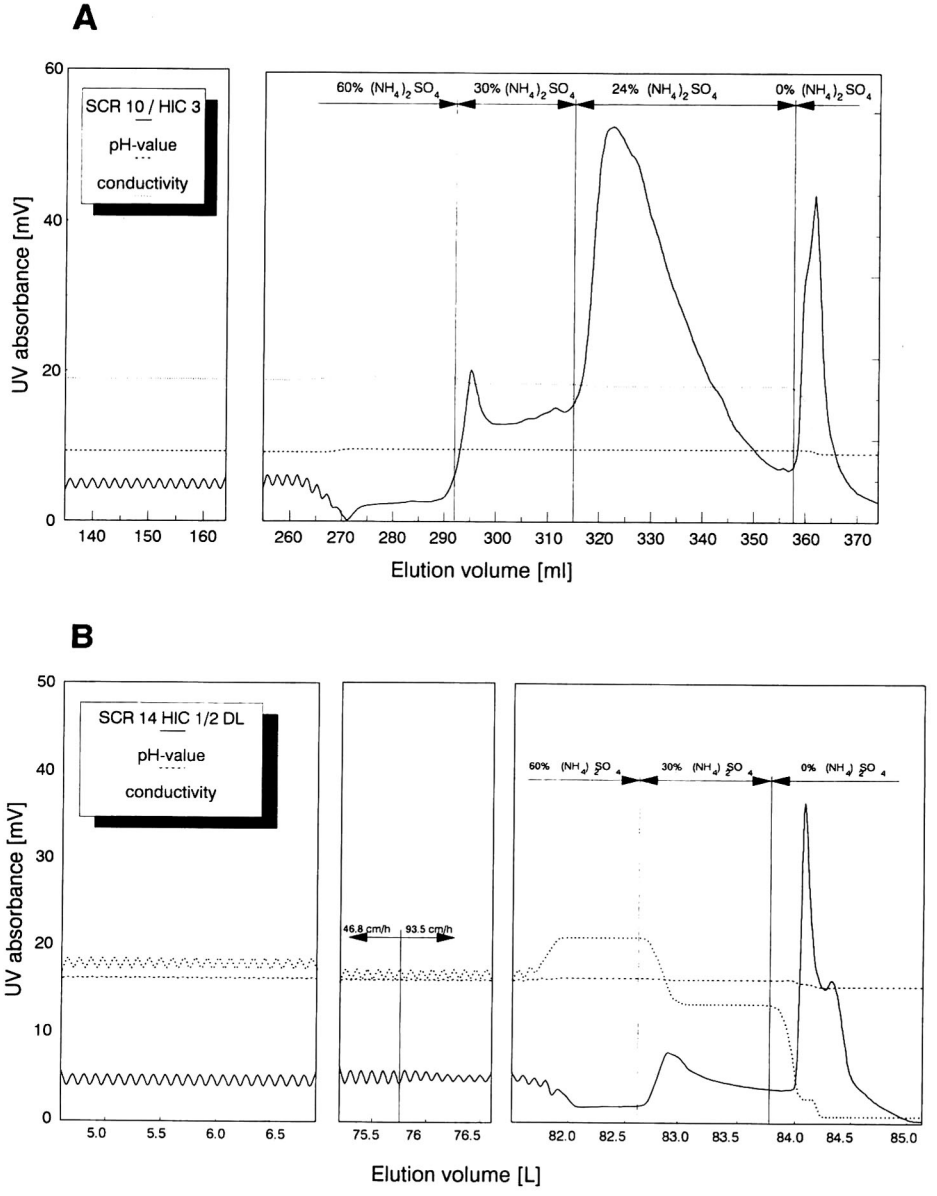

Fig. 8. Pilot-scale purification of a recombinant single-chain antibody by phenyl-Sepharose fast flow using repetitive injections of protein solution at low salt concentration followed by 60 m*M* ammonium sulphate buffer. (A) Laboratory-scale experiments; $H = 13.5$ cm and $D = 0.9$ cm. (B) Pilot-scale experiment; $H = 14$ cm and $D = 7$ cm. Solid line, UV absorbance; dashed line, pH; dotted line, conductivity.

TABLE 4

SCALE-UP OF DEAE-SEPHAROSE FAST FLOW CHROMATOGRAPHY OF HUMAN SOD FROM CLARIFIED ERYTHROCYTE LYSATE [27]

Elution was effected stepwise with 100 m*M* salt. v_t = Total column volume; *H* was kept constant for both pilot- and large-scale runs.

Run	Column volume (l)	Loading volume (l)	Peak volume (l)	Peak volume (% v_t)	Recovery (%)
Pilot 1	20	50	4.0	20	90
Pilot 2	20	57	3.8	19	92
Pilot 3	20	69	2.7	13.5	95
Large 1	159	576	26.3	17.5	89
Large 2	150	500	37.0	24.6	96

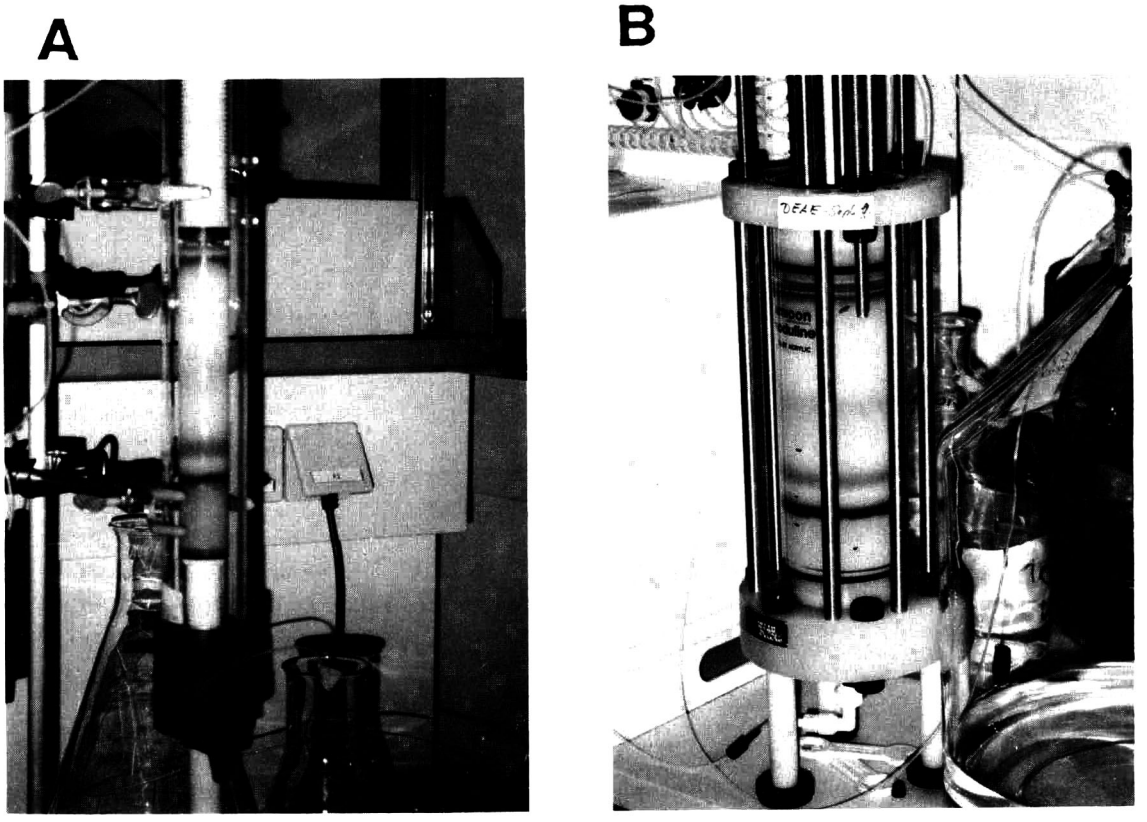

Fig. 9. Purification of SOD on the laboratory scale (18 cm × 2.6 cm I.D. and 18 cm × 9 cm I.D.). The small column was connected to an FPLC set-up and the large column to a Biopilot. A linear flow-rate of 25 cm/h was kept constant. The runs were carried out simultaneously with the same starting material. Identical patterns on the columns were observed.

scale. If different particles sizes are used on the small and large scales, additional experiments are necessary below the process size can be increased. Owing to the decrease in resolution when using larger particles or a broader distribution of particle sizes, the height also must be adjusted to the new packing material. For pilot- and industrial-scale chromatography it is necessary to optimize the resolution and height on the bench scale [60], then further scale-up is performed simply by increasing the column diameter (Table 4). In Fig. 9, scale-up by increasing the column diameter is demonstrated with a laboratory-scale and a pilot-scale column connected to an FPLC system or a Biopilot (Pharmacia Biotechnology). The resolution pattern and the peak profiles are identical with one exception: the scale.

The construction of the column inlet is an important consideration if this mode of scale-up is to be feasible. An even distribution can be achieved by nets and grids placed on the inlet adapter or by small radially arranged buffels or frit profiles [61].

IEX and AC operated with stepwise elution are very insensitive to changes in column geometry. The finding that the shape of the column has no influence on either the shape of the breakthrough curve [62,63] or the peak shape and volume indicate the use of short, wide columns to minimize the pressure drop and process time [24,64,65]. Linear gradients in conjunction with binding characteristics (Fig. 1) should be investigated more carefully prior to a change of column geometry [66]. Also, the use of the salt (often called displacer) has an influence on band broadening. The effect of the displacer ion appears to be dependent on the relative position of the ion in the lyotropic series [67,68]. The overall advantage of this mode of scale-up is a constant cycle time on all scales.

3.5. Increase column size by constant height-to-diameter ratio

In RPLC, single-point injection is commonly employed. In that event, the column size must be increased by a constant height-to-diameter ratio (H/D). In single-point injection, a certain height of a valuable packing is sacrificed for an even distribution of the sample over the cross-sectional area of the column.

Using line elution chromatography (*e.g.*, size-exclusion chromatography or frontal chromatography), an efficient scale-up is achieved by increasing the column size to a constant H/D.

In some instances, scale-up processing by just increasing the column diameter is not successful. The loss of resolution may be caused by irregularities in the bed, an unequal distribution of the sample over the gel surface or propagation of disturbances in the gel. HIC columns are more sensitive than other packings to such phenomena. Vorauer *et al.* [44] reported the purification of recombinant superoxide dismutase (SOD) as a model for scale-up of HIC with constant H/D.

The cycle time (θ_1, θ_2) increases with increasing size of the column according to

$$\theta_1/\theta_2 = H_1/H_2 \quad (6)$$

where H_1 and H_2 are the heights of the small and large column, respectively. The volumes of the columns, V_1 and V_2 change by the factor

$$V_1/V_2 = (H_1/H_2)^3 \quad (7)$$

This mode of scale-up should be avoided if at all possible.

3.6. Scale-up by dynamic similarity

Columns with constant H/d_p are called isochronic. Scale-up processing by dynamic similarity is not carried out in practice. Experiments with larger particles must be carried out on the laboratory scale to ascertain that the resolution is sufficient. For theoretical considerations and optimization of economics in a process, scale-up with constant H/d_p plays an important role. Wall effects are negligible in large-scale chromatography. On the 1–100-ml scale, wall effects influence the performance of chromatography. The larger the column, the smaller are the wall effects.

4. PRACTICAL ASPECTS OF SCALE-UP OF PROTEIN CHROMATOGRAPHY

4.1. Flow-rate, dynamic capacity

Flow-rate and dynamic capacity (defined as

adsorbed solute at a particular flow-rate) are important parameters in preparative chromatography using rigid gels, which allow a linear flow-rate up to 1500 cm/h (*e.g.*, SP-Sepharose fast flow). The mechanical stability allows a high flow-rate but the dynamic capacity decreases with increasing flow-rate. Therefore, an optimum must be found at which the column should be operated.

Conventional soft gels and rigid gels are available for a broad range of applications. The relationship between flow-rate and dynamic capacity is valid for all types listed in Table 1. Conventional soft gels continue to be used owing to their inertness. The introduction of cross-linkers also changes the surface hydrophobicity or charge of the surface of a gel particle. As a result of the enhanced mechanical stability to some extent the inertness of the gel is sacrificed.

4.2. Column packing

The packing of a soft gel with a broad distribution of particle size is very time consuming and the packing quality may differ from experiment to experiment. One rule should be obeyed: the particles must be held in motion to avoid separation of particles according to Stoke's law. Using rigid particles, the packing procedures are very simple. A slurry is poured or pumped into the column [69], then the particles are allowed to settle. Separation of particles into different sizes will not occur because the settling velocity is very high. The adapter is placed on the liquid surface and a flow is started at a higher rate than used during separation. When the gel bed does not change, the adapter is lowered on to the gel surface. For optimum distribution of the sample, the adapter should not touch the surface. This type of packing is a mixed procedure of a method called semi-constant-pressure packing and gravitational packing [70,71].

The packing quality is easily checked by a pulse response experiment, applying a small volume of salt to the column, or by the frontal curve of salt when the column is equilibrated.

4.3. Gradient formation

Reproducible gradient formation is an obvious requirement when a preparative chromatographic scheme is elaborated. For shallow gradients, one should bear in mind that continuous mixing of solutes is very imprecise at a mixing ratio of less than 1:20. If highly accurate mixing is necessary, elution buffer should be prepared beginning with 5% buffer B and elution should be completed within 95% B. The upper limit of precision was well demonstrated by Kaltenbrunner *et al.* [72]. A linear pH gradient produced with an LCC 500 gradient programmer and two P500 pumps and a 1-ml mixer varied by less than 0.1 pH/ml flow.

4.4. On-line measurement of pH and conductivity

On-line measurements of pH and conductivity are recommended, as pH and salt concentration are the most important criteria for protein chromatography. All steps can be examined quickly and accuracy is improved owing to the continuous monitoring.

4.5. Yield and productivity

The performance of different processing steps should be compared on the basis of solute productivity (P). Yamamoto and co-workers [73,74] defined productivity by

$$P = [(\text{recovery ratio})(\text{sample feed volume})]/[(\text{column volume/cycle time})]$$

Productivity has an optimum range (Fig. 10). At very high flow-rates the productivity again decreases. The recovery ratio, better known as the yield or recovery, is influenced by the operating conditions, stability of the protein, source of the protein and the presence of degrading enzymes. A general rule is that the faster the sequence of purification steps is performed, the higher is the yield. Often this rule is not too valid when considering a single step. Also, operation at 4°C does not guarantee higher recovery. Rapid oper-

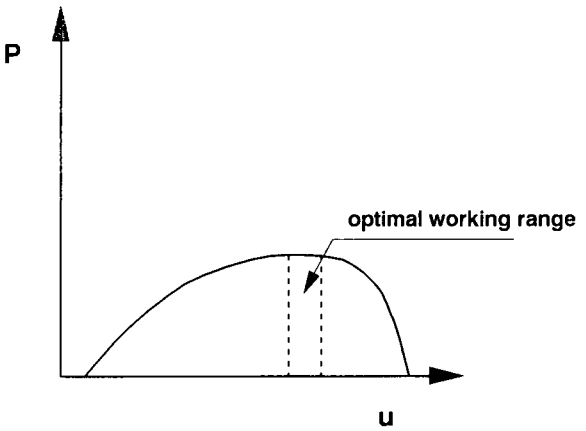

Fig. 10. Productivity *versus* velocity according to the definition by Yamamoto and Sano [73].

ation using FPLC strategies can circumvent cool room operation, however.

5. CONCLUSIONS AND FUTURE PROSPECTS

Preparative chromatography retains an indisputable position in the purification of biomolecules and the preservation of their activity. A variety of specific adsorption/desorption systems with high yield productivity and simple performance make this technique superior to others such as counter-current extraction and preparative electrophoreis. Improvements of the kinetic by the introduction of through-pores in the particle [75] or by membrane adsorption are current trends. Nevertheless, one should not forget that kinetics in preparative chromatography are not the only parameter. The overall story depends more on productivity, which includes resolution, dynamic capacity and resistance to fouling and clogging. Preparative chromatography continues to serve well "when one wants more" of precious biomolecules serving mankind.

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Application of non-size-related separation effects to the purification of biologically active substances with a size-exclusion gel

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ABSTRACT

Most reports on the size exclusion of biomolecules describe chromatographic conditions designed to ensure a pure size-exclusion process. However, other retention mechanisms are also associated with size-exclusion chromatography, resulting in non-size-related separation effects. Applications of the size-exclusion gel Sephacryl S-100 High Resolution (HR) employing non-size-related separation effects, are described. With the help of non-size-related effects, purification and desalting of a biologically active, low-molecular-mass substance (<1000 g/mol) was achieved. To gain a better understanding of the non-size-related effects, the retention behaviour of the S-100 HR gel was examined with low-molecular-mass substances (amino acids and metabolites) and the K_{AV} values of the substances, chromatographed with high- and low-ionic-strength eluents, were compared. Coulombic interactions were most prominent at basic pH values and with low-ionic-strength eluents. Electrostatic interactions led to cation-exchange and anion-exclusion separation effects. The results demonstrate that, utilizing non-size-related separation effects, the application range of the S-100 HR gel is not limited to the fractionation of macromolecules, but may also be useful for the purification of low-molecular-mass biomolecules.

INTRODUCTION

Size-exclusion chromatography (SEC) has become a routine technique for biopolymer separation and characterization [1]. Typically, mild, non-aggressive mobile phase conditions are used, which preserve the native structure and functionality of the biopolymers [2]. Ideally solute molecules are fractionated in terms of size by equilibrium partitioning via diffusion between the mobile, fluid phase and gel pores [3]. However, the migration of the biopolymers on SEC columns is in fact influenced by electrostatic, hydrophobic, hydrogen bonding and steric effects [4]. For the analytical characterization of macromolecules it is desirable to minimize these effects. For preparative purposes non-size-related effects have been demonstrated to improve the separation of molecules of similar hydrodynamic volume [5]. Especially for the separation of peptides, non-ideal SEC has attracted increasing interest [6–9].

The utilization of non-size-related separation effects appeared to be a suitable solution for a separation problem in our laboratory. A biologically active substance with a molecular mass between 300 and 1000 g/mol [10], present in a perchloric acid extract from human platelets, had to be separated from other low-molecular-mass organic molecules and inorganic salts. To detect the biological activity a bioassay was used [11]. The bioassay only yields reproducible results if the compounds in the fractions to be tested for their vasoactive action do not exceed special physiological limits. Because of these conditions it is impossible to purify the bioactive substances chromatographically with eluents that contain

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non-volatile components. Aqueous 1 *M* acetic acid is completely volatile and therefore fitted to the needs of the bioassay. With this eluent, the size-exclusion gel Sephacryl S-100 High Resolution (HR) shows remarkable separation results, especially if low-molecular-mass substances with high affinities to proteins have to be separated from the proteins [12]. A study of Johansson and Gustavsson [13] on the interactions of some proteins with the gel matrix suggested solving the separation problem with S-100 HR employing non-size-related separation conditions. Therefore, in this study, the elution behaviours of small organic molecules with similar molecular masses, diverse aliphatic and aromatic amino acids and metabolities, chromatographed with different eluents, were examined. The results of this investigation should be of help in finding suitable separation conditions for purifying the biologically active substance from platelets.

EXPERIMENTAL

Materials

Sephacryl S-100 HR was supplied by Pharmacia Biosystems (Freiburg, Germany), HPLC-grade water by J.T. Baker (Gross-Gerau, Germany), aspartic acid (Asp), arginine (Arg), glycine (Gly), phenylalanine (Phe), vanillylmandelic acid (VMA), norepinephrine (NE), bovine insulin and tris(hydroxymethyl)aminomethane (Tris) by Sigma (Deisenhofen, Germany) and ferritin, ovalbumin, cytochrome *c* and vitamin b_{12} by Fluka (Neu-Ulm, Germany). All other chemicals were purchased form Merck (Darmstadt, Germany).

Equipment

All chromatographic equipment was obtained from Pharmacia Biosystems unless specified otherwise. The HPLC equipment consisted of a Model 2248 HPLC pump, coupled to a Rheodyne injector (Latek, Heidelberg, Germany), a Uvicord S II spectrophotometer, a flowcell for conductivity measurement (LE-191, WTW, Germany), a two-channel compensation recorder and a RediFrac fraction collector. A column with 1000 mm × 16 mm (C16/100) and a 600 mm × 10 mm column (Superformance 600-10; Merck) were used.

Eluents

Five eluents were used (see Figs. 1 and 2): (1) 1 *M* acetic acid in HPLC-grade water; (2) 30 m*M* Tris buffer (pH 9) in HPLC-grade water; (3) 20 m*M* Tris buffer (pH 9) with 200 m*M* sodium chloride in HPLC-grade water; (4) 10 m*M* hydrochloric acid; and (5) HPLC-grade water. Prior to chromatography all eluents were filtered through a 0.2-μm filter (Anotop; Merck) and degassed.

Packing procedure

The columns were packed according to the packing instructions [14]. Briefly, the gel suspension was degassed under vacuum, fines were removed and the flow-rates for packing the C16/100 column were 1 ml/min (step 1, 2 h) and 2.5 ml/min (step 2, 1 h) and for the Superformance 600-10 column 0.4 ml min (step 1, 2 h) and 1.2 ml/min (step 2, 1 h). The efficiency of the column packing procedure was confirmed by passing 200 μl of acetone (10 mg/ml in water) through the C16/100 column at 0.5 ml/min or 80 μl of acetone (10 mg/ml in water) through the Superformance 600-10 column at 0.2 ml/min. The column was repacked if the peak shape of the eluting acetone was not symmetrical.

Chromatography

The flow-rate used for the C16/100 column was 1 ml/min and that for the Superformance 600-10 column was 0.4 ml/min. UV absorbance and conductivity were measured continuously. More details are given in the captions on the figures.

Determination of partition coefficients (K_{AV})

The void volume (V_0) was determined with ferritin in eluents 1 and 3 and the total volume of the packed bed (V_t) with acetone (5 μl/ml in water). Acetone was chosen for the determination of V_t because acetone had the same elution volume with all the eluents used. The partition coefficient (K_{AV}) was calculated with the equation $K_{AV} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the individual substances.

Preparation of platelet extract

The preparation of the platelets was described by Agha *et al.* [10]. Briefly, the isolated, washed, frozen and rethawed platelets from 200 ml of blood were deproteinized with 0.6 *M* perchloric acid, followed by neutralization using potassium hydroxide. The clear supernatant (2 ml) was applied directly to the Sephacryl S-100 HR size-exclusion column (see Fig. 2).

Bioassay

Vasoactivity was measured in an isolated perfused rat kidney [11]. The isolated perfused kidney was prepared as described by Hagel [1]. As the perfusion system, a single-pass system at a constant flow-rate of 9 ml/min with Tyrode solution kept at 37°C and equilibrated with CO_2–O_2 (5:95) was used as described previously [15]. A typical dose–response curve was given by Schlüter *et al.* [16].

RESULTS

Fig. 1 shows the calibration graph for S-100 HR. The eluent used for the calibration was 1 *M* acetic acid. Within the molecular mass range 1000–100 000 g/mol S-100 HR exhibits linear elution behaviour when the logarithm of molecular mass is plotted *versus* the partition coefficient.

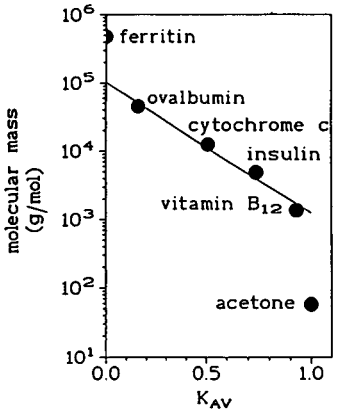

Fig. 1. Calibration graph for Sephacryl S-100 HR. Plot of molecular mass of typical calibration substances *versus* the corresponding partition coefficients (K_{AV}). Molecular masses of the substances: ferritin, 440 000; ovalbumin, 44 000; cytochrome *c*, 12 600; insulin (bovine), 4800; vitamin b_{12}, 1350; acetone 58 g/mol. Column, 1000 × 16 mm; bed size, 920 mm; eluent, 1 *M* acetic acid; flow-rate, 1 ml/min.

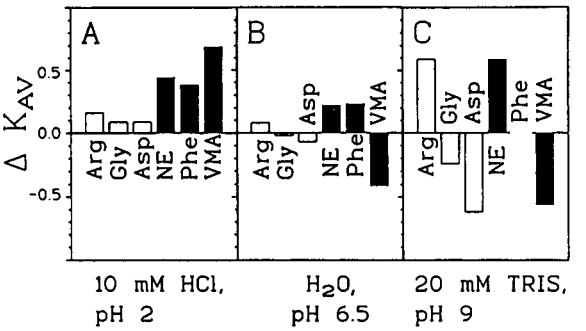

Fig. 2. Elution behaviour of standard molecules chromatographed on Sephacryl S-100 HR under non-ideal size-exclusion conditions. Plot of the deviation of the partition coefficients K_{AV} from the partition coefficients K_{AV} under ideal size-exclusion conditions. Standard molecules: open bars, aliphatic substances; filled bars, aromatic substances. (A) 10 m*M* hydrochloric acid (pH 2); (B) distilled water; (C) 20 m*M* Tris buffer (pH 9). Column, 600 × 10 mm; bed size, 550 mm; flow-rate, 0.4 ml/min; detection, UV absorbance at 280 nm or conductivity; amount of substance injected, 0.1 mg; volume injected, 100 μl. For the determination of K_{AV} single substances were injected.

In Fig. 2 the deviation from ideal size-exclusion retention behaviour of the standard molecules aspartic acid, vanillylmandelic acid, glycine, phenylalanine, arginine and norepinephrine chromatographed on S-100 HR in Tris buffer (pH 9), in distilled water and in 10 m*M* hydrochloric acid, is summarized. The retention values under ideal size-exclusion conditions were measured with 20 m*M* Tris buffer (pH 9) with electrostatic interaction suppressing 200 m*M* sodium chloride. In this buffer system all standard molecules eluted at $K_{AV} = 1$, owing to their molecular masses. In Fig. 2 negative differences from the origin reflect exclusion effects whereas positive differences reveal retention effects. In the acidic, low-ionic-strength eluent (0.01 *M* HCl, Fig. 2A) the K_{AV} values of the aliphatic amino acids glycine, arginine and aspartic acid differ only slightly from unity. The aromatic substances were retained significantly more strongly. In Fig. 2B, the deviation of the K_{AV} values of the low-molecular-mass standard substances, chromatographed in doubly distilled water, from ideal values is shown. Arginine, glycine and aspartic acid revealed only slight

differences from the ideal K_{AV} of 1. Norepinephrine and phenylalanine were clearly retained. The elution of vanillylmandelic acid was influenced by exclusion effects. In the low-ionic-strength Tris buffer (pH 9) (Fig. 2C), the retention of the standard molecules differs greatest from ideal retention behaviour. The basic molecules arginine and norepinephrine were retained whereas the neutral and acidic molecules eluted earlier than expected under ideal size-exclusion conditions.

The chromatogram in Fig. 3 demonstrates the resolving power of S-100 HR under non-ideal size-exclusion conditions. It was possible to separate vanillylmandelic acid, sodium chloride and phenylalanine from each other in water, whereas under ideal size-exclusion conditions no separation of these molecules was achieved.

The first attempt to chromatograph the platelet extract with S-100 HR was carried out in 1 *M* acetic acid (Fig. 4A). According to the calibration graph (Fig. 1), the UV absorption elution pattern indicates that most of the substances in the platelet extract have a low molecular mass. The biological activity (Fig. $4A_2$) eluted together with the main conductivity peak (Fig. $4A_1$). As this separation was not satisfactory with respect to further purification steps, the question arose of whether changes in the composition of the eluent can improve the separation.

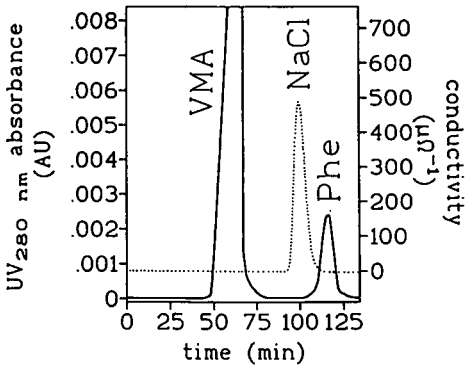

Fig. 3. Chromatogram of the separation of vanillylmandelic acid, sodium chloride and phenylalanine on Sephacryl S-100 HR. Column, 600 × 10 mm; bed size, 550 mm; flow-rate, 0.4 ml/min; left ordinate = UV absorbance at 280 nm (AU = arbitrary units) (solid line); right ordinate = conductivity (dotted line); sample, 0.25 mg of vanillylmandelic acid, 10 mg of sodium chloride and 5 mg phenylalanine, dissolved in 1 ml of water; volume injected, 100 μl.

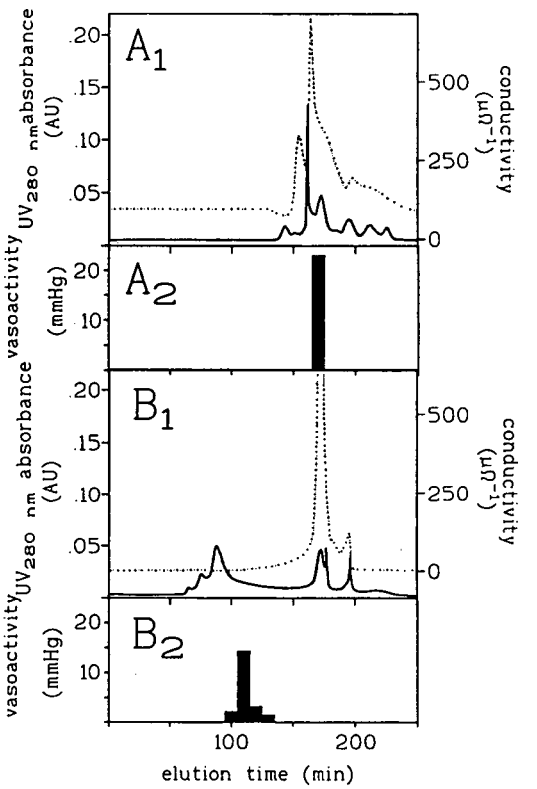

Fig. 4. (A_1, B_1) Chromatograms of typical separations of human platelet extracts in (A) 1 *M* acetic acid and (B) water and (A_2, B_2) corresponding profiles of the vasopressor actions of the fractions. Column, Sephacryl S-100 HR (1000 × 16 mm); bed size, 90 cm; flow-rate, 1 ml/min; samples, perchloric acid extract from human platelets from 200 ml of blood, dissolved in (A) 1 *M* acetic acid or (B) water; sample volume, 2 ml; A_1 and B_1, left ordinate = UV absorbance at 280 nm (solid line) (AU = arbitrary units), right ordinate = conductivity (dotted line); A_2 and B_2, ordinate = vasoactivity, measured as changes in perfusion pressure (mmHg; 1 mmHg = 133.322 Pa) after injection of the individual fractions into an isolated perfused rat kidney; abscissa, elution time.

Fig. 4B depicts the chromatography of the platelet extract with S-100 HR in water. The biologically active substance elutes between a pair of UV-absorbing peaks and the increase in conductivity (Fig. $4B_2$).

DISCUSSION

The Sephacryl S-100 HR matrix consists of allyldextran cross-linked with N,N'-methylenediacrylamide [17]. Dextrans contain small

amounts of carboxylic groups [18]. Johansson- and Gustavsson [13] demonstrated that these anionic groups are responsible for the non-size-related elution behaviour of proteins. The electrostatic interactions of the gel matrix with the proteins result in an ion-exchange and ion-exclusion mechanism. The question arose of how the electrostatic interactions affect the elution behaviour of low-molecular-mass substances and if these effects can be utilized for the purification of the biologically active platelet-derived substance. It could be considered that the biologically active substance, which binds to anion exchangers, interacts with the Sephacryl support via ion-exclusion.

Further, to evaluate the extent of interactions between molecules smaller than 1000 g/mol and the Sephacryl support, aliphatic and aromatic amino acids and metabolites were chosen as standard substances and the elution behaviours of these standard substances in different eluents were compared. Johansson and Gustavsson [13] assumed that proteins with intermediate p*I* values between 5 and 9 may not interact with the Sephacryl support when the pH of the mobile phase was 7 or 10 and the sodium concentration was higher than 0.2 *M*. According to these findings, an aqueous Tris buffer with a pH of 9 and containing 200 m*M* sodium chloride was chosen as the eluent, which guarantees minimum support–solute interactions. Because the electrostatic interactions are most prominent at basic pH values in the absence of sodium chloride, 20 m*M* Tris buffer (pH 9) was chosen as an eluent that promotes electrostatic interactions. The comparison of the retention of the low-molecular-mass substances in the two buffer systems revealed significant differences (Fig. 2C). Acidic molecules undergo ion exclusion. These molecules eluted about 0.5 K_{AV} units earlier under conditions where electrostatic interactions were present. In the case of basic molecules without suppression of the electrostatic interactions an increased retention was observed owing to ion-exchange effect.

The next question that had to be answered was whether water used as the eluent exhibits similar separation effects (Fig. 2B). Although the pH of water is lower than pH 7 it could be assumed that ion-exclusion and ion-exchange mechanisms are still present. Fig. 2B verifies this assumption. The deviation of the elution behaviour from the size-related separation in water is not as distinct as in the basic eluent with low ionic strength, but still the elution order is comparable, indicating an interaction between the standard molecules and the negative groups of the S-100 HR support. In an acidic eluent with low ionic strength (Fig. 2A) it seems that only minimal electrostatic interactions were present, but it is obvious that the aromatic molecules interact with the S-100 HR support. This interaction may be due to interactions between the aromatic systems and the ether bridges introduced by the cross-linker [19].

The calibration graph (Fig. 1) suggests that non-specific interactions of the standard proteins with the S-100 HR packing were minimal in 1 *M* acetic acid. With this eluent the purification of the platelet extract was tried first. As Fig. 4A indicates, in 1 *M* acetic acid a separation of the biologically active substance from the salts was not achieved. This result was expected because a previous study [10] showed that the biologically active substance has a molecular mass smaller 1000 g/mol. Molecules smaller 1000 g/mol, which do not interact with the S-100 HR matrix, elute near V_t, the total liquid volume of the column. With regard to the purification of the platelet extract, water as eluent seemed advantageous because it is completely volatile. Fig. 4B shows the chromatogram of the separation of the platelet extract in water. The K_{AV} value of the biologically active substance decreased from 1 in 1 *M* acetic acid (Fig. 4A) to 0.5 in water (Fig. 4B). This behaviour suggests an ion-exclusion effect. This observation corresponds to the previous finding [10] that the biologically active substance has an anionic group.

CONCLUSIONS

The utilization of non-size-related effects in the purification of biomolecules with similar size may be beneficial with size-exclusion supports, as this study demonstrates. Therefore, the knowledge of the possible interactions between support and solute is most important. With this knowledge the chromatographic conditions can be manipulated to achieve separations that are

not possible under ideal size-exclusion conditions. With the S-100 HR support the most prominent separation effects observed in this study can be obtained with low-ionic-strength buffers at basic pH values. These effects result in ion exclusion and ion exchange of the solutes. To work with low-ionic-strength eluents offers the opportunity to use even water as the eluent, which may be beneficial if complete volatility of the eluent is necessary.

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Comparison of three activated agaroses for use in affinity chromatography: effects on coupling performance and ligand leakage

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ABSTRACT

Three commercially available activated supports, N-hydroxysuccinimide (NHS)-, tresyl chloride- and hydrazide-activated agarose, were compared with respect to coupling rate and coupling efficiency for the ligand and ligand leakage during both storage and chromatography. A monoclonal antibody against the E_1 protein of the rubella virus was used as a ligand. For each support the monoclonal antibody (mab) was immobilized at three concentrations: 0, and *ca.* 2.5, 5.0 and 10 mg IgG per ml gel. The NHS-activated support showed very fast and complete binding of the ligand. Moreover, using this support the ligand leakage was considerably less both during storage and chromatography as compared with the other two supports. It was also shown that the static binding capacity was comparable for the NHS- and tresyl chloride-derivatized agaroses and it was about a factor of two lower for the hydrazide-derivatized agarose.

INTRODUCTION

Affinity chromatography based on antigen–antibody interactions, called immunosorption is an extremely powerful technique and has been increasingly successful since the advent of monoclonal antibodies (mabs). When there is a suitable monoclonal antibody at hand, immunosorption is an especially attractive technique for protein purification. Purification factors of 2000–20 000-fold are often achievable, and it is sometimes possible to achieve purification to homogeneity in a single step.

The extensive use of antibody-containing affinity columns in the purification of biologically active compounds is severely hampered by the leaching of antibody or portions thereof from the immunoaffinity support during elution of the target antigen. Part of the problem is caused by the combined use of reducing agents (*i.e.*, thiols) and chaotropic agents (*e.g.*, detergents and denaturants) in the elution step, which causes the dissociation of heavy and/or light chains from the immobilized antibody. This part of the leakage problem can be diminished by, amongst other things, intramolecular cross-linking of the antibody chains at their sites of disulphide interlinkage using bifunctional SH-specific reagents [1] or via the lysine groups using glutaraldehyde. A decrease in the problems in this context can also be obtained by the selection of conjugation methods that yield a more stable chemical linkage between the matrix and the spacer and between the spacer and the antibody [2].

Many activated gel matrices ready for the reaction with a ligand are commercially available [3]. They differ in, amongst other things, the reactive group, the extent of activation, introduced spacer length and type, particle size and porosity. In practice, most of these gel matrices are based on beaded agarose.

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In spite of some disadvantages, activation by cyanogen bromide (CNBr) remains a popular method. The main disadvantages of CNBr-activated agarose stem from the isourea linkage [4] between the gel and the amino groups of lysine. The isourea derivative introduces an extra positive charge at neutral pH ($pK \approx 9.5$), causing the gel to act as a weak ion exchanger at low salt concentrations. This does not usually present a problem. More serious is the fact that the isourea bond is reversible and can be cleaved, *e.g.*, by hydrolysis at weakly alkaline pH (>8) and by aminolysis with low-molecular-mass amines. In addition, very slow leakage of the (protein) ligand from the column occurs over a period of months to years [5].

Proteins may also be coupled to agarose which has been derivatized by spacer arms with N-hydroxysuccinimide ester at their ends [6]. Succinimide esters are very susceptible to nucleophilic attack by the ϵ-amino groups of lysine, resulting in the formation of a stable amide bond between the protein and the spacer arm. Limitations of NHS esters in affinity chromatography and protein immobilization were described by Wilchek and Miron [2]. On reaction with ligands containing an amino group, the columns were unstable to alkali and were plagued by constant leakage during use. However, they also described an alternative two-step method for the preparation of NHS esters, based first on the reaction of a carboxyl-containing matrix with a carbodiimide, that yields stable affinity columns. Tresyl chloride-activated gel matrices are suitable for immobilization of amino- and thiol-containing ligands and allow efficient immobilization even at neutral pH. The ligand becomes immobilized to the matrix by stable $-CH_2-S-$ (thioether) or $-CH_2-NH-$ (amine) linkages. The thiol groups are more reactive than amines and also imidazole and tyrosine hydroxyl groups can displace the sulphonate ester [7].

A hydrazide matrix can be used for immobilization of ligands containing aldehyde and ketone groups [8]. The reaction occurs at low pH (*ca.* 5) and the chemical bond that is formed is a stable hydrazone, obviating the need for reduction (although this can be performed if desired) [9]. In the case of antibodies, carbohydrate residues in the oligosaccharide chains, mainly at the Fc-part of the molecule, are oxidized at the vicinal hydroxyls to form aldehydes. This should have the advantage that antibodies are immobilized with optimum orientation [10].

The purpose of this investigation was to evaluate three commercially available products, hydrazide-, tresyl chloride- and N-hydroxysuccinimide (NHS)-activated agarose, with respect to their performance with regard to ligand leakage during storage and immunoaffinity chromatography. Moreover, the coupling speed and coupling efficiency for a mab and the performance of the immunosorbents with respect to static binding capacities at three ligand densities were determined. These factors can be influenced by, amongst other things, temperature, pH, type and concentration of coupling buffer, use and type of spacer arm and nature of the ligand [7,11]. Because investigations according to these factors were beyond the scope of this work, preparation of the immunosorbents was performed according to the manufacturer's protocol.

The purification of the E_1–E_2 glycoprotein complex (M_r 300 000) of the rubella virus from culture fluid was used as a model for this study. A mab directed against the E_1 protein of the antigen complex was immobilized on the three gels at three different concentrations. It has been reported that rubella virus proteins are unstable at low pH [12] and that they can be purified by immunochromatography using 0.5 *M* diethanolamine (pH 11.5) [13]. Also in this investigation basic conditions were used to elute the bound antigen from the column. Because the isourea bond is not stable under these conditions, CNBr-activated agarose was excluded from this evaluation.

EXPERIMENTAL

Materials and reagents

Tresyl-activated Sepharose 4 fast flow (FF) and protein A Sepharose 4 FF were purchased, and NHS-activated Sepharose 4 FF (a prototype gel containing 22 μmol of NHS groups per ml gel stored in 2-propanol) was obtained as a gift from Pharmacia (Woerden, Netherlands). Affi-Gel Hz

was purchased as a complete kit from Bio-Rad (Veenendaal, Netherlands). A Micro BCA protein assay kit was purchased from Pierce (Oud-Beijerland, Netherlands). A glycan detection kit was purchased from Boehringer (Mannheim, Germany). All other chemicals were of analytical-reagent grade.

The liquid chromatographic system was supplied by Pharmacia and consisted of two P-500 piston pumps, an MV-7 valve, a UV-M II monitor, a FRAC-200 fraction collector and a flat-bed recorder. A microwell system (model 510 reader, model 500 incubator and model 500 washer) was obtained from Organon Teknika (Turnhout, Belgium).

Protein purification

Monoclonal antibodies. Mab OT-Ru-5, 27 and 28, all specific for the E_1 protein of the rubella virus, were produced under protein-free conditions in a hollow-fibre dialysis system [14,15] and were purified by means of protein A affinity chromatography [16].

Antigen. Rubella virus was produced in baby hamster kidney (BHK) cells persistently infected with the vaccine strain HPV-77. The protein fraction was extracted with Tween–diethyl ether and concentrated 20-fold by ultra-filtration using an M_r 100 000 filter. Phenylmethylsuphonyl fluoride (PMSF), sodium azide and cinnamaldehyde were added to the antigen solution at concentrations of 2, 3 and 5 m*M*, respectively. The pH was adjusted to 7.3 using 1.0 *M* sodium phosphate–potassium phosphate buffer (pH 6.5). The solution was centrifuged for 15 min at 4000 *g* and the supernatant was filtered through a 0.45-μm pore size filter. It should be noted that the amount of active protein was only 0.02% (w/w) of the total amount of protein in the starting material.

Preparation of immunosorbents

Mab OT-Ru-28 was coupled to the activated supports according to the manufacturer's protocol. A schematic illustration of the bonds formed between the activated supports and the mab is given in Fig. 3.

Hydrazide-activated support. The solvent in which the mab was dissolved was exchanged for coupling buffer (pH 5.5) using an Econo-Pac 10 DG column. The column and buffer were included in the Affi-Gel Hz kit. Oxidation of IgG carbohydrates was performed by adding 10% (v/v) of 0.1 *M* aqueous $NaIO_4$ to the mab solution. This mixture was incubated for 1 h by end-over-end rotation in the dark at ambient temperature. The solvent was exchanged for coupling buffer by means of gel filtration using an Econo-Pac 10 DG column. The final IgG concentration was determined by UV measurement [A_{280} (1 cm, 1 mg/ml) = 1.45].

A portion of 6 ml of gel in 2-propanol was washed twice with 12 ml of coupling buffer. Mab in coupling buffer was added in amounts of 0, 3.4, 7.4 and 14.8 mg to portions of 1.5 ml of gel. The volume of the incubation mixtures was adjusted to *ca.* 6 ml by adding coupling buffer. Incubation was performed by end-over-end rotation for 24 h at ambient temperature. After incubation for 2, 4 and 24 h samples were taken from the supernatant to determine the coupling performance. To remove non-covalently bound antibody [17] the gels were washed on a sintered-glass filter with *ca.* 25 bed volumes of 7 m*M* phosphate–0.1 *M* NaCl (pH 7.3) [phosphate-buffered saline (PBS)], PBS–1 *M* NaCl, cold water, 0.1 *M* Na_2CO_3–0.5 *M* NaCl (pH 11), 0.1 *M* sodium acetate–0.5 *M* NaCl (pH 4), 0.1 *M* Na_2CO_3–0.5 *M* NaCl (pH 11), 0.1 *M* sodium acetate–0.5 *M* NaCl (pH 4) and again PBS. The gels were stored at 4°C in PBS containing 3 m*M* sodium azide.

Before each incubation with antigen solution, the gels were washed with approximately four bed volumes of 10 m*M* Na_2CO_3 (pH 11) and PBS.

Tresyl-activated support. Lyophilized tresyl-activated Sepharose (1.8 g) was swollen for 1 h in 1 m*M* HCl. The swollen gel was washed on a sintered-glass filter successively with 360 ml of 1 m*M* HCl and 150 ml of 9 g/l NaCl. The gel was suspended in an equal volume of 0.1 *M* $NaHCO_3$–0.5 *M* NaCl (pH 8.3) (coupling buffer). To 3-ml portions of this gel suspension, mab in coupling buffer was added in amounts of 0, 4.2, 8.4 and 16.8 mg. The volume of the incubation mixtures was adjusted to *ca.* 6 ml with coupling buffer. Incubation was performed by

end-over-end rotation for 24 h at ambient temperature. During and at the end of incubation samples were taken from the supernatant to determine the coupling performance. After coupling the supernatant was removed, 3 ml of 0.1 *M* Tris–HCl (pH 8.0) were added to the gels and incubation was continued for 4 h at ambient temperature. The gels were washed, stored and equilibrated as described for the hydrazide-activated support.

NHS-activated support. Approximately 12 ml of a 66% (v/v) slurry was washed on a sintered-glass filter with 120 ml of 1 m*M* HCl at 4°C. The gel was suspended with an equal volume of 0.2 *M* $NaHCO_3$–0.5 *M* NaCl (pH 8.2) (coupling buffer). Immediately afterwards, mab in coupling buffer was added in amounts of 0, 4.3, 8.6 and 17.2 mg to 3-ml portions of gel suspension. The volume of the incubation mixtures was adjusted to *ca.* 6 ml with coupling buffer. Incubation was performed by end-over-end rotation for 5 h at ambient temperature. During and at the end of incubation samples were taken from the supernatant to determine the coupling performance. Although not prescribed, the gels were incubated with 3 ml of Tris–HCl (pH 8.0) for 17 h at 4°C. The gels were washed, stored and equilibrated as described for the hydrazide-activated support.

Determination of static binding capacity and binding efficiency

The static binding capacity was measured in batch experiments, for which it was determined that 16–20 h of incubation was ample for equilibrium to be established. Six different amounts of antigen were incubated with 40 μl of a 25% (v/v) gel suspension of each immunosorbent. Incubation was performed by end-over-end rotation for 16–20 h at ambient temperature. After settling of the gel, the amount of antigen in the supernatant was determined by means of an enzyme-linked immunosorbent assay (ELISA).

Determination of ligand leakage

In the supernatants obtained during a period of up to 3 months after preparation of the gels and storage at 4°C and in preparations obtained during purification, the IgG content was determined by means of ELISA.

Chromatography

Purification was performed with the immunosorbents containing 3.0, 2.6 and 2.9 mg of IgG/ml gel based on the hydrazide-, tresyl- and NHS-activated supports, respectively. Portions of 1 ml of a 25% (v/v) gel suspension (duplicates) were incubated with antigen solution (1000 U/ml gel). Incubation was performed batchwise by end-over-end rotation for 16–20 h at ambient temperature. After incubation the gel was transferred to a C10/10 column (Pharmacia) and washed with PBS (75 cm/h) until the original baseline (absorbance at 280 nm) was reached. The adsorbed antigen was eluted with 10 m*M* Na_2CO_3 (pH 11) (15 cm/h). The pH of the eluted antigen fraction was neutralized with 1 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 6.5).

Determination of rubella E1-antigen activity using ELISA

Polystyrene microtitration strip-plates were coated overnight at ambient temperature with a 2.5 μg/ml solution of mab OT-Ru-5. The plates were washed with PBS containing 0.05% (w/v) of Tween 20 (PBST), dried and stored at 4°C. Samples and standards were diluted with PBST and incubated (100 μl per well) for 1 h at 37°C. The wells were washed with PBST (300 μl per well) and incubated with 100 μl of conjugate solution (HRP conjugated to mab OT-Ru-27) in PBST for 1 h at 37°C. The wells were washed again and incubated for 0.5 h at ambient temperature with 100 μl per well of substrate (urea peroxide)–chromogen (tetramethylbenzidine) solution. The reaction was stopped by addition of 1 *M* sulphuric acid (100 μl per well). Absorbances at 450 nm were determined using a microtitration plate reader. The measuring range of the assay was 0.025–0.1 U antigen/ml. Units are obtained by defining an in-house rubella antigen solution arbitrarily as 10 U/ml.

Determination of mouse IgG using ELISA

Polystyrene microtitration strip-plates were coated with sheep anti-mouse IgG at a concen-

tration of 0.7 μg/ml in 50 m*M* sodium carbonate buffer (pH 9.6) at ambient temperature for 24 h. The coated plates were washed, dried and stored at 4°C. A 100-μl volume of diluted sample or standard (mab OT-Ru-28) was pipetted into each well and incubated for 1 h at 37°C. The wells were washed and incubated with sheep anti-mouse IgG conjugated with HRP for 30 min at 37°C. The wells were washed again and incubated for 0.5 h at ambient temperature with 100 μl per well of substrate–chromogen solution. The reaction was stopped by addition of 1 *M* sulphuric acid (100 μl per well). Absorbances at 450 nm were determined using a microplate reader. The measuring range of the assay was 1–10 ng IgG/ml.

Determination of protein content

Samples and standard [bovine serum albumin (BSA)] solutions were diluted in 0.15 *M* NaCl and 100 μl were pipetted into the well of a microtitration strip-plate. A 100-μl volume of Micro BCA working reagent was added per well and the plate was incubated for 1 h at 50°C. Absorbances at 540 nm were determined using a microplate reader. The measuring range of the assay was 1–20 μg/ml.

RESULTS AND DISCUSSION

Determination of coupling performance

The amount of mab OT-Ru-28 IgG in the samples taken during and at the end of the coupling and taken during the wash cycles (see preparation of the immunosorbents) was determined by means of ELISA. It was confirmed that periodate oxidation of the mab had no effect on the reactivity in the ELISA for mouse IgG. The ligand density, defined as mg of IgG coupled per ml of gel, and the coupling efficiency, defined as (amount of IgG coupled/amount of IgG offered) × 100%, were calculated and are given in Table I. The amount of IgG coupled was calculated by subtracting the total amount of IgG found in the supernatant after coupling and in all wash fractions from the amount of IgG offered.

For hydrazide- and tresyl-activated supports, coupling efficiencies based on the ELISA results were confirmed by measurements of the absorbance at 280 nm (A_{280}) of the supernatant (results not shown). Because of the high A_{280} of NHS, which is released during coupling, supernatants obtained after coupling were analysed for IgG content using HPSEC [Zorbax GF-250 column, 20-μl injection volume, 0.2 *M* phosphate buffer (pH 7.0) and detection at 206 nm]. No IgG could be detected, which confirmed the results obtained from the ELISA.

TABLE I

LIGAND DENSITY AND COUPLING EFFICIENCY

Immobilization of mab OT-Ru-28 to hydrazide-, tresyl- and NHS-activated agarose matrices were performed by overnight coupling at ambient temperature, followed by an extensive washing procedure as described in detail under Experimental. The amount of uncoupled mab was determined using ELISA for mouse IgG and used to calculate coupling efficiency and ligand density.

Immunosorbent	Ligand density (mg IgG/ml gel)	Coupling efficiency (%)
Hydrazide	0	0
	1.3	54
	3.0	60
	7.8	79
Tresyl	0	0
	2.6	93
	5.3	96
	8.9	81
NHS	0	0
	2.9	100
	5.4	100
	11.1	100

Washing the gel directly after coupling to remove non-covalently bound IgG resulted with the tresyl- and hydrazide-activated agarose only in a minimal loss of ligand compared with the amount of IgG coupled. No IgG could be detected in the wash fractions of the NHS-activated gels.

The coupling of mab OT-Ru-28 to NHS-activated agarose is completed within 30 min for all three concentrations tested (Fig. 1). This fast and efficient coupling of mab agreed very well with the results obtained by Matson and Little [18]. After incubation for 4–5 h, coupling of mab to the hydrazide- and tresyl chloride-activated supports is almost at its maximum (81–96%);

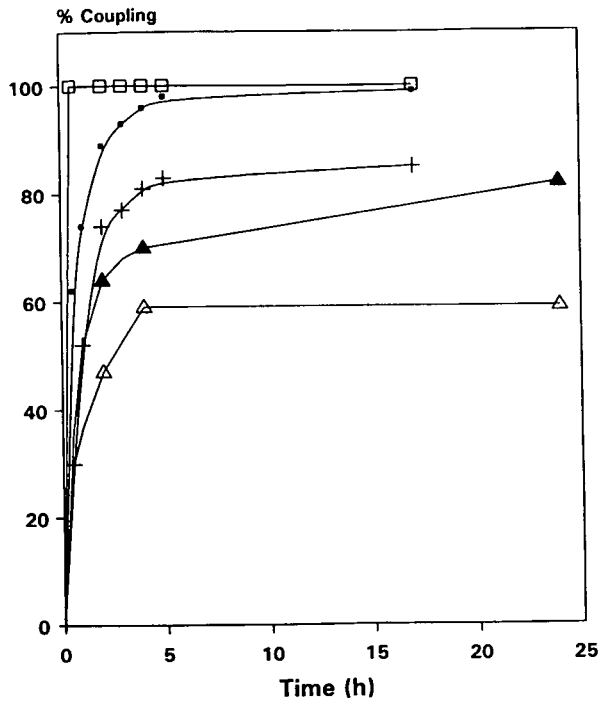

Fig. 1. Determination of coupling rate and coupling efficiency of mab OT-Ru-28 to NHS-activated agarose offering 2.9, 5.4 or 11.1 mg/ml gel [all the same curve (□)], hydrazide-activated agarose offering (△) 2.3 or (▲) 9.9 mg/ml and tresyl-activated agarose offering (■) 2.8 or (+) 11.2 mg/ml.

longer incubation times result only in a slightly higher ligand density. These results were also in good agreement with those reported [7,19].

A relatively low binding efficiency of 54–79% after incubation for 24 h was observed for the hydrazide-activated support. This may be due to the degree and type of glycosylation of the antibody molecule, causing a limiting amount of aldehyde groups on the oxidized antibody, or to sub-optimum coupling conditions for this particular mab.

Determination of ligand leakage during storage

The leakage of ligand from the gel during storage is the strongest for the tresyl-activated gel followed by the hydrazide-activated matrix. Detailed results are given in Table II. With the NHS-activated gel almost no IgG could be detected in the supernatants.

TABLE II

LIGAND LEAKAGE DURING STORAGE

IgG leakage was determined by ELISA for mouse IgG on the supernatants of the immunosorbents after a storage period of 3 months in PBS + 0.02% NaN_3 at 4°C.

Immunosorbent	Ligand density (mg IgG/ml)	Ligand leakage (μg/ml gel)
Hydrazide	1.3	11
	3.0	21
	7.8	31
Tresyl	2.6	15
	5.3	104
	8.9	>120
NHS	2.9	0.008
	5.4	0.028
	11.1	0.291

Static binding capacity

The static binding capacities of the immunosorbents are summarized in Table III, and were determined as described under Experimental. They were read from the binding efficiency curve at the 80% point. This definition gives a practically useful binding capacity and is not the maximum binding capacity of the immuno-

TABLE III

DETERMINATION OF BINDING CAPACITY

Binding capacities of the immunosorbents were determined using batchwise incubation (16–20 h, ambient temperature) with antigen. The amount of unbound antigen was determined by ELISA. The binding capacity was defined as the amount of antigen (U) bound per ml of immunosorbent with an efficiency of 80%.

Gel	Ligand density (mg IgG/ml)	Binding capacity (U/ml gel)
Hydrazide	1.3	325
	3.0	500
	7.8	555
Tresyl	2.6	1130
	5.3	1115
	8.9	1235
NHS	2.9	1060
	5.4	1083
	11.1	1080

sorbent. The binding efficiency curves for the three gels which were formerly used in purification experiments are shown in Fig. 2.

No binding was observed when the antigen was incubated with the gels containing no ligand, which excludes non-specific adsorption.

Static binding capacities for immunosorbents based on the tresyl- and NHS-activated gels were about equal and increasing the ligand density from 2.6 to 11.1 mg IgG/ml did not result in an increase in static binding capacity. Instead of an improved binding capacity as expected by oriented coupling of the mab molecules, immunosorbents based on the hydrazide-activated gels had binding capacities that were only half or less those that of the other two supports.

Orthner *et al.* [20] found that their mabs against human plasma proteins factor IX or protein C also contained carbohydrates in the Fab′ region. Carbohydrate analysis of intact IgG and $F(ab')_2$ fragments of mab OT-Ru-28B under reducing and non-reducing conditions showed that sugars were only present on the Fc part. This excluded coupling via the antigen-binding site.

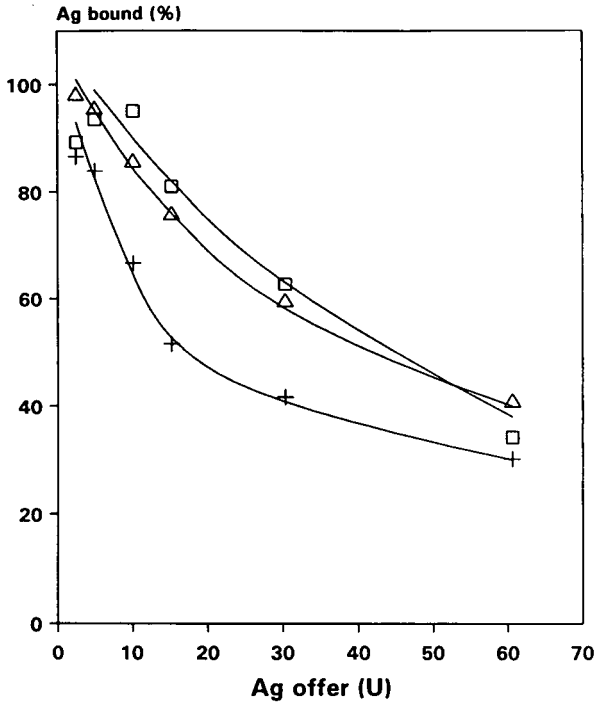

Fig. 2. Determination of the binding efficiency of the immunosorbents based on (□) NHS-activated agarose, (+) hydrazide-activated agarose and (△) tresyl-activated agarose with ligand densities of 2.9, 3.0 and 2.6 mg mab/ml gel, respectively.

Whether the reduced activity is due to the oxidation step was not investigated. Orthner *et al.* [20] found for their mabs no reduced activity caused by oxidation. However, Fleminger *et al.* [21] showed for several mabs a loss of activity up to 26% after incubation with 10 m*M* sodium periodate for 1 h in the dark at 4°C. They also showed that increasing the temperature during the oxidation step to ambient temperature resulted in a rapid inactivation of the more sensitive antibodies. Another reason for the reduced activity may be that sodium periodate oxidizes not only the carbohydrate moieties, but also certain amino acid residues, particularly N-terminal serine, threonine and methionine. Whenever these residues are essential for the antigen-binding activity of an antibody, their oxidation may harm its activity.

Because the immunosorbents were useful for isolation of rubella virus proteins and optimization of the binding capacity, which was outside the scope of this investigation, has been thoroughly discussed in the literature [22,23], no further attention was devoted to this subject.

Determination of ligand leakage during chromatography

Ligand leakage during chromatography using an immunosorbent based on NHS-activated agarose is about a factor of fifteen lower than that using the sorbent prepared from tresyl-activated agarose and more than a factor of twenty lower than that using the sorbent based on hydrazide-activated agarose (see Table IV). This means that instability towards alkali of ligands coupled to an NHS group-containing support, as been noted by Cuatrecasas and Parikh [6] and Wilchek and Miron [2], is almost overcome.

Because the amine linkage formed after reaction of tresyl with amino groups is a stable bond, the higher ligand leakage for the tresyl-activated agarose in comparison with the NHS-activated gel can be explained by the lower stability at high pH of the bonds probably formed by side-reactions with imidazole or

TABLE IV

LIGAND LEAKAGE DURING CHROMATOGRAPHY

The amount of mouse IgG was determined using ELISA and the amount of total protein was determined using the Micro BCA protein assay. For details, see Experimental. Contamination of the purified antigen fraction with anti-rubella mab was determined in duplicate. Contamination was expressed as (amount of mouse IgG/amount of total protein) × 100%.

Immunosorbent	Ligand density (mg IgG/ml gel)	Contamination (%, w/w)	
		Run 1	Run 2
Hydrazide	3.0	0.44	0.50
Tresyl	2.6	0.26	0.33
NHS	2.9	0.02	0.02

tyrosine hydroxyl groups. As IgG has no free thiol groups, the presence of a thioether bond is not likely.

The relatively high contamination of the final product with mouse IgG (expressed as %, w/w) observed for the hydrazide gel can partly be explained by the lower binding capacity of this immunosorbent. Other explanations may be the lower stability of the hydrazone bond at high pH as compared with the amine and amide bonds (see Fig. 3), or that leakage is related to the mode of introduction of the hydrazide group into the support.

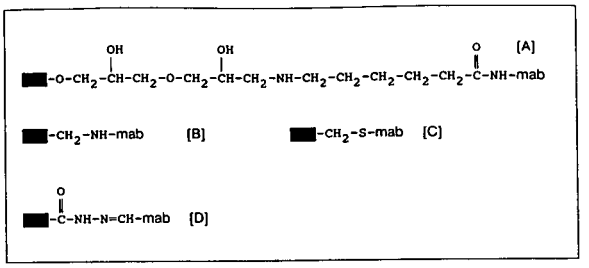

Fig. 3. Immobilization of an amino-containing ligand (mab-NH_2) to the (A) NHS- and (B) tresyl-activated support. Coupling of a thiol-containing ligand (mab-SH) to the (C) tresyl-activated support and coupling of an aldehyde-containing ligand (mab-CHO) to the (D) hydrazide-activated support [D].

CONCLUSIONS

It was concluded that of the three activated supports evaluated, NHS-activated Sepharose 4 fast flow is the most suitable activated matrix with respect to (mab) ligand leakage both during storage at neutral pH and during chromatography using alkaline (pH 11.0) desorption conditions. An improvement in ligand leakage for this gel in comparison with earlier available NHS-activated agaroses was obtained by introducing the spacer arm via epoxy activation to the polysaccharide matrix [24], which results in a stable ether bond, followed by a suitable method for introduction of the NHS ester. For the mab used in this investigation it was shown that the desired ligand density can be exactly achieved because coupling is complete within 30 min of incubation.

Two minor practical disadvantages of this gel are that the succinimide ester gives COOH groups after hydrolysis, which introduce an extra charge, causing the gel to act as an ion exchanger at low salt concentrations, and that the progress and efficiency of coupling of protein cannot be followed by simple ultraviolet absorption measurement because NHS also absorbs strongly.

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Continuous buffer exchange of column chromatographic eluates using a hollow-fibre membrane module

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ABSTRACT

A method was developed to remove continuously the concentration gradient of any low-molecular-mass substance in the eluate during adsorption liquid column chromatography. For this purpose, a hollow-fibre membrane module (HFMM) was directly connected to the outlet of the chromatographic column and on-line buffer exchange was achieved by cross-flow ultradialysis. It could be demonstrated that the HFMM when used during liquid chromatography has some additional advantages. It is an ideal tool to concentrate continuously column eluates by dialysis against polyethylene glycol solutions. Moreover, it is also possible to detect specifically enzyme activities on-line using an HFMM.

INTRODUCTION

When liquid column chromatography is performed for biochemical purposes, it is often indispensable to exchange the buffer, reduce the volume or determine enzymatic activities of the eluate obtained. All these procedures are batch techniques which often cause many inconveniences or problems.

Interaction chromatography in principle requires alteration of the buffer composition during the elution phase in order to desorb bound macromolecules [1]. For that purpose, the concentration of low-molecular-mass substances (protons, salts or competitors) is altered in most of the applications such as affinity, ion-exchange or hydrophobic interaction chromatography [2]. The presence (or absence) of those desorbants in the eluate may cause the following disadvantages. Detection of separated macromolecules after or during chromatography by UV–Vis spectrophotometry can be impaired [3]. Distorted band patterns, such as pinching or flaring of lanes, can be caused by salts in the samples prior to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) [4]. Activity measurements are often disturbed or even rendered impossible when not performed at well defined salt concentrations [5]. Further, the stability of several macromolecules strictly depends on defined salt or buffer compositions [6]. In the literature there are several examples of the necessity for buffer exchange after chromatographic steps. Buffers were altered by dilution [7], tube dialysis [8] or molecular sieve chromatography using PD 10 columns (Pharmacia) [9]. As these methods are batch procedures, it may be necessary to exchange the buffer of every single fraction after column chromatography has been performed. Further, performing enzymatic assays with every fraction is very time consuming. In many instances, sample concentration is regarded as useful.

In order to overcome all these problems, we developed a method to exchange the buffer

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continuously, measure enzymatic activities or reduce the volume of the eluate during liquid column chromatography by cross-flow ultradialysis, using a hollow-fibre membrane module (HFMM) connected directly to the outlet of the column.

EXPERIMENTAL

Chemicals

Lactate dehydrogenase (LDH), bovine serum albumin (BSA), reduced nicotinamide adenine dinucleotide (NADH) and pyruvate were purchased from Boehringer (Mannheim, Germany) and polyethylene glycol 20 000 was obtained from Fluka (Buchs, Switzerland). All other chemicals used were of analytical-reagent grade appropriate for biochemical use. Water purified with a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout and all experiments were performed at room temperature.

Hollow-fibre membrane module

All experiments were performed using an HFMM packed with Cuprophan C1 capillaries, which was a generous gift from Akzo (Obernburg, Germany) and is now commercially available from the authors. The specifications of the module are given in Table I.

TABLE I

SPECIFICATIONS OF THE HFMM

Parameter	Value
Module length	270 mm
Capillary length	230 mm
Active capillary length	215 mm
Case diameter	10 mm
Packing diameter	8 mm
Packing cross-section	50 mm^2
Module fill-grade	29.7%
Capillary I.D.	0.2 mm
Capillary O.D.	0.222 mm
Number of capillaries	50
Molecular mass cut-off value	5 kDa
Total intracapillary surface area	68 cm^2
Total extracapillary surface area	75 cm^2
Intracapillary (void) volume	0.34 ml

Conductivity measurements and volume determinations

The efficiency of cross-flow ultradialysis in the HFMM was determined by measuring the decrease of intracapillary (IC) NaCl concentration using a flow-through conductivity electrode. For sample volume reduction experiments, 0.715 mg/ml BSA was used IC, 20% (w/w) polyethylene glycol 20 000 was used in the extracapillary (EC) space and the IC volume reduction was monitored by determining the increase in BSA concentration in the IC eluate according to Bradford [10].

Anion-exchange chromatography

In order to measure the peak broadening caused by the void volume of the HFMM, anion-exchange chromatography was performed using Q-Sepharose fast flow (Pharmacia, Uppsala, Sweden) in an 8×1 cm I.D. column (Amicon, Witten, Germany). A 500-μg amount of LDH was applied in 1 ml of 50 m*M* triethanolamine (TEA) (pH 7.8) and 30 ml of a linear NaCl gradient (0–1 *M*) were run at 1 ml/min in order to desorb bound protein.

Covalent chromatography

To give an example of the extreme detection problems caused by low-molecular-mass light-absorbing molecules, we performed covalent chromatography using thiol-Sepharose 4B (Pharmacia). A column (8×1 cm I.D.) (Amicon) was equilibrated with 50 m*M* TEA (pH 7.8) and loaded with 400 μl of a crude cell extract (5 mg/ml) prepared from *Euglena gracilis* as described previously [11]. Covalently bound sample (200 μg) was removed from the column using 30 ml of a linear gradient (0–50 m*M*, 0.2 ml/min) of β-mercaptoethanol in the same buffer.

On-line detection of enzymatic activity

In order to show the possibility of using the HFMM as a sensor for detecting enzymatic activity in a continuous buffer flow, we selected the reduction of pyruvate to lactate, as catalysed by LDH [12]. Potassium phosphate (50 m*M*, pH 7.5) containing 3.1 m*M* pyruvate plus 1.5 m*M* NADH was circularly pumped EC in a total volume of 250 ml at 1 ml/min. A sample of 5 μg

of LDH in 100 μl was injected during a continuous IC flow of 0.8 ml/min of 50 m*M* potassium phosphate (pH 7.5). LDH activity was detected spectrophotometrically by measuring the decrease in NADH at 340 nm in a 1-mm flow-through quartz cuvette.

RESULTS

On-line desalting and buffer exchange

As can be seen in Fig. 1, the efficiency of buffer exchange depends on both the EC and IC flow-rates. Using an IC buffer flow-rate of 0.5–1.0 ml/min, a flow-rate usually applied for fast protein liquid chromatographic (FPLC) experiments and an EC buffer flow of 30 ml/min, the efficiency of desalting a 1 *M* NaCl solution is better than 95%.

Peak broadening

The effect of peak broadening caused by passing the IC void volume of the HFMM is demonstrated in Fig. 2. A sample of LDH was separated using a column packed with Q-Sepharose fast flow either with or without the HFMM. Peaks were delayed by *ca.* 1 ml in the case of desalting the eluate with the HFMM but peak broadening was below 20% (half-width of the main LDH peak). The partial disappearance of the very first small peak could be due to low-molecular-mass compounds being eliminated by the HFMM.

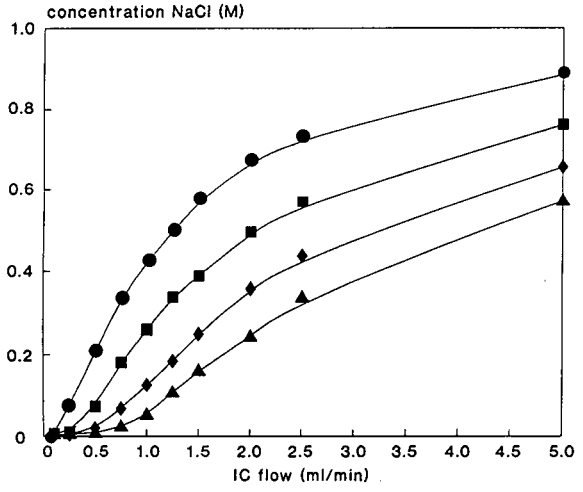

Fig. 1. Efficiency of desalting 1 *M* NaCl at EC flow-rates of (●) 1, (■) 5, (◆) 15 and (▲) 30 ml/min.

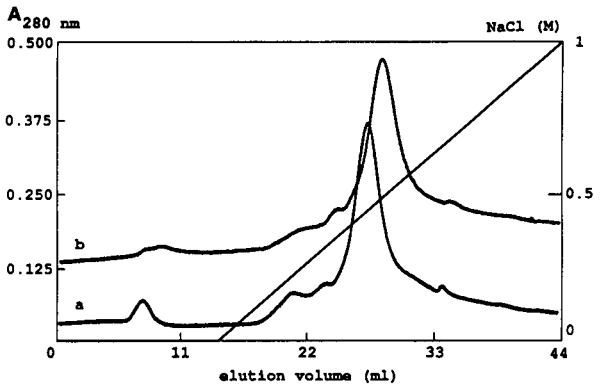

Fig. 2. Anion-exchange chromatography of LDH using a Q-Sepharose fast flow column either (a) without or (b) with desalting of the eluate at a flow-rate of 5 ml/min (EC).

Improvement of detection

In many applications of liquid column chromatography, continuous detection of macromolecules in the eluate by measuring the absorbance is interfered with or is even impossible because of the presence of light-absorbing low-molecular-mass substances. In order to demonstrate the ability to overcome such detection problems, covalent chromatography using thiol-Sepharose [13] was performed with a crude cell extract prepared from *Euglena gracilis*. As shown in Fig. 3a, detection of proteins at 280 nm during chromatography is impossible owing to 2-thiopyridyl groups replaced by both protein binding to the column and thiol-reducing agents during elution. In addition, a baseline shift due to the absorption of β-mercaptoethanol at 280 nm occurs. By using the HFMM during chromatography (Fig. 3b) it was possible to detect two small protein peaks without any baseline shift. The absence of those peaks in the control experiment without injecting a sample indicates that the 2-thiopyridyl groups indeed were completely removed by cross-flow ultradialysis under the described experimental conditions. The first peak, eluting during sample injection, should contain both non-bound proteins and 2-thiopyridyl groups replaced by bound protein. Therefore, protein determination by peak inte-

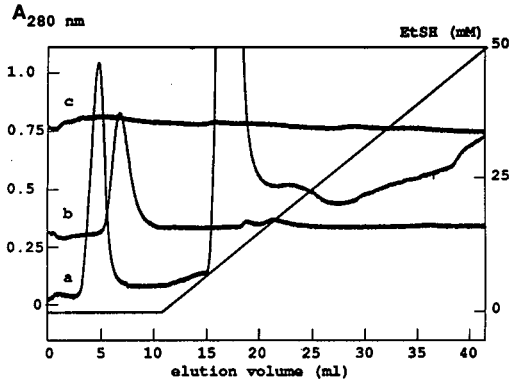

Fig. 3. Covalent chromatography of a crude cell extract derived from *Euglena gracilis* using a thiol-Sepharose column. Sample was eluted with 30 ml of a linear gradient of β-mercaptoethanol (0–50 m*M*). (a) Conventional chromatography; (b) chromatography using the HFMM with an EC flow-rate of 30 ml/min; (c) control, performed as in (b) but without sample.

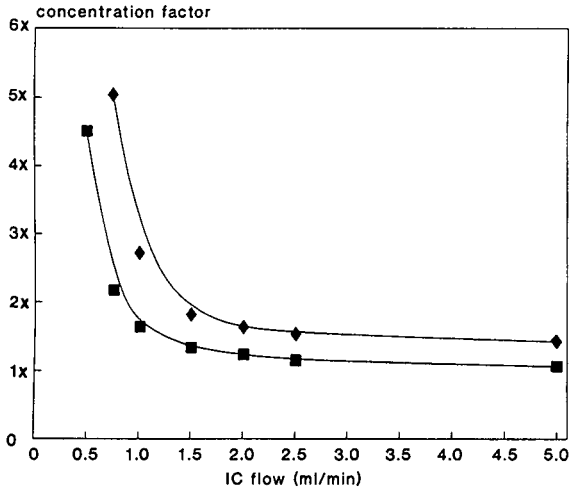

Fig. 4. Concentration of 0.715 mg/ml BSA IC using 20% polyethylene glycol 20 000 at EC flow-rates of (■) 1 and (◆) 5 ml/min.

gration is not possible. The total area under this peak decreases when the HFMM is used, indicating that 2-thiopyridyl groups are now removed, and consequently the amount of protein can be determined.

On-line concentration

Installing the HFMM between the outlet of the column and the detection unit not only solves desalting problems. Using hygroscopic solutions EC, it should be possible to create osmotic pressure gradients useful for concentrating solutions of macromolecules by IC volume reduction. In Fig. 4 the capability of such an arrangement is demonstrated with a BSA solution IC and 20% (w/w) polyethylene glycol 20 000 EC. As can be seen, we were able to concentrate up to fivefold, but the efficiency to concentrate proteins is not limited in principle, *e.g.*, by the choice of flow-rates, but rather by IC protein precipitation, membrane adsorption or inaccuracy of pump velocity, responsible for exact and constant IC flow-rates.

On-line detection of enzymatic activity

The HFMM also appeared to be suitable for detecting enzymatic activities within a column eluate. Consequently, a low-molecular-mass substrate was pumped continuously EC and transferred into the IC and conversion was detected by measuring the reaction product spectrophotometrically. When an NADH–pyruvate-con-

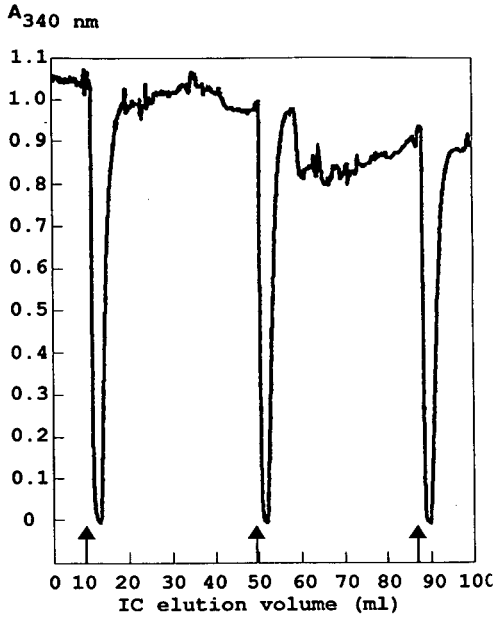

Fig. 5. On-line detection of LDH activity: 50 m*M* potassium phosphate (pH 7.5) containing 3.1 m*M* pyruvate and 1.5 m*M* NADH was pumped circularly EC. The NADH decrease was detected spectrophotometrically at 340 nm directly at the outlet of the HFMM. Arrows indicate injection of 5 μg LDH per 100 μl into a continuous IC flow-rate of 0.8 ml/min.

taining buffer was used EC and small amounts of LDH were injected into a continuous IC flow, clearly negative peaks indicating the corresponding NADH turnover were detectable (Fig. 5). No significant positive protein peaks were found at 280 nm with the same sensitivity (data not shown). The continuous, slow decrease of the baseline is caused by the loss of NADH, as the EC flow was pumped circularly in order to recycle and thus minimize the NADH–pyruvate consumption.

DISCUSSION

Neither the principle of constructing HFMMs nor their application in order to perform cross-flow ultradialysis is new. However, pumping a continuous sample flow, which contains sequentially appearing different macromolecules through a small IC space and obtaining the same pattern within the flow-through after passing an HFMM, has not previously been reported. Moreover, combining this newly discovered principle of application with liquid chromatographic procedures, by connecting HFMMs to the outlet of chromatographic columns, appeared to provide an advance for liquid chromatography in general: the method allows desalting, concentration and enzymatic activity detection of column eluates to be performed on-line without significantly altering the separation profiles.

Hydrophobic interaction, ion exchange, hydroxyapatite and some applications of affinity chromatography essentially are accompanied by the formation of salt gradients in the eluate. There are several circumstances that make desalting of such a column eluate indispensable. It is well known that SDS-PAGE requires samples that contain low concentrations of salt. On-line desalting therefore makes it possible to avoid separately desalting every single fraction collected after liquid column chromatography, when subsequent SDS-PAGE analysis is planned. During purification procedures, in many instances desalting steps are necessary to achieve protein binding to matrices of further chromatographic columns. On-line desalting is therefore suitable for saving as much time as possible, which is often a crucial condition when sensitive proteins are to be purified. In addition, detection problems that often occur when continuous-flow absorbance spectrometry is performed, *e.g.*, with light-absorbing salts or low-molecular-mass compounds present in a column eluate, can be obviated by on-line desalting. Free-flow electrophoresis (FFE), a method which requires very low ionic strengths, is capable of continuously separating biomolecules during purification procedures [14]. Thus on-line desalting over an HFMM is a helpful tool prior to FFE. Not only is desalting of the column eluate a useful application, but also the transfer of salts into the eluate in order to increase the ionic strength. It is known that the proteins derived from halophilic bacteria require high concentrations of salt, which makes the application of salt-dependent purification steps such as ion-exchange chromatography impossible [5]. Lanyi [15] reported that the deactivation of such proteins by exposure to low salt concentrations is a time-dependent process. Two HFMMs, the first connected to the inlet of the column in order to desalt the sample prior to ion-exchange chromatography and the second connected to the outlet to increase again the ionic strength should minimize the exposure time at low ionic strengths and therefore may solve some purification problems.

The use of on-line HFMM applications is not limited to buffer exchange designs. Another time-consuming step during purification procedures is the reduction of buffer volume. Therefore, mostly batch procedures made use of ammonium sulphate precipitation [16], ultrafiltration [17,18] or lyophilization [19]. As demonstrated above, an HFMM pumped with polyethylene glycol EC is sufficient to concentrate macromolecules on-line without simultaneously concentrating low-molecular-mass compounds, as occurs during lyophilization. We used polyethylene glycol 20 000 in a 20% (w/w) solution (non-saturating conditions) without solubility or viscosity problems. This method allows the fractionation of column eluates after concentration and further applies simultaneously to the chromatographic procedure without consuming additional time. The resolving power of gel filtration depends strictly on the sample volume and

consequently on-line concentration should in general be useful prior to molecular sieve chromatography. It is known that the flatter the salt gradient selected in absorption chromatography, the more the separation of peaks is improved but the concentration of macromolecules in the eluate is decreased. Therefore, one would expect better overall separations by running flat salt gradients and compensating for the indispensable eluate dilution by applying an HFMM to reconcentrate continuously the eluate obtained.

In order to purify enzymes, it is necessary to determine the enzymatic activity after each chromatographic step. In most instances many fractions have to be assayed. There are approaches in the literature for mechanizing this detection procedure by flow-injection analysis [20]. Nevertheless, 2 min are required for the determination of each data point in the case of quasi-on-line detection of LDH. In contrast, the properties of an HFMM used on-line as mentioned above give the opportunity to detect enzymatic activity and record a continuous enzyme elution profile. In combination with a flow-through detection of protein concentration at 280 nm and a suitable computer program, it should be possible to record the specific activity during chromatography and thereby to optimize any automatic collection of fractions.

It should be noted that the three principles of using HFMMs in the mode described here (buffer exchange, concentration and enzyme detection) can also be combined. For example, a column eluate containing a highly diluted enzyme can be concentrated on-line by a first HFMM with the activity being detected continuously by a second HFMM. Another example of combining more HFMMs could be the preparation of pyridoxal 5′-phosphate-dependent apoenzymes, *e.g.*, tryptophan synthase [21]. The first HFMM directly mounted on the last chromatographic column of a purification procedure could be used for detecting enzymatic activity, the second to transfer hydroxylammonium chloride into the IC in order to obtain apoenzymes and a third to remove the resulting oxime of the reaction.

The principle of cross-flow ultradialysis depends on pore size, molecular mass of sample compounds, temperature, total membrane surface and EC and IC flow rates. When HFMMs are used in the manner described here, they must obey a new, crucial principle of construction: in comparison with conventional HFMMs, an on-line desalting HFMM should have an increased ratio of capillary length to total number of capillaries. This principle of construction is necessary because the production process of HFMMs causes significant deviations of the fibre inner diameters directly at the inlet and outlet of the module, implying different flow-rates in the capillaries and consequently peak broadening. Further, the longer the capillaries are, the more the concentration gradient is extended and therefore the more the efficiency of dialysis is increased. It is also important to minimize the void volume, because of sample dilution and consequent peak broadening. On the other hand, minimizing the void volume implies a reduction in the total exchange surface and therefore a decrease in efficiency. This disadvantage can be compensated for by warming the EC buffer, as the time of sample exposure in the IC normally is very short.

In this study, an HFMM suitable for FPLC as well as HPLC applications and standard chromatography using columns with matrix volumes not more than 10 ml was investigated. The only minor restrictions of using HFMMs during column chromatography are peak broadening caused by the void volume of the IC space and velocity limitations in sample flow. As we could demonstrate, the HFMM works with sufficient efficiency at the flow-rates usually used for FPLC or standard chromatography with columns of the described sizes. The peak broadening was below 20% and therefore readily tolerable. Consequently, the use of HFMMs should become an advance in column chromatography in general and HFMMs of different sizes can be used for any scale of chromatographic columns.

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Isoprotein analysis by ion-exchange chromatography using a linear pH gradient combined with a salt gradient

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ABSTRACT

Isoproteins of human monoclonal antibodies with a p*I* range between 8.45 and 8.70 or 8.15 and 8.65 were separated by ion-exchange chromatography with a linear ascending pH gradient combined with a linear descending salt gradient using borax, mannitol and salt. The isoproteins were eluted according to their isoelectric points as demonstrated by conventional isoelectric focusing. Preparative purification and monitoring of the isoprotein composition of human monoclonal antibodies during a purification process is also presented to demonstrate the applicability of the method.

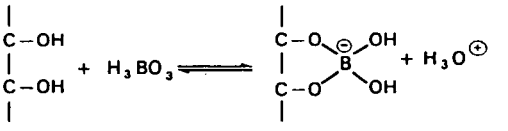
INTRODUCTION

Owing to post-translatorial modifications, monoclonal antibodies exhibit strong micro-heterogeneity [1]. Several methods, such as isoelectric focusing (IEF) [2,3], chromatofocusing (CF) [4], hydrophobic interaction chromatography (HIC) [5] and capillary zone electrophoresis (CZE) [6], have been described for the analysis and preparation of their isoforms. Isoelectric focusing is mainly used for the analytical determination of isoform patterns [7], whereas HIC and CF are the methods of choice for preparative purposes.

Ion-exchange chromatography is a powerful tool for the purification of monoclonal antibodies. In most applications step gradients are used to accomplish elution [8]. In principle, ion-exchange chromatography can also be used for separating the various isoforms of monoclonal antibodies. By generating a linear pH gradient the isoforms can easily be eluted near their isoelectric points. Shukun *et al.* [9] used borax–mannitol solution in free-flow electrophoresis for generating a pH gradient. The addition of mannitol to borate buffer [10] changes the pH to an acidic milieu. The low conductivity of this buffer allows the use for ion-exchange chromatography. In this work, linear pH–salt gradients were used for the analytical and preparative separation of isoproteins. As model proteins human monoclonal antibodies were investigated.

THEORY

A linear pH gradient is achieved by the reaction of the *cis*-diol of mannitol with borate, which liberates H_3O^+ in a stoichiometric manner:



A stable complex is formed, which does not undergo ageing. The theory of the formation of new acids by association of boric acids and planar diols is due to the fact that the "ionization

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constants" of such acids often obey an equation of the form

$$K^{**} = C_{2n}K_1K_n + K_1$$

The "ionization constant" K^{**} varies with the concentration of the ligand C_{2n} and is independent of the concentration of the boric acid at concentrations below 0.1 *M*. K_n is the association constant of the boric acid and the diol and K_1 is the first ionization constant of boric acid.

To generate a linear pH gradient for ion-exchange chromatography, a strong ion exchanger must be used. The dissociation constant of the functional group is not significantly affected by the pH of the mobile phase [11]. This assumption is valid for a working pH range between 4 and 9. Therefore, the continuous pH change in the mobile phase is not affected by ionizable groups of the ion exchanger. In contrast to chromatofocusing, the ion exchanger is not involved in the formation of the pH gradient.

EXPERIMENTAL

Model proteins

Human monoclonal antibodies [12] were purified as described in detail by Unterluggauer *et al.* [13]. Briefly, the clarified culture supernatant was concentrated by ultrafiltration, desalted by diafiltration and chromatographed on protein A Sepharose fast flow and S-Sepharose fast flow and desalted with Sephadex G-25 coarse. Both antibodies, "3D6" and "Virgil", belong to isotype IgG1. "3D6" has an isoelectric range between p*I* 8.45 and 8.70 and "Virgil" between p*I* 8.15 and 8.65.

Ion-exchange chromatography

Mono S HR5/5 and Mono S HR16/10 columns (Pharmacia, Uppsala, Sweden) were used. The columns were connected to a fast protein liquid chromatographic system. UV absorbance at 280 nm, pH and conductivity were monitored continuously. For elution a linear pH gradient was generated by mixing the starting buffer consisting of 5 m*M* borate, 45 m*M* mannitol and different concentrations of NaCl (depending on the binding behaviour of the model proteins) with 5 m*M* borate.

Isoelectric focusing

Isoelectric focusing was performed with carrier ampholytes in the pH range 3–10 according to Westermeier [14]. The gel was developed by

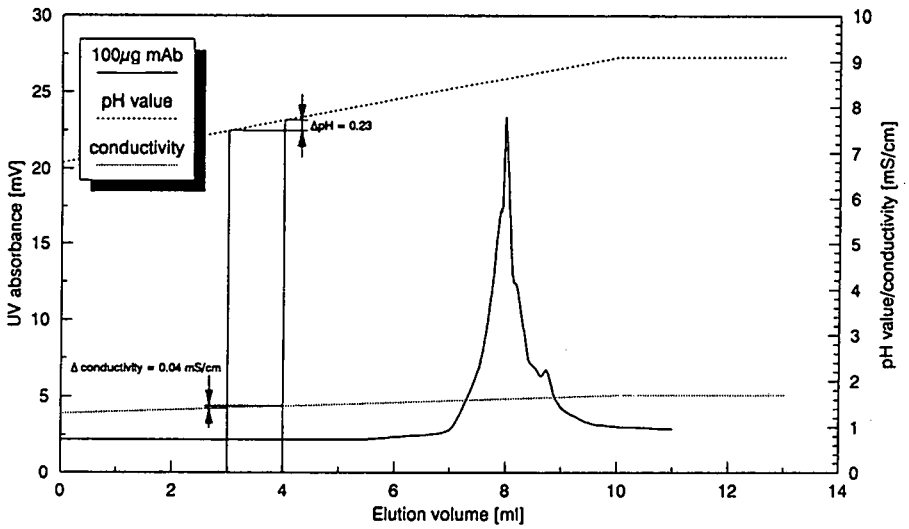

Fig. 1. Separation of isoproteins of human monoclonal antibody "3D6" by ion-exchange chromatography on a Mono S column (HR 5/5); column volume (v_t) = 1 ml; 5 cm × 0.5 cm I.D. Sample, 100 µg of monoclonal antibody dissolved in 25 µl of starting buffer and loaded on a 1-ml column; starting buffer, 10 m*M* borate, 70 m*M* mannitol (pH 6.8), κ = 1.3 mS/cm; elution buffer, 10 m*M* borate (pH 9.1), κ = 1.7 mS/cm. Elution was effected with a linear pH gradient over ten column volumes at a flow-rate of 0.1 ml/min (30.6 cm/h).

silver staining according to Heukeshoven and Dernick [15].

Chromatography data acquisition

UV absorbance, pH and conductivity signals were processed on a Nelson Analytical (Cupertino, CA, USA) chromatography data system.

RESULTS

Two human monoclonal antibodies with p*I* ranges between 8.45 and 8.70 and between 8.15 and 8.65 were used as model proteins. To resolve the isoproteins with linear pH gradients an elution procedure analogous to chromatofocusing was designed. The sample was transferred into the starting buffer by gel permeation on Sephadex G-25 in PD-10 columns (Pharmacia). Elution was performed with a linear gradient from pH 7.0 to 9.1.

Development of the linear gradient

The antibody "3D6" with p*I* 8.45–8.70 was used as model antibody for the development of a linear gradient. To improve the resolution the following experiments were carried out. To increase the insufficient resolution obtained with a linear pH gradient (Fig. 1), salt was added to the starting buffer in an amount that still allowed binding of the antibody to Mono S. A combined ascending pH and a descending salt gradient (Fig. 2) was generated by this procedure. However, the resolution was still unacceptable. A steeper salt gradient was generated by decreasing the borax concentration from 10 to 5 m*M* to obtain a conductivity equal to the conditions described in Fig. 2. The results of this run are shown in Fig. 3. Subsequently optimum resolution was achieved when the gradient volume was doubled (Fig. 4).

Preparative purification

For preparative purification of the various isoforms, the human monoclonal antibody "Virgil" of p*I* 8.15–8.65 was used. Prior to scale-up, the resolution was optimized with a 1-ml column applying the same conditions as developed for antibody "3D6" (Fig. 5). The preparative purification was carried out on a Mono S HR16/10 column with a total column volume of 22 ml. The partially separated isoforms were collected in fractions (Fig. 6) and analysed by IEF (Fig. 7)

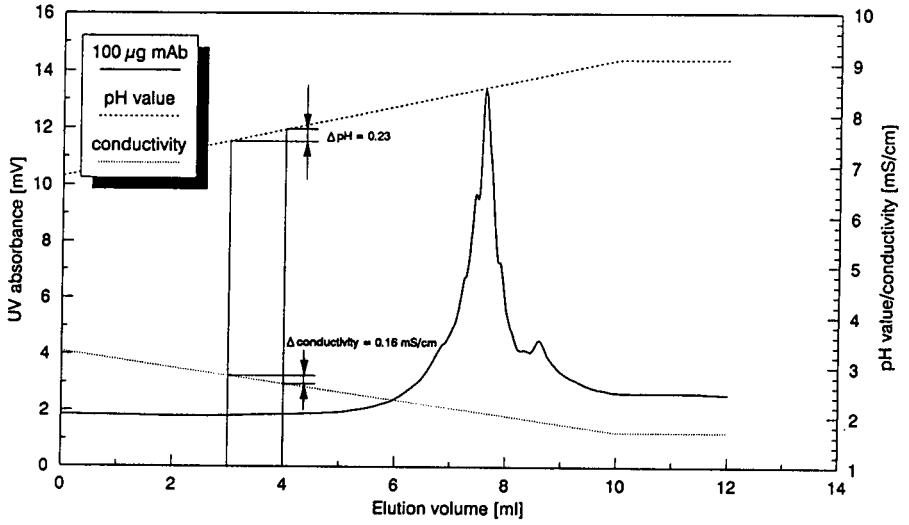

Fig. 2. Separation of isoproteins of human monoclonal antibody "3D6" by ion-exchange chromatography on a Mono S column (HR 5/5). Sample, 100 µg of monoclonal antibody dissolved in 25 µl of starting buffer and loaded on a 1-ml column; starting buffer, 10 m*M* borate, 70 m*M* mannitol, NaCl (pH 6.8), $\kappa = 3.3$ mS/cm; elution buffer, 10 m*M* borate (pH 9.1), $\kappa = 1.7$ mS/cm. Elution was effected with a linear pH–salt gradient over ten column volumes at a flow-rate of 0.1 ml/min. Other conditions as in Fig. 1.

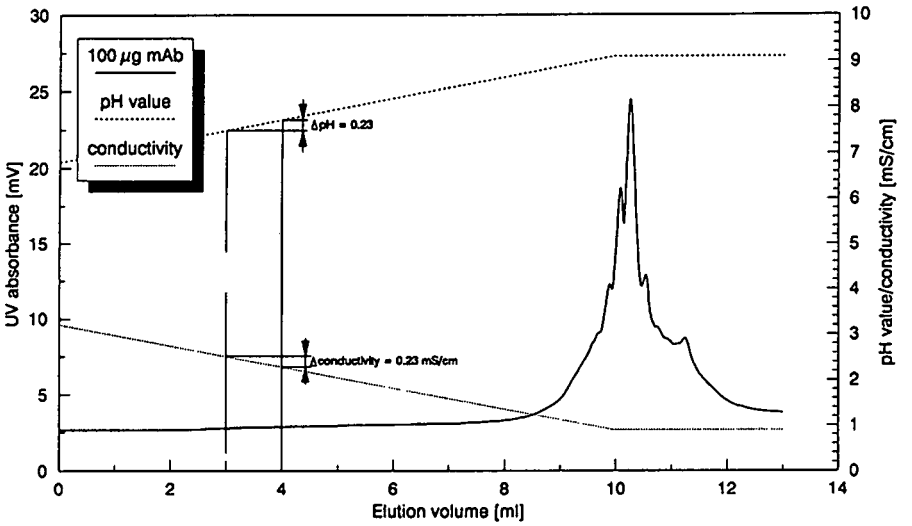

Fig. 3. Separation of isoproteins of human monoclonal antibody "3D6" by ion-exchange chromatography on a Mono S column (HR 5/5). Sample, 100 μg of monoclonal antibody dissolved in 25 μl of starting buffer and loaded on a 1-ml column; starting buffer, 5 mM borate, 40 mM mannitol, NaCl (pH 6.8), $\kappa = 3.2$ mS/cm; elution buffer, 5 mM borate (pH 9.1), $\kappa = 0.9$ mS/cm. Elution was effected with a linear pH–salt gradient over ten column volumes at a flow-rate of 0.1 ml/min. Other conditions as in Fig. 1.

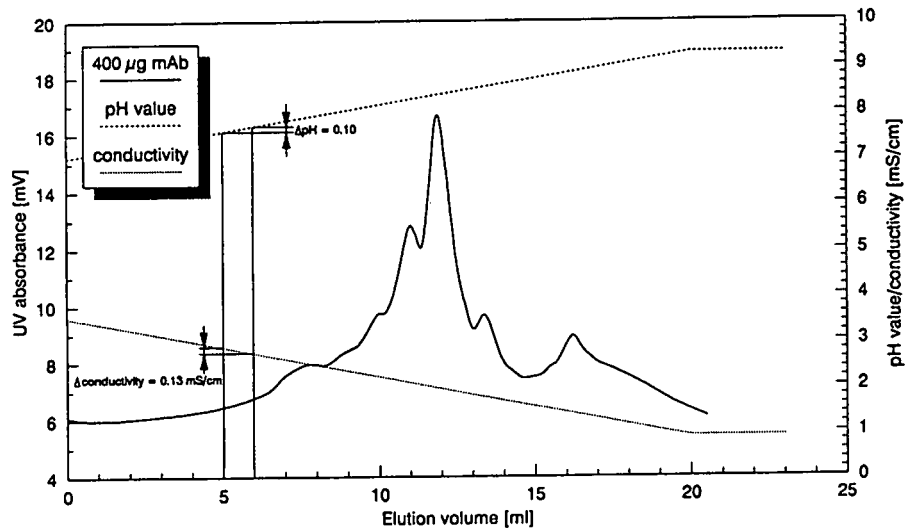

Fig. 4. Separation of isoproteins of human monoclonal antibody "3D6" by ion-exchange chromatography on a Mono S column (HR 5/5). Sample, 400 μg of monoclonal antibody dissolved in 100 μl of starting buffer and loaded on a 1-ml column; starting buffer, 5 mM borate, 40 mM mannitol, NaCl (pH 7.1), $\kappa = 3.5$ mS/cm; elution buffer, 5 mM borate (pH 9.1), $\kappa = 0.9$ mS/cm. Elution was effected with a linear pH–salt gradient over twenty column volumes at a flow-rate of 0.2 ml/min (61.1 cm/h). Other conditions as in Fig. 1.

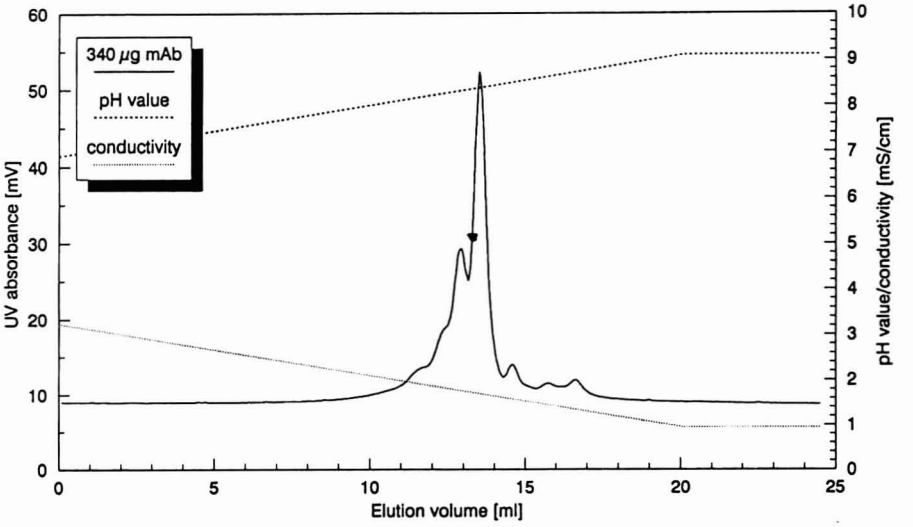

Fig. 5. Analytical separation of isoproteins of human monoclonal antibody "Virgil" by ion-exchange chromatography on a Mono S column (HR 5/5). Sample, 340 µg of monoclonal antibody dissolved in 31.0 ml of starting buffer and loaded on a 1-ml column; starting buffer, 5 m*M* borate, 45 m*M* mannitol, 0 m*M* NaCl (pH 6.9), $\kappa = 3.2$ mS/cm; elution buffer, 5 m*M* borate (pH 9.1), $\kappa = 0.9$ mS/cm. Elution was effected with a linear pH–salt gradient over twenty column volumes at a flow-rate of 0.2 ml/min. Other conditions as in Fig. 4.

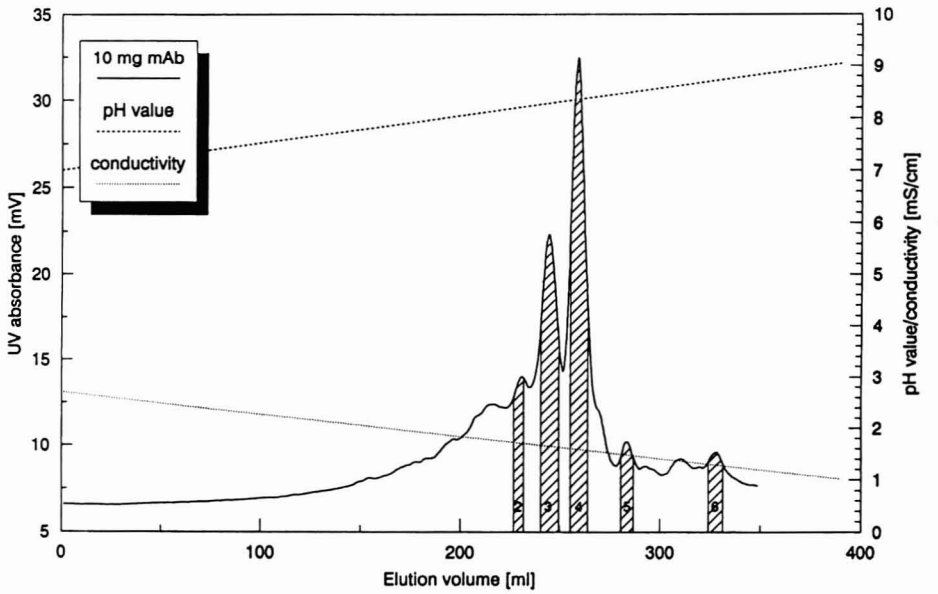

Fig. 6. Preparative separation of isoproteins of human monoclonal antibody "Virgil" by ion-exchange chromatography on a Mono S column (HR 10/16). Sample, 10 mg of monoclonal antibody dissolved in 23.0 ml of starting buffer and loaded on a 20-ml column; starting buffer, 5 m*M* borate, 45 m*M* mannitol, 20 m*M* NaCl (pH 7.0), $\kappa = 2.8$ mS/cm; elution buffer, 5 m*M* borate (pH 9.1), $\kappa = 0.9$ mS/cm. Elution was effected with a linear pH–salt gradient over twenty column volumes at a flow-rate of 2.0 ml/min.

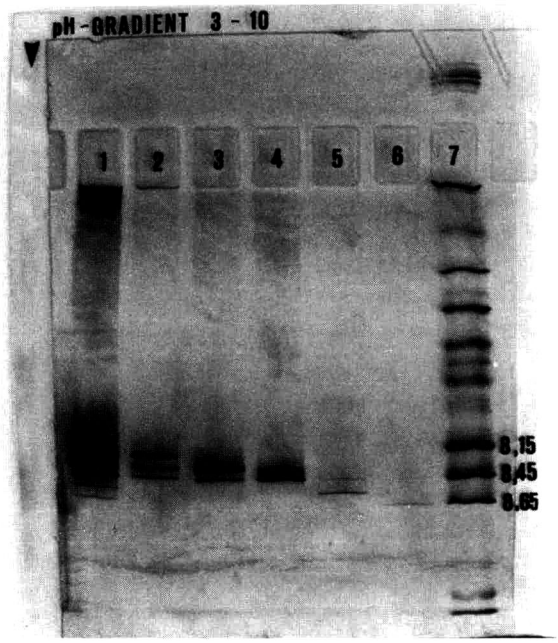

Fig. 7. Isoelectric focusing of the fractions in Fig. 6. Samples: lane 1 = starting material (human monoclonal antibody "Virgil"); lanes 2–6 = isoprotein fractions; lane 7 = p*I* 3–10 marker.

to demonstrate that the isoforms elute according to their isoelectric points. Aliquots of the collected fractions were desalted on Sephadex G-25 in PD-10 columns and rechromatographed on Mono S HR5/5 (Fig. 8).

Process monitoring

This method was also used for monitoring the purification process of human monoclonal antibody "Virgil". The antibody was produced by animal cell culture using RPMI medium containing 2% foetal calf serum.

The isoform pattern in the particular purification steps of "Virgil" is demonstrated in Fig. 9A and B. Peak areas were integrated by a Nelson chromatography data system using force dropline integration and a project horizontal baseline rearward setting. The area of the most dominant isoprotein was arbitrarily defined as 100%, and peak areas of the other isoproteins were expressed as a percentage relative to this. The experiments showed that the purification step

TABLE I

QUANTITATIVE ISOFORM DISTRIBUTION OF HUMAN MONOCLONAL ANTIBODY ("VIRGIL") DURING A PURIFICATION PROCESS SHOWING A LOSS IN AMOUNT OF ISOPROTEIN 4 AFTER PROTEIN A SEPHAROSE

The samples of single antibody purification sequence were analysed in duplicate.

Sample	Area (%)			
	Peak 1	Peak 2	Peak 3	Peak 4
Diaretentate 1	41.0	100	10.0	17.1
Diaretentate 2	42.2	100	7.8	16.2
Eluate protein A Sepharose 1	45.4	100	9.8	4.9
Eluate protein A Sepharose 2	41.9	100	8.2	4.4
Eluate S-Sepharose 1	43.2	100	8.7	3.7
Eluate S-Sepharose 2	45.6	100	9.9	4.2

with protein A Sepharose fast flow causes a loss of the most basic isoform (Table I).

DISCUSSION

A linear pH gradient of 20 column volumes formed by mixing 5 m*M* borate, 45 m*M* mannitol and 20 m*M* NaCl with 5 m*M* borate gives sufficient resolution on Mono S for separating the isoproteins of human monoclonal antibodies with a p*I* range between 8.45 and 8.70 or 8.15 and 8.65. Identical resolutions were obtained on 1-ml and 22-ml columns (Figs. 5 and 6). Further, ascending pH gradients in combination with descending salt gradients can improve the resolution of isoprotein separation (compare Figs. 1 and 4). The various isoforms are resolved according to their isoelectric points as demonstrated by IEF (Fig. 7). The antibodies elute in order of their p*I* values. Isoproteins are very homologous with each other, differing only in several charged carbohydrate moieties. These moieties are at the surface of the protein and elution in the ion-exchange mode is coincidental with the p*I* values. The oligosaccharide might also form a complex with borate, like mannitol and borax in the mobile phase. Such complex

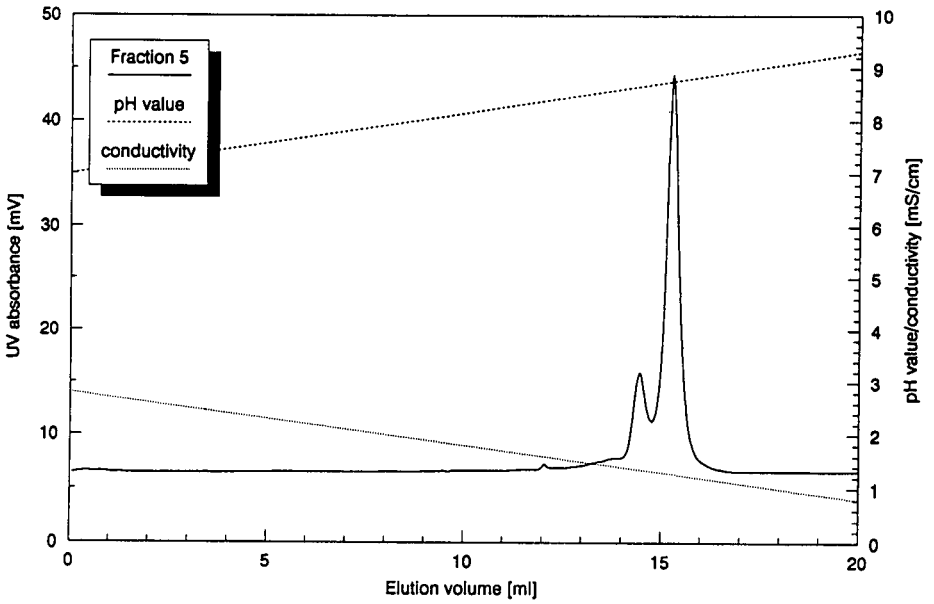

Fig. 8. Rechromatography of separated isoproteins of "Virgil" by ion-exchange chromatography on a Mono S column (HR 5/5). Sample, fraction 5 from Fig. 6 dissolved in 21.0 ml of starting buffer and loaded on a 1-ml column; starting buffer, 5 m*M* borax, 45 m*M* mannitol, 20 m*M* NaCl (pH 7.0), $\kappa = 2.8$ mS/cm; elution buffer, 5 m*M* borax (pH 9.1), $\kappa = 0.9$ mS/cm. Elution was effected with a linear pH–salt gradient over twenty column volumes at a flow-rate of 0.2 ml/min.

formation may contribute to the separation. Additional charges might be introduced into the surface of the antibody molecule. Brena *et al.* [18] also reported the selective adsorption of immunoglobulins on phenylboronate–agarose. An interaction between the sugars from the IgG molecule and H_3BO_3 was confirmed by this observation. Complex formation has also been used for the separation of polyhydroxy compounds such as sugars by ion-exchange chromatography [16]. Further investigations to explain this involvement of complex formation between IgG and H_3BO_3 are in progress. The complex formation between IgG and H_3BO_3 can enhance the separation but the combined pH–salt gradient seems to be the major contributor to the resolution of isoproteins. Without salt, just with the linear pH gradient, the resolution is lower than with the combined pH–salt gradient (compare Figs. 1 and 3).

The advantages of ion-exchange chromatography with linear pH–salt gradients are as follows. The buffer composition (borax, NaCl and mannitol) is simple and inexpensive. After separation the isoforms can be used for immunochemical, *in vivo* and *in vitro* experiments because the problem of ampholine removal is circumvented. In chromatofocusing and conventional IEF, ampholines are needed for the generation of the pH gradients. Ion-exchange chromatography is rapid, in contrast to IEF and CF, but slower than CZE. Compared with IEF, the recovery of isoforms is high. For preparative purposes in IEF the proteins have to be eluted from the polyacrylamide matrix either with a high salt concentration or an electric current. CZE is not scaleable and can therefore only be used for analytical purposes. Ion-exchange chromatography with a pH–salt gradient also allows the quantitative determination of the isoprotein pattern (Table I) in a very accurate manner. IEF fails completely in detecting small quantitative changes in isoform pattern. Coomassie Brilliant Blue is too insensitive to detect these small changes. Unlike Coomassie Brilliant Blue dye, which binds stoichiometrically to most proteins, the silver staining method results in bands which show much greater variation [17].

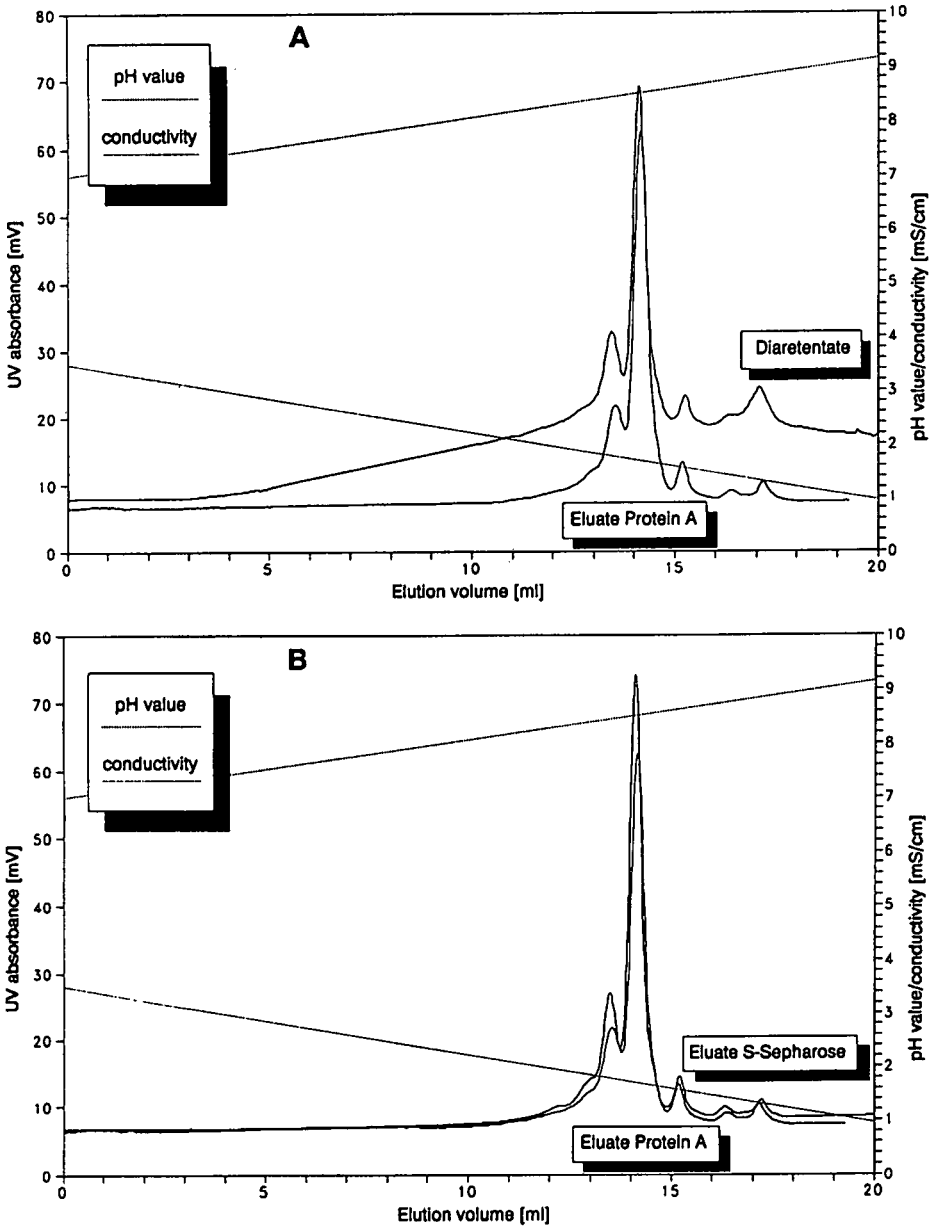

Fig. 9. Comparison of isoform pattern during a purification process of human monoclonal antibody "Virgil" by ion-exchange chromatography on a Mono S column (HR 5/5). Samples: (A) diaretentate and eluate of protein A Sepharose fast flow; (B) protein A Sepharose fast flow and S-Sepharose fast flow. Each sample was diluted to an IgG concentration of 13.5 μg/ml with starting buffer. The sample volume was 20 ml.

The separation of isoforms is essential for the investigation of the heterogeneous nature of native and recombinant proteins and for quality control of recombinant proteins. In this work an attempt was made to demonstrate that ion-exchange chromatography with linear pH–salt gradients could be an alternative method to preparative IEF, CF and CZE. The isoforms of two human monoclonal antibodies could be partially separated according to their isoelectric points. The separation was sufficient to obtain homogeneous samples after rechromatography. Further,

the quantitative determination of isoproteins for process monitoring was possible.

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Separation of polysaccharide-specific human immunoglobulin G subclasses using a Protein A Superose column with a pH gradient elution system

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ABSTRACT

Protein A Superose was employed to separate affinity-purified anticarbohydrate antibodies according to immunoglobulin G (IgG) subclass. Separation was achieved with a novel buffer system (disodium phosphate–sodium acetate–sodium chloride–glycine), which allowed the generation of a linear pH gradient from pH 8 to 3. Protein A-bound anti-carbohydrate antibodies were eluted as three peaks, two of them mainly containing IgG2 and one consisting of highly enriched IgG1. The enriched antibody preparations retained their functional activity. This separation procedure can be considered as an alternative to the preparation of IgG subclasses with subclass-specific monoclonal antibodies and could be employed whenever contamination with immune complexes has to be avoided.

INTRODUCTION

Antibody preparations enriched in immunoglobulin G (IgG) subclasses constitute an important tool for studying the biological role of the various IgG isotypes. Several methods for the separation of human IgG subclasses have been developed, mainly utilizing immunoaffinity chromatography with monoclonal antibodies [1–3] or based on differences in the isoelectric points of IgG subclasses [4,5]. The aim of this study was to obtain an IgG preparation enriched in polysaccharide-specific IgG2 without the use of monoclonal antibodies. To achieve this aim we employed Protein A Superose, a new column chromatographic material that allows high flow-rates and is therefore especially applicable for fast protein liquid chromatography (FPLC). By using a novel buffer system that led to the generation of a linear gradient from pH 8 to 3, separation of *Haemophilus* capsular polysaccharide-specific and pneumococcal polysaccharide-specific human IgG into its subclasses could be achieved. The separated IgG fractions retained their functional activity.

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EXPERIMENTAL

Materials

Pneumococcal polysaccharide serotype 3 was obtained from American Type Culture Collection (Rockville, MD, USA). *Haemophilus influenzae* type b capsular polysaccharide polyribosyl ribitol phosphate (HIB-PRP) was prepared by Dr. A. Mitterer (Immuno, Vienna, Austria). Hyperimmunoglobulins specific for pneumococcal polysaccharides and for *Haemophilus influenzae* type b polysaccharide (HIB-PRP) were obtained from Immuno. EAH-Sepharose 4B was obtained from Pharmacia–LKB (Uppsala, Sweden). All other reagents were from Merck (Darmstadt, Germany). The Protein A Superose HR 10/2 column was obtained from Pharmacia–LKB and was connected to a Pharmacia FPLC system (consisting of an LCC-500 control unit, two P-500 pumps and a Frac-100 fraction collector). The column effluent was monitored for absorbance at 280 mm with a 2-mm flow-through cell and a UV-1 monitor (Pharmacia–LKB), and for pH with a flow-through cell connected to a pH monitor (Pharmacia–LKB). Buffer solutions for the FPLC runs were sterile-filtered and degassed.

Affinity purification of antigen-specific antibodies

Haemophilus influenzae type b capsular polysaccharide (HIB-PRP) and pneumococcal polysaccharide serotype 3 were coupled to EAH-Sepharose 4B according to the method of Munson *et al.* [6]. Gel materials were packed into low-pressure columns.

HIB-PRP and pneumococcal hyperimmunoglobulins were dissolved in PBS and loaded on to the respective affinity columns equilibrated with the same buffer. Antigen-specific antibodies were eluted with 3.5 *M* magnesium chloride in phosphate-buffered saline (PBS). Eluted antibodies were dialysed against PBS and stored at −20°C.

Protein A Superose chromatography

Affinity-purified antibodies were dialysed against buffer A [0.1 *M* disodium phosphate–0.1 *M* sodium acetate–0.1 *M* glycine–0.15 *M* NaCl, (pH 8.1)]. Chromatography was performed at room temperature by the following procedure: 5–10 mg of antigen-specific antibodies were loaded on to a Protein A Superose HR10/2 column connected to an FPLC system. Unbound material was removed by washing with 16 ml of buffer A. Bound IgG was eluted with a linear gradient (40 ml) starting with buffer A and ending with a 0.1 *M* sodium acetate–0.1 *M* glycine–0.15 *M* NaCl buffer (pH 2.8) (buffer B). The column was then washed with an additional 24 ml of pure buffer B before regeneration with buffer A. A flow-rate of 1 ml/min was maintained throughout sample application, washing and elution. The fractions from the peak maxima (2–3 ml each) were collected, neutralized immediately and then dialysed against PBS. Up to six runs with each of the antibody preparations were performed, and the corresponding peak fractions were pooled for protein analysis. For chromatography of serum samples, the various sera were diluted at least sixfold with buffer A, and material corresponding to 5–10 mg of IgG, as determined by radial immunodiffusion, was applied to the column and treated as described above.

Myeloma proteins were purified from patients' sera by ethanol precipitation [7] and chromatography on DEAE Affi Gel Blue (Bio-Rad, Richmond, CA, USA) [8]. The proteins were obtained as 20–50 mg/ml solutions and were diluted to 0.4–0.6 mg/ml with buffer A. Aliquots of 2 ml were loaded on to the column and pH gradient elution was performed as described.

Protein analysis

Protein concentration was determined by the use of the IgG extinction coefficient $E_{280}^{1\%} = 13.5$ [9] or by the bicinchoninic acid method (BCA-Protein Assay Kit; Pierce, Oud-Beijerland, Netherlands) using bovine γ-globulin (Bio-Rad) as a standard.

To determine total IgG, commercial radial immunodiffusion (RID) plates (EP-RID; Immuno) and a human standard serum (Immuno) were used. IgG subclass concentrations were determined by radial immunodiffusion with poly-

clonal RID kits (BINDARID; Binding Site, Birmingham, UK).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with reduced samples using ExcelGel SDS gradient 8–18 ready-to-use gels (Pharmacia–LKB) according to the supplier's instructions.

Determination of antigen-specific antibody titres by ELISA

Pneumococcal polysaccharide and HIB-PRP were coupled to tyramine as described by Barra *et al.* [10]. Enzyme-linked immunosorbent assay (ELISA) plates (NUNC, Kamstrup, Denmark) were coated with the coupled material (1 μg per well) by incubation at 4°C for 16 h. After blocking residual binding sites with 2% bovine serum albumin (4 h at 37°C), sample and standard dilutions were applied and incubated overnight at 4°C. A human serum pool (PL30; Immuno) with 10 000 arbitrary units per ml was used as a standard. Bound IgG was detected with biotinylated anti-human IgG (Vector, Burlingame, CA, USA) (diluted 1:1000; 2 h at 37°C) and with the Vectastain ABC kit [avidin-biotinylated peroxidase complex (Vector), 40 min at 25°C] according to the supplier's instructions. Colour reaction was performed using ABTS tablets (Boehringer, Mannheim, Germany) and was measured at 405 nm with an Immuno Reader (NUNC).

RESULTS

Separation of polysaccharide-specific immunoglobulin preparations into IgG subclasses

Anti-Haemophilus influenzae PRP antibodies. An anti-*Haemophilus* hyperimmunoglobulin was affinity-purified using Sepharose-immobilized *Haemophilus* PRP. This purification step resulted in both an increase in the IgG2 content and a marked increase in the ELISA titre per μg protein (Table I). IgG3 and IgG4 were not detectable or comprised less than 1% of the total IgG in this isolate. This affinity-purified material was loaded on to the Protein A Superose column. Bound IgG was eluted with a gradient that started with a phosphate–acetate–glycine–NaCl buffer (pH 8.1) and ended with an acetate–glycine–NaCl buffer (pH 2.8). By using this buffer system, a linear pH gradient from pH 8 to 3 was generated, which allowed the separation of the polysaccharide-specific IgG into three peaks with maxima differing by 0.5–0.7 pH units (Fig. 1A and Table I). Two of the peaks (peaks 1 and 3) consisted primarily of IgG2, whereas in peak 2 IgG1 was predominant. The lowest anti-PRP titre per μg of protein was seen in the IgG1 material of peak 2. The IgG2 eluting at the lower pH (peak 3) had a higher anti-PRP titre than the IgG2 eluting at the higher pH (peak 1). When the three isolated peak fractions were subsequently rechromatographed on Protein A

TABLE I

FRACTIONATION OF ANTI-HIB-PRP ANTIBODIES

Amounts of 5–10 mg of affinity-purified antibodies obtained from hyperimmunoglobulin preparations were loaded on to a Protein A Superose HR 10/2 column and eluted with a linear pH gradient as described under Experimental. Fractions (2–3 ml) were collected at the peak maxima, neutralized and dialysed against PBS. Six runs were performed, and the corresponding peak fractions were pooled and used for further analysis.

	Protein (mg/ml)	IgG1 (%)	IgG2 (%)	Titre (units/μg protein)	Elution pH
Hyperimmunoglobulin	80.02	63	37	7	
Affinity-purified antibodies	10.78	45	55	694	
Protein A, peak 1	0.40	14	86	812	4.8
Protein A, peak 2	0.51	75	25	411	4.1
Protein A, peak 3	0.30	28	72	1501	3.5

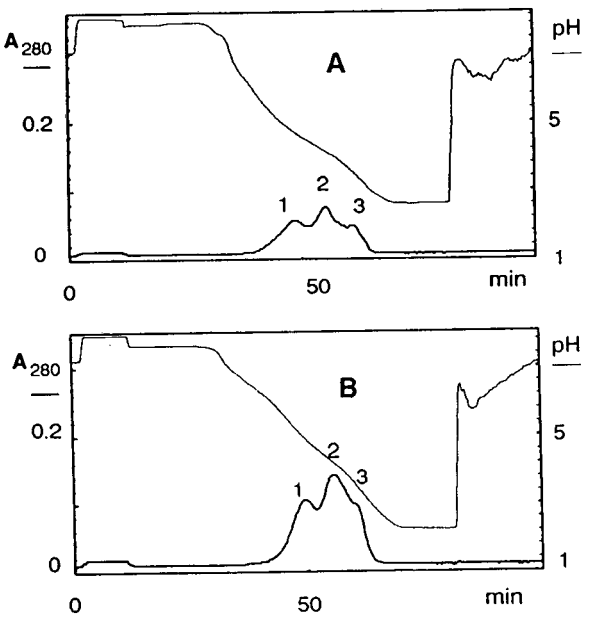

Fig. 1. Fractionation of anti-polysaccharide immunoglobulin preparations into IgG subclasses. (A) Affinity-purified anti-*Haemophilus* PRP antibodies or (B) anti-pneumococcal polysaccharide antibodies were loaded on to a Protein A Superose HR 10/2 column and eluted at 1 ml/min with a pH gradient as described under Experimental. Absorbance was measured at 280 nm.

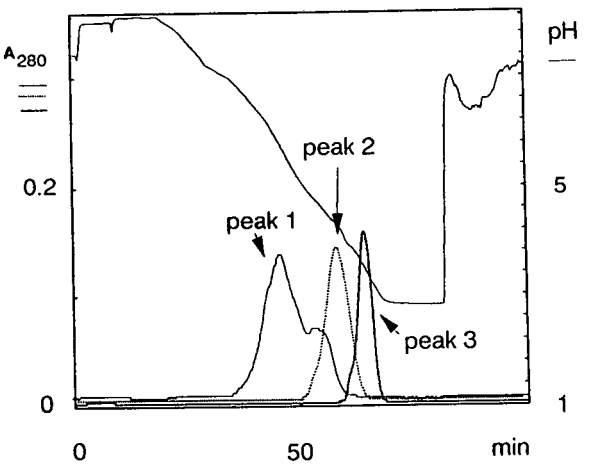

Fig. 2. Rechromatography of the isolated peak fractions of Fig. 1A on Protein A Superose The pooled peak fractions obtained with Protein A Superose chromatography of affinity-purified anti-PRP antibodies (shown in Fig. 1A) were rechromatographed separately on Protein A Superose. The elution patterns of the three runs have been superimposed graphically (peak 1, thin line; peak 2, broken line; peak 3, thick line). Absorbance was read at 280 nm.

Superose, single prominent peaks and, for peak 1, an additional small peak due to the respective impurities from the neighbouring peak were observed (Fig. 2). Their location within the pH gradient was identical with that of their predecessors, indicating that IgG2 can in fact be separated according to differences in the affinity to Protein A Superose. When analysed in reduced form by SDS-PAGE, the three IgG subclass preparations presented the same band pattern as did the hyperimmunoglobulin starting material (data not shown).

Anti-pneumococcal antibodies. As described for the isolation of anti-*Haemophilus* PRP antibodies, anti-pneumococcal antibodies were affinity-purified from an anti-pneumococcal hyperimmunoglobulin (containing 56% IgG1 and 43% IgG2) using immobilized pneumococcal polysaccharide serotype 3. The affinity-isolated material consisted of 52% IgG1 and 48% IgG2, with IgG3 and IgG4 contents of less than 1%. The affinity-purified material was separated on the Protein A Superose column with a pattern (Fig. 1B) nearly identical with that for the separation of anti-*Haemophilus* antibodies, *i.e.*, the three protein peaks obtained had similar IgG subclass distributions (peak 1, 19% IgG1, 81% IgG2; peak 2, 82% IgG1, 18% IgG2; peak 3, 35% IgG1, 65% IgG2). These data show that the separation of IgG into two different IgG2 peaks was not a property of anti-*Haemophilus* PRP antibodies.

Separation of IgG subclasses in normal human sera

When normal human serum from individual donors was passed over the Protein A Superose column and eluted with the pH gradient described above, the bound material was resolved into three peaks (Fig. 3). The first peak (1) consisted of nearly (94%) pure IgG2, the second peak (2) contained 94% IgG1 and 6% IgG2, and the third peak (3) consisted of approximately equal amounts of IgG1 and IgG2. This demonstrates that a biphasic elution pattern is a common property of polyclonal IgG2. IgG3 could not be detected in the eluted peaks, as protein A does not bind IgG3 [11]. IgG4 levels in the protein peak fractions were below the detection limit of our assay system (0.02 mg/ml). Similar

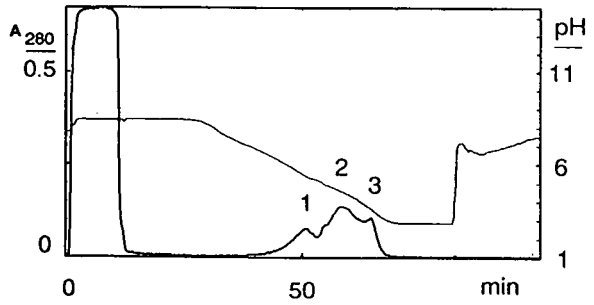

Fig. 3. Separation of human IgG subclasses in normal human serum. A 1-ml volume of human serum from a single donor was diluted with column buffer, loaded on to a Protein A Superose HR 10/2 column and eluted with the pH gradient described.

results were obtained with sera from two other donors (data not shown).

Elution of monoclonal IgG2 as a single peak

In contrast to polyclonal IgG2, three different purified myeloma IgG2 proteins eluted from Protein A Superose as single peaks at distinct pH values (4.9–5.1); the elution profile of one of them is shown in Fig. 4.

DISCUSSION

In pursuing the goal of isolating human IgG2 antibodies against carbohydrate antigens without the use of immunoaffinity chromatography with IgG2-specific monoclonal antibodies, the recently developed Protein A Superose column was employed. A simple buffer system consisting of only two components was developed to generate a linear pH gradient between pH 8 and 3. This allowed the separation of affinity-purified anti-*Haemophilus* capsular polysaccharide antibodies (HIB-PRP) into fractions with highly enriched IgG subclass contents.

The affinity-purified anti-HIB-PRP preparation used as the starting material for IgG subclass purification consisted of nearly equal amounts of IgG1 and IgG2 and only traces of IgG3 and IgG4. This is in contrast to antibody responses to other bacterial polysaccharides, such as group A streptococcal carbohydrates or dextran from Lactobacteriaceae or levan from Corynebacteriaceae, which predominantly result in the production of IgG2 [12]. Our data, however, are consistent with results reported by Goodall *et al.* [3], who also found IgG1- and IgG2-containing anti-HIB fractions following separation of HIB-specific IgG with immobilized mouse monoclonal anti-IgG subclass antibodies. Further, analysis of anti-HIB sera by IgG subclass-specific ELISA also revealed the presence of both HIB-specific IgG1 and IgG2 [13].

When this affinity-purified material was loaded on a Protein A Superose column and eluted with the described buffer system, the IgG preparation was resolved into three peaks. Two of these peaks contained IgG2 and were separated by a central peak consisting of IgG1. This elution pattern was not specific for anti-HIB antibodies, as the separation of anti-pneumococcal antibodies and also of normal serum IgG yielded a corresponding elution pattern and similar IgG subclass distribution of the isolated fractions (see Figs. 1B and 3).

The finding of a second IgG2 peak during elution of polyclonal IgG from the column points to a different protein A affinity for distinct IgG2 moieties and is obviously due to the high resolution of Protein A Superose (even at the relatively high flow-rate of 1 ml/min) in combination with the linear pH elution system. In this respect, the system described is superior to the biphasic pH gradient published by Duhamel *et al.* [14], which resulted in the separation of

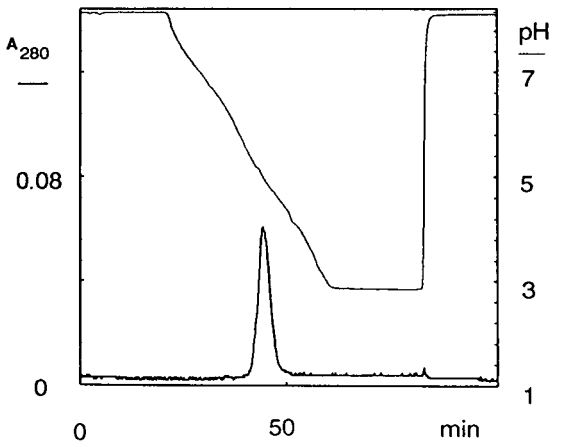

Fig. 4. Elution of purified IgG2 myeloma proteins from Protein A Superose as single peaks. Purified IgG2 myeloma protein diluted in column buffer A was loaded on to a Protein A Superose HR 10/2 column and eluted with the pH

into two overlapping peaks composed primarily of IgG1 and IgG2, respectively. The distinctiveness of the two IgG2 peaks obtained by the present procedure was indicated by rechromatography of the IgG subclass fractions, which resulted in the elution of single prominent peaks eluting at exactly the same pH as their predecessors (Fig. 2). The reasons underlying the separation of IgG2 into two fractions with different binding capacities to staphylococcal protein A have yet to be elucidated. The data presented here, however, suggest that the two peaks contain IgG2 specific for different epitopes on the polysaccharide antigen. Monoclonal IgG2 which reacts only with one antigenic epitope elutes from the column as a single peak (Fig. 4).

The novel IgG fractionation procedure described here yielded two functionally active and structurally unmodified IgG2 preparations. Their capacity to bind to their respective antigen demonstrates the presence of a functional antigen-binding region, and both preparations contained largely unmodified IgG molecules, as shown by unimpaired interaction with the second-step antibody. Further, preliminary studies (data not shown) also demonstrated that the complement-binding properties of both IgG2 fractions remained intact.

Although more highly purified IgG subclass preparations can be obtained by monoclonal antibody-immunoaffinity chromatography, the use of Protein A Superose for isolating IgG subclass anti-polysaccharide antibodies has certain advantages. Ready-to-use chromatographic columns are available with comparable lot-to-lot properties. The column can be cleaned with denaturing detergents and even with some organic solvents, making reliable removal of pyrogens possible. Further, the column matrices coupled with monoclonal antibodies against IgG subclasses may leak protein, which will then lead to the formation of immune complexes with sample proteins. These immune complexes may cause unwanted side-effects if present in preparations used for long-term therapy. Even traces of immune complexes present as contaminants have been shown to exert immune-modulating effects which lead to impairment of certain monocyte functions [15–17]. Hence the procedure described here can be employed for the preparation of IgG2 subclass-enriched products in which contamination with immune complexes has to be avoided.

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Membrane chromatography for rapid purification of recombinant antithrombin III and monoclonal antibodies from cell culture supernatant

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ABSTRACT

The task of purifying monoclonal antibodies (MAbs) and human recombinant antithrombin III (rATIII) from cell culture supernatant was carried out using two different approaches, both based on the use of membraneous matrices. The first approach employed a strongly acidic and a strongly basic membrane ion exchanger, which were evaluated for their ability to purify monoclonal antibodies and the human active recombinant antithrombin III from cell culture supernatant. Within minutes gram amounts of product could be purified in a high-flux system, specially developed for this purpose, achieving purities of 80% for MAbs and 75% for rATIII, respectively. The capacity of the acidic membrane ion exchanger for MAbs was found to be 1 mg/cm^2 with recoveries up to 96% and that of the basic membrane ion exchanger for rATIII was 0.15 mg/cm^2 with recoveries up to 91%. The second approach consisted of using heparin, a mucopolysaccharide with a strong affinity towards ATIII, coupled to amine-modified or epoxy-activated membranes by reductive amination, for the purification of rATIII. The ATIII binding capacities of the membranes were found to be 91 μg/cm^2 for the amine-modified and 39 μg/cm^2 for the epoxy-activated membrane, achieving purities of 75%. The coupling proved to be fairly stable over a period of 5 months and the membranes remained operable even after steam sterilization and treatment with sodium dodecyl sulphate. Final purification in both instances was carried out by gel filtration.

INTRODUCTION

Ion-exchange and affinity chromatography are common techniques for the purification and preparation of proteins, peptides and enzymes. However, using classical soft gels or porous particle media as a matrix results in a limitation arising from the restriction of mass transport in these diffusion-dominated systems [1,2]. A different approach is the attachment of functional groups to the inner surfaces of synthetic microporous membranes (Table I). The main advantage of using membranes as a matrix is that the mass transport is convective and therefore avoids the aforementioned limitation of classical matrices [1,2]. Other interesting features in addition to the high flow-rate include high adsorption rates and binding velocities, no problems arising from channelling, bed shift or bed collapse and the ease of scale-up and the comfort of using a prepacked stabilized matrix. Kroner and coworkers [1,2] showed the purification of monoclonal antibodies (MAbs) using protein A/G and acidic membrane ion exchangers on an analytical scale (single sheets of 13.4 and 42 cm^2), using the same type of membranes that are discussed in this investigation. The isolation of microbial enzymes with affinity membranes was described by Champluvier *et al.* [3], Briefs and Kula [4] and Kroner *et al.* [5].

The aim of this work was to test new prototypes of membrane ion exchangers and heparin affinity membranes. Analytical-scale purification was carrried out using a 100 cm^2 membrane in a fast protein liquid chromato-

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			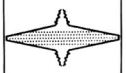	
	syringe top	filter holder	membrane module	pleated module
Area: (cm^2)	5.4	12.56	100	1400, 2400
Flow-rate: (ml/min)	6 *	10 *	30 *	850 **

* FPLC, ** High flux system

Fig. 1. Schematic diagrams of the different housings containing the membranes. The syringe top and the membrane holder were equipped with single membrane sheets. The 100-cm^2 unit consisted of a stack of five membrane sheets. These three units were used with an FPLC or LC system. The membrane in the preparative-scale ion exchanger units is pleated, and was used with the HFS.

graphic (FPLC) system. For preparative-scale purification, 1400 and 2400 cm^2 membrane ion exchanges were used (Fig. 1).

A special high-flux system (Fig. 2) was developed for optimum use of the preparative membrane ion exchanger units (MIEX Q, S). This system allows high flow-rates (up to 1200 ml/min) and a change in flow direction, which is very important for reducing the elution volume and also helps to prevent fouling of the membrane. Corresponding controlling software, allowing complete automation of the whole process, has been developed and is currently being tested.

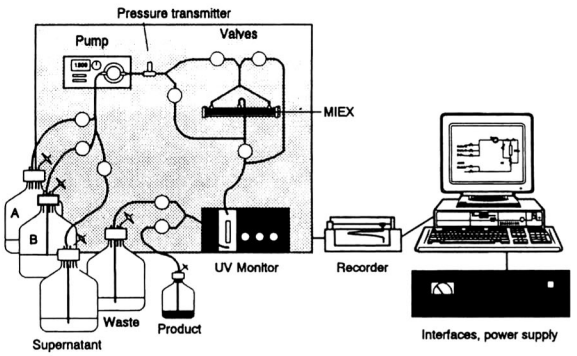

Fig. 2. High-flux system (HFS) consisting of a gear-wheel pump, a UV monitor, a pressure transmitter and special valves. A personal computer was equipped with interfaces for A/D, D/A and TTL signal transfer. Specially developed software offers the opportunity to control the valves and to monitor the UV and pressure signals, thus permitting complete automatisation. The system was developed for optimum use of the preparative membrane ion exchanger units and allows flow-rates up to 1200 ml/min and a maximum pressure of 1.2 bar.

Heparin, a highly sulphated surface mucopolysaccharide (M_r 5000–30 000) shows affinity interactions with many enzymes [6], namely enzymes of the nucleic acid metabolism [6], low- and high-density lipoproteins [7], lysosomal hydrolases [6] and many factors of the blood coagulation cascade [8]. In our case we exploit the particularly high affinity interaction that exists between heparin and ATIII [9] by covalently coupling the former to a membrane. The method of reductive amination [10] was chosen because of its reported ability to couple heparin in an oriented and stable manner [11].

EXPERIMENTAL

Cell culture

Cells. A rat/mouse hybridoma (HB 58; ATCC) and a mouse/mouse hybridoma were cultivated. Both cell lines secreted monoclonal antibodies type IgG_1 with an isoelectric point $pI \geq 7$. The recombinant Chinese hamster ovary (rCHO) cell line produced human active recombinant antithrombin III (rATIII) [12], a glycoprotein which is an important regulator of blood coagulation.

Medium. A serum-free standard medium [13] containing human transferrin (10 mg/l), bovine insulin (10 mg/l) and bovine albumin (1 g/l) complexed with oleic acid as the main protein components was used. A lipoprotein fraction (Excyte I; Bayer Diagnostic, Munich, Germany) was added (1 ml/l). On the 100-l scale and for the cultivation of the CHO cell line, albumin was omitted.

Bioreactors. The cell lines were cultivated in a 2-l bench-scale perfusion system (Biostat BF 2) and in a 100-l bioreactor (Biostat 100 L) (Braun Biotech International, Melsungen, Germany). pO_2 was set at 40% air saturation, pH at 7.1, stirrer speed at 30 rpm and temperature at 37°C.

Membrane ion exchangers

The studies were performend with Sartobind membrane ion exchangers, which were kindly supplied by Sartorius (Göttingen, Germany). A characterization of the membranes is given in Table I.

The *high-flux system (HFS)* (Fig. 2) consists of a gear-wheel pump (Watson Marlow 5003 U), a UV monitor (Uvicord; Pharmacia, Uppsala, Sweden), a pressure transmitter (Kistler Instruments, Winterthur, Switzerland) and special valves (Type 700; Bürkert, Ingelfingen, Germany). A personal computer was equipped with interfaces for A/D, D/A and TTL signal transfer. Specially developed software offering the opportunity to control the valves and to monitor the UV and pressure signals, thus allowing complete automation, has been developed and is currently being tested. The system was developed for optimum use of the preparative membrane ion exchanger units and allows flow-rates up to 1200 ml/min and a maximum pressure of 1.2 bar. In order to reduce the elution volume and to prevent fouling, the flow direction was changed between loading and the other steps (Fig. 3).

Sample preparation. In order to bind proteins to the ion exchanger, the conductivity of the supernatant had to be reduced from 13 mS/cm (normal cell culture medium) to 1.3–1.8 mS/cm. In addition, some of the supernatants were first concentrated by ultrafiltration. To achieve this reduction in conductivity, several methods were used, including a diafiltration cross-flow system (S10Y30, SP20; Amicon, Beverly, MA, USA), an electrodialysis system (StanTech, Geesthacht, Germany) and simple dilution with water (Fig. 4). After adjusting the pH to 5.8 for MAbs and pH 7 for rATIII, the supernatant was passed through the S- and Q-type membrane ion ex-

TABLE I

CHARACTERISTICS OF THE SARTOBIND MEMBRANES S, Q AND A

Characteristic	Value
Membrane material	Synthetic copolymer
Filtration area	12.6, 100, 1400, 2400 cm^2
Type	S = strongly acidic (sulphonic acid); Q = strongly basic (quaternary ammonium); A = amine modified
Membrane thickness	170–190 μm
Maximum operation pressure	4 bar (400 kPa)
Pore size	0.45 μm
pH stability	pH 2–13
Thermal stability	121°C
Binding capacity	S ≈ 1 mg IgG/cm^2; Q ≈ 0.15 mg ATIII/cm^2; A ≈ 91 μg ATIII/cm^2

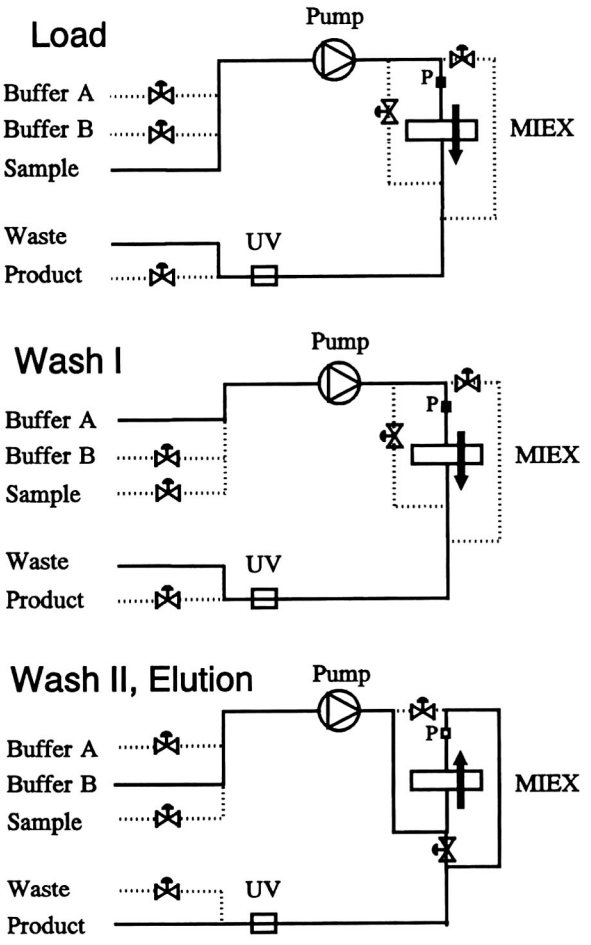

Fig. 3. Process diagram of the HFS. Load: the sample is filtered through the membrane. Wash I: the membrane is washed with buffer A to remove unbound proteins. Wash II: a change in the flow direction (back-flush) of buffer A is used to prevent fouling of the membrane. Elution: buffer B eluates the bound protein.

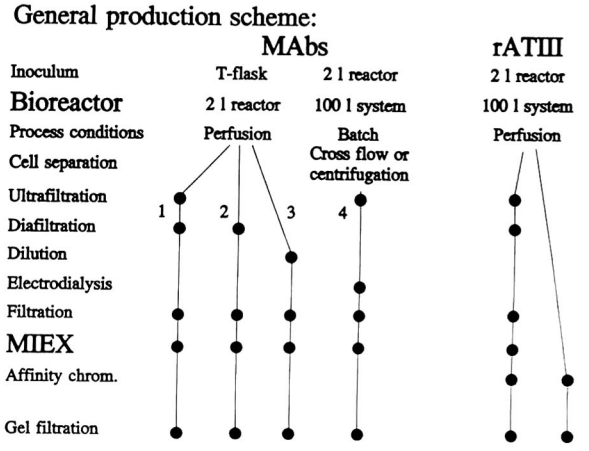

Fig. 4. Schematic diagram of the general processes used in the production and purification of MAbs and rATIII. The different sample preparation steps for the membrane ion-exchange chromatography are presented together with the purification process employing the amine-modified affinity membranes.

changers, respectively, using the HFS or FPLC system. The product was eluted from the membrane by changing the salt concentration.

Buffers. For the acidic ion exchanger, buffer system I was (A) 25 m*M* 2-(N-morpholino)ethanesulphonic acid) (MES)–10 m*M* NaCl (pH 5.8) and (B) 25 m*M* MES–250 m*M* NaCl (pH 5.8); buffer system II was (A) 10 m*M* sodium-phosphate–10 m*M* NaCl p(H 5.8) and (B) 10 m*M* sodium-phosphate–250 m*M* NaCl pH 5.8. For the basic ion exchanger, the buffer system was (A) 20 m*M* Tris–HCl (pH 7) and (B) 20 m*M* Tris–HCl–250 m*M* NaCl (pH 7).

Cleaning and regeneration. Using 0.2 *M* NaOH (60°C, 20 min), the membranes could be cleaned without loss of binding capacity [14] and were regenerated by washing with buffer A.

Heparin affinity membranes

Material. An LC system (Pharmacia Biosystems) consisting of a fraction collector, a UV monitor, a recorder and a peristaltic pump was used for these studies. The matrix was epoxy- or amine-modified synthetic copolymer membranes (Sartobind; Sartorius).

Coupling procedure with amine-modified membrane. The amine-modified membrane, a single sheet 4.7 cm in diameter, was washed thoroughly with 0.2 *M* phosphate buffer (pH 7.2), placed in a sealed test-tube containing 20 mg of heparin (sodium salt, from porcine intestinal mucosa, 180 USP units/mg; Sigma, St. Louis, MO, USA) and 15 mg of sodium cyanoborohydride ($NaCNBH_3$) (Fluka, Buchs, Switzerland) in 5 ml of 0.2 *M* phosphate buffer (pH 7.2) and then allowed to mix end-over-end for 8 days at room temperature. Completion of the coupling was determined by analysing the heparin content of the coupling solution by the method of Dubois *et al.* [15].

The remaining NH_2 groups were acetylated according to Baues and Gray [16] with 10 ml of acetic anhydride and 20 ml of 0.2 *M* sodium acetate, starting at −20°C and mixed end-over-end for 60 min at room temperature. After thorough washing in buffer A the membrane was placed in a membrane holder (Sartorius), which reduced the effective surface area of the membrane to 12.56 cm^2, and equilibrated with 200 ml of buffer A for the first run.

Coupling procedure with epoxy-modified membrane. The 100 cm^2 epoxy-modified membrane module was placed in a coupling array consisting of a peristaltic pump and a reservoir allowing the respective solutions to be circulated through the membrane. The membrane was washed with 300 ml of 0.2 *M* phosphate buffer (pH 7.2) (without circulation) and then treated for 3 days with 200 ml of 0.2 *M* ethylendiamine (EDA), with the pH adjusted to 8.5 in order to prevent damage to the casing of the module. The membrane was then washed one way with 500 ml of 0.2 *M* phosphate buffer (pH 7.2). The coupling solution, consisting of 30 mg of heparin (sodium salt, from ovine intestinal mucosa, 182 IU/mg; Sigma) and 30 mg of $NaCNBH_3$ in 9 ml of 0.2 *M* phosphate buffer (pH 7.2) was circulated over the membrane for 5 days. After 3 days an additional 50 mg of $NaCNBH_3$ in 1 ml of 0.2 *M* phosphate buffer (pH 7.2) were added. The remaining NH_2 groups were again removed by acetylation.

Sample run. A typical sample run consisted of equilibration with 100 ml of buffer A (10 ml/min) followed by loading of the membrane with supernatant, pH adjustment to 5.8 (1–10 ml/min), a wash step with 100 ml of buffer A and finally elution with buffer B (both 10 ml/min).

Buffers. The buffers were (A) 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)–150 m*M* NaCl (pH 5.8) and (B) 20 m*M* HEPES–2.5 *M* NaCl (pH 7).

Cleaning. In order to prevent fouling of the membrane equilibration, washing and elution were always performed in a different flow direction to loading, resulting in backflushing of the system. Additional cleaning of the membrane with 2.5% sodium dodecyl sulphate (SDS) solution was also successful and resulted in removal of clogging material from the membrane, so that the original flow-rates were restored.

Final purification. This was carried out using a preparative gel filtration column (Superdex 200 PG; Pharmacia) with a 600-ml gel volume. A 20 m*M* phosphate buffer containing 150 m*M* NaCl was used. The flow-rate was set at 6 ml/min.

Analytical methods

Antibody concentrations were determined using a standard kinetic sandwich enzyme-linked immunosorbent assay (ELISA) method or an ABICAP affinity column (Abion, Jülich, Germany) for rapid analysis. rATIII concentrations were also determind by kinetic ELISA (Behring Werke, Marburg, Germany). Purity was determined by SDS–polyacrylamide gel electrophoresis (PAGE) [17] either automatically (Phast System; Pharmacia) using silver staining [18] or manually (Multiphor II; Pharmacia) using Sensi-quant Coomassie Brilliant Blue staining [19]. The silver-stained gels were scanned using the Gel-Image 1DEVA software (Pharmacia).

The concentration of heparin in the coupling solution was determined using the phenol-based method described by Dubois *et al.* [15].

Conversion factor. In order to compare membraneous and gel matrices with respect to ligands and protein bound, a conversion factor, obtained from Sartorius [20], equalling 50 cm^2 of membrane surface with 1 ml of gel matrix was used. It is also important to note that the 17.35-cm^2 membrane was placed in a filter holder that reduced the effective surface area to 12.56 cm^2. All calculations made refer to these values unless stated otherwise.

RESULTS AND DISCUSSION

Purification of MAbs with membrane ion exchangers 100-cm^2 membrane ion exchanger

In order to find the optimum conditions for protein binding to the membrane ion exchanger, supernatant feed solutions of various pH and ionic strength were applied to a 100-cm^2 module (Fig. 5). It was found that a pH of 5.8 and a corresponding ionic strength of 1.8 mS/cm^2 yielded the best results (96% recovery). During the runs conducted so far over 50 mg of IgG per module could be bound employing a maximum flow-rate of 30 ml/min. The concentration factor was about 50-fold, which is comparable to that of other ion-exchange matrices. Most of the albumin could be separated by gradient elution; human transferrin, however, was concentrated under the conditions stated above (Fig. 6).

Preparative purification of MAbs using the HFS

The capacity of the membrane ion exchanger of 1 mg IgG/cm^2 (equivalent to 50 mg IgG/ml bed volume), as determined with a single filter

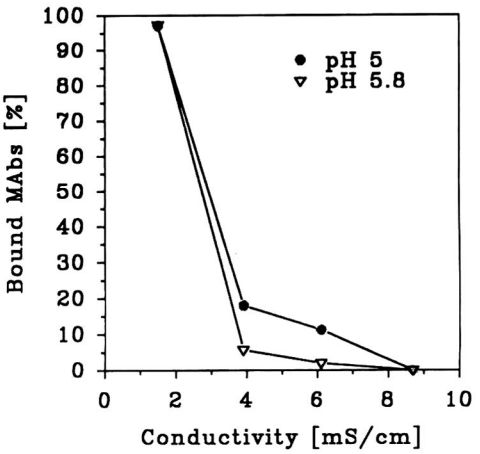

Fig. 5. Relationship between conductivity of the supernatant and binding of MAbs using the 100-cm^2 membrane ion exchanger with a flow-rate of 10 ml/min. In each run 7 mg of MAbs were applied to the membrane. In order to bind more than 90% it was necessary to reduce the conductivity to values below 1.8 mS/cm. Buffer systems: ● = pH 5, 25 m*M* acetic acid–10 m*M* NaCl; ▽ = pH 5.8, 25 m*M* MES–10 m*M* NaCl; the MAbs were eluted with the corresponding buffer with 250 m*M* NaCl.

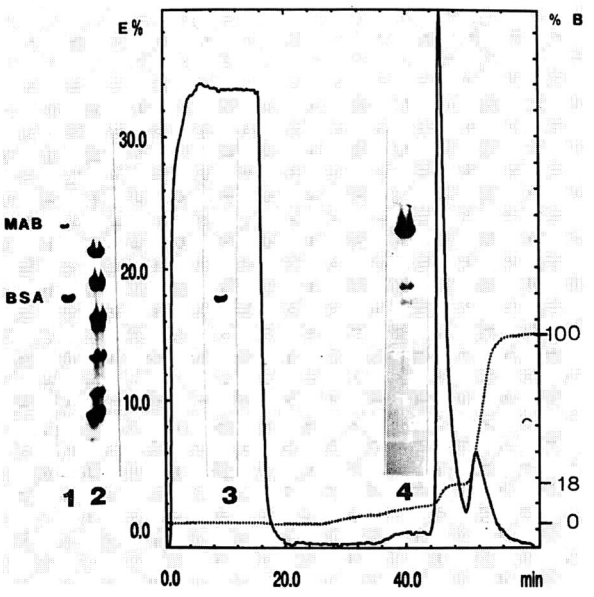

Fig. 6. Purification of monoclonal antibodies from cell culture supernatant. Following the diafiltration step the supernatant (9 mg of MAbs) was applied to the membrane ion exchanger at a flow-rate of 6 ml/min [buffer system I, MES (pH 5.8)]. The binding rate was 95%. Using a gradient from 0 to 8% B for 20 min, impurities were separated. With an 18% step of buffer B, 90% of the MAbs could be eluted. The membrane was flushed with 100% B and washed with buffer A. SDS-PAGE (unreduced) shows the purification process. Samples were diluted 1:1 with buffer. Lanes: 1 = crude cell culture supernatant (150 ml); 2 = low-molecular-mass marker (reduced); 3 = breakthrough (180 ml); 4 = MAbs eluted with 18% B (23 ml); the last peak contains unknown components but no proteins.

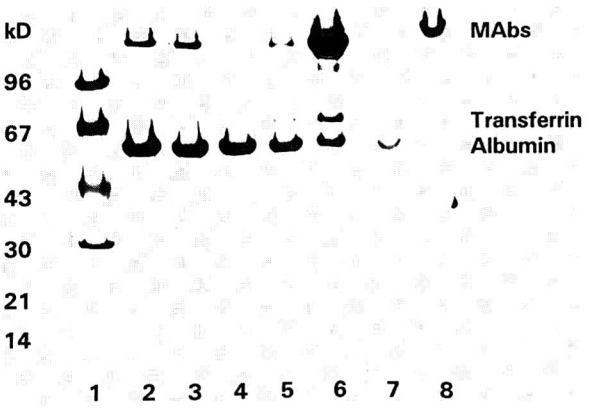

Fig. 7. SDS-polyacrylamide gradient gel (8–25%) using a modified silver stain under unreduced conditions. Isolation of MAbs with strongly acidic membrane ion exchanger and gel filtration. Lanes: 1 = marker, M_r 14 000–96 000 (reduced); 2 = supernatant; 3 = supernatant after salt reduction; 4 = unbound proteins (MIEX); 5 = first wash (MIEX); 6 = concentrated IgG (MIEX); 7 = gel filtration 1 peak; 8 = gel filtration 2 peak, purified MAbs. kD = kilodalton.

sheet (12.56 cm^2 effective surface), is very high compared with filter materials from other suppliers. The preparative units could bind up to 910 mg without any decrease in recovery (larger amounts were not determined). Lowering the ionic strength of the supernatant by method I, II or IV (Fig. 4) resulted in binding rates of about 95% and recovery rates in excess of 80% (Table II). The recovery was found to be independent of the concentration of the supernatant. The only exception occurs if the relationship between the concentration of the MAbs and the volume in which they are dissolved becomes too unfavourable. A decrease in the ionic strength by simple 1:8 dilution with water (III) resulted in lower binding capacities if the feed solution exceeded a certain volume (>6 l), but in this instance no expensive membrane step such as diafiltration was necessary. The concentration factor achieved by the MIEX is still unsatisfactory in comparison with a common ion exchanger (Table III), but optimization of the ion exchanger unit towards smaller elution volumes could change that. Up to a flow-rate of 571 $l/m^2 \cdot h$ (maximum achieved by the system) the binding of the MAbs on the MIEX was better than 90%. The concentration of 5 g of IgG in several cycles with one unit was possible; sub-

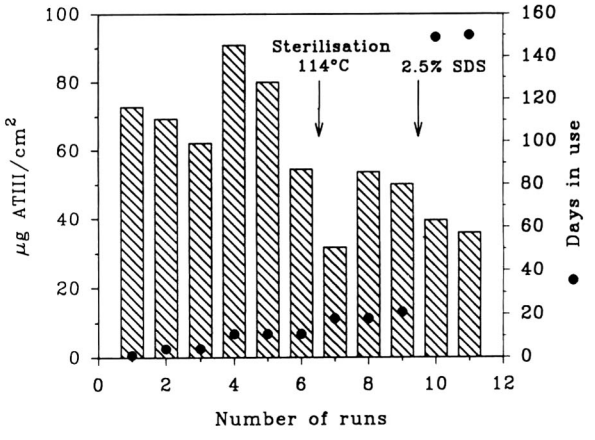

Fig. 8. Repeated use of the amine-modified membrane over a period of 150 days. Effects of steam sterilization and treatment with 2.5% SDS.

TABLE II

SUMMARY OF DIFFERENT SAMPLE PREPARATION STEPS AND THE SUBSEQUENT MEMBRANE ION-EXCHANGER RUNS

Within minutes gram amounts of product could be purified in the HFS [buffer system I, sodium phosphate (pH 5.8)]. The concentration factor was calculated by dividing the IgG concentration in the elution by the IgG concentration in the supernatant. The recovery was found to be independent of the concentration of the supernatant. Lowering the ionic strength of the supernatant by method I, II or IV resulted in binding rates of about 95% and recoveries in excess of 80%.

Method	No.	IgG (mg)	Volume (ml)	IgG bound (%)	Flow-rate (ml/min)	Recovery (%)	Concentration factor	Process time (h)	Throughput (g/h)	IgG species
(I) Ultrafiltration and diafiltration	1	320	2010	99	750	81	13.6	0.08	4.0	Rat
	2	187	5200	95	866	93	10.7	0.14	1.3	Mouse
	3	319	5500	99	845	81	12.7	0.15	2.1	Mouse
	4	910	1000	97	843	84	1.8	0.06	14.3	Mouse
	5	910	1000	99	843	94	2.6	0.06	14.3	Mouse
(II) Diafiltration only	6	187	5200	95	866	93	10.7	0.14	1.3	Mouse
	7	166	4600	99	836	96	14.3	0.13	1.3	Mouse
	8	43	3300	95	843	81	8.9	0.11	0.4	Mouse
(III) Dilution of the supernatant with water	9	146	3500	91	845	91	10.3	0.11	1.3	Mouse
	10	139	5600	94	845	91	17.7	0.15	0.9	Mouse
	11	37	16700	73	843	68	17.2	0.37	0.1	Rat
	12	110	14300	68	843	66	22.1	0.32	0.3	Rat
	13	240	34500	63	843	57	122	0.72	0.3	Rat
(IV) Ultrafiltration and electrodialysis	14	456	1400	91	845	90	3.1	0.11	4.2	Mouse
	15	489	1500	91	845	84	4.0	0.11	4.3	Mouse
	16	391	1200	98	845	75	3.0	0.11	3.6	Mouse
	17	267	1100	97	1060	80	4.4	0.08	3.3	Mouse

TABLE III

COMPARISON BETWEEN A GEL-MATRIX CATION EXCHANGER (S-SEPHAROSE FF) AND THE STRONGLY ACIDIC MEMBRANE ION EXCHANGER (SARTOBIND S)

Parameter	Matrix	
	S-Sepharose FF	Sartobind S
Bed volume/area	28 ml (XK 50 × 30)[a]	1400 cm^2
Supernatant volume (ml)	4700	5500
Flow-rate (ml/min)	80	845
MAbs applied (mg)	359	319
MAbs bound (%)	69	99
Recovery (%)	67	81
Concentration factor	58	13
Process time (h)	1.50	0.15
Throughput (g/h)	0.16	2.10

[a] Pharmacia, S.

sequently a treatment with 0.2 *M* NaOH (60°C, 20 min) restored the flow. So far one MIEX unit has been used 25 times without decreases in binding capacity, recovery or flow-rate. The main advantage of the MIEX in comparison with an ion exchanger based on gel matrices is the much higher throughput (>13-fold) (Table III). Final purification of the MAbs was carried out using a preparative gel filtration column. The elutions from the MIEX were applied directly, without prior concentration, to the gel filtration column and resulted in two distinct peaks. The second peak contained the purified MAbs (Fig. 7).

Purification of rATIII

Affinity membranes. The 17.35-cm^2 amine-modified membrane covalently coupled 5.2 mg of heparin (porcine), corresponding to *ca.* 0.3 mg heparin/cm^2 matrix, which is slightly higher

than that obtained by Sasaki *et al.* (0.2 mg/cm^2) [11]. The coupling took fairly long to complete (8 days), but this is not uncommon for reductive amination [10,21] and the optimized conditions that were taken from the paper by Sasaki *et al.* [11], which were obtained using amino-Sepharose as a matrix, might not apply completely to the membraneous matrix used in this instance. The maximum amount of rATIII that could be adsorbed to the amine-modified membrane was 1.14 mg (Fig. 8), which amounts to a binding capacity of 90.8 μg/cm^2 (effective surface area 12.56 cm^2). This value equals or exceeds that of other commercially available heparin affinity matrices. The stability achieved by the method of reductive amination used in this instance was very satisfactory especially when compared with the known shortcomings [10] of products coupled by the cyanogen bromide method. The amine-based heparin-affinity membrane was used eleven times over a period of about 5 months, purifying a total of 8.2 mg of rATIII. It was also sterilized by steam sterilization (15 min at 114°C) after six runs, losing about 38% of its capacity (average of the first six runs, 71.7 μg rATIII/cm^2; average of the last five runs, 43.9 μg/cm^2) and treated sucessfully at one time with 2.5% SDS solution to remove clogging material from the membrane surface.

The 100-cm^2 module containing an epoxy-activated membrane, which was first treated with ethylenediamine in order to produce sufficient spacers, coupled 12 mg of heparin (ovine) in 5 days, which corresponds to 0.18 mg heparin/cm^2. The best run resulted in the purification of 3.95 mg of rATIII, which denotes a binding capacity of 39.5 μg/cm^2. The 100-cm^2 module purified a total of 18 mg of rATIII in eight runs over a period of almost 3 months (Fig. 9). The 100-cm^2 module lost considerable binding capacity (about 65% when comparing the average of the first three runs with that of the last five) (Fig. 9). This loss might be due to storing the filter without first removing the clogging material. Treatment with 2.5% SDS after the fifth run, however, resulted in an improvement of the rATIII binding capacity (Fig. 9).

The initial purity of the eluates was about 75%

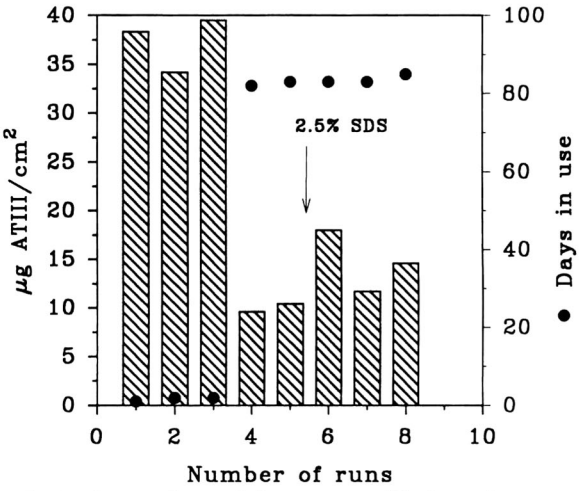

Fig. 9. Repeated use of the epoxy-modified membrane over a period of 85 days. Effect of treatment the membrane with 2.5% SDS.

(as determined with the Gel-Image system), with impurities mainly in the lower-molecular-mass range. These non-specific bindings might be due to the many affinity interactions of heparin [6], or the fact that heparin as a highly sulphated saccharide polyanion has some of the properties of a cation exchanger. The low-molecular-mass protein impurities were readily removed by gel filtration (Fig. 10).

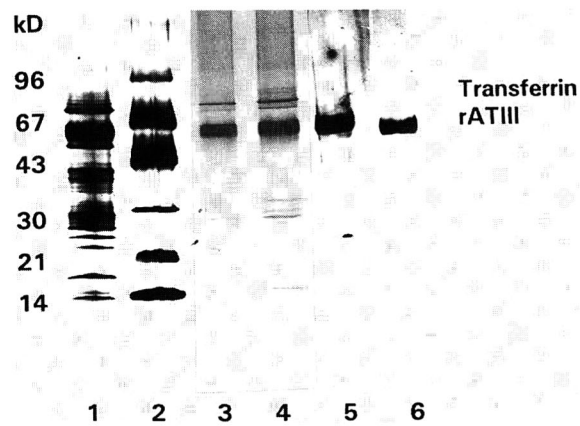

Fig. 10. SDS polyacrylamide gradient gel (8–25%) using a modified silver stain under unreduced conditions. Isolation of rATIII with basic preparative ion exchanger, amine-modified affinity membranes and gel filtration. Lanes: 1 = supernatant after ultra- and diafiltration (concentrated tenfold); 2 = marker, M_r 14 000–96 000 (reduced); 3 + 4 = elution of rATIII from MIEX Q; 5 = elution of rATIII from affinity membrane; 6 = pure rATIII (after affinity membrane and subsequent gel filtration.)

TABLE IV

CONCENTRATION OF rATIII WITH THE STRONGLY BASIC MEMBRANE ION EXCHANGER (2400 cm^2) USING THE HFS

No.	rATIII (mg)	Volume (ml)	rATIII bound (%)	Flow-rate (ml/min)	Recovery (%)	Concentration factor	Process time (h)	Throughput (g/h)
1	210	2000	100	560	99	3.9	0.08	2.6
2	240	2000	100	560	99	2.5	0.15	1.5
3	140	550	98	380	98	1.6	0.51	0.3
4	344	7500	100	580	96	14.4	0.51	0.6
5	366	8000	100	600	79	11.8	0.47	0.8
6	113	5000	93	610	79	13.1	0.31	0.5
7	110	3500	85	620	82	11.9	0.22	0.5
8	68	4500	100	620	98	22.0	0.28	0.3
9	18	3500	100	620	92	17.8	0.36	0.05

The affinity of the heparin membranes towards human transferrin, the most critical contaminant in this instance, is very weak. Ionic interactions with the matrix were further diminished by adjusting the pH of the supernatant to 5.8, the isoelectric point of human transferrin.

Preparative purification of rATIII using the HFS. The 2400-cm^2 units could be run with flow-rates up to 620 ml/min, achieving binding rates of 97% and recoveries of about 91% (Table IV). The estimated capacity of the anion exchanger membrane for rATIII of 0.15 mg/cm^2 (360 mg/unit) is low compared with the capacity of the S type for MAbs; this might be due to the competive binding of DNA and other negatively charged biomolecules under these conditions. The purity of the product was 75%, with human transferrin as one of the main impurities (Fig. 10). In comparison with standard ion exchangers (Table V), MIEX shows similar binding and recovery rates. Only the concentration factor (elution volume) could be improved in the future. Purification with MIEX resulted in a throughput more than four times higher than Q-Sepharose FF.

TABLE V

COMPARISON OF A GEL-MATRIX ANION EXCHANGER (Q-SEPHAROSE FF) AND THE STRONGLY BASIC MEMBRANE ION EXCHANGER (SARTOBIND Q)

Parameter	Matrix	
	Q-Sepharose FF	Sartobind Q
Bed volume/area	30 ml (XK 50 × 30)[a]	2400 cm^2
Supernatant volume (ml)	4200	7500
Flow-rate (ml/min)	60	580
rATIII applied (mg)	246	344
rATIII bound (%)	100	100
Recovery (%)	95	96
Concentration factor	42	14
Process time (h)	1.70	0.51
Throughput (g/h)	0.15	0.60

[a] Pharmacia, S.

CONCLUSIONS

Membrane ion exchanger and affinity membranes are versatile tools for the concentration and purification of MAbs and rATIII. The binding capacity, the recovery and the concentration factor (only by the 100-cm^2 unit) are similar to those for gel matrices. The main advantage is the very high throughput. They are easy to handle and can be used repeatedly.

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Increased yield of homogeneous HIV-1 reverse transcriptase (p66/p51) using a slow purification approach

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ABSTRACT

A chromatographic procedure to purify recombinant reverse transcriptase (RT) from human immunodeficiency virus-1 is reported. A bacterial system which expressed large amounts of p66 RT polypeptide was used. The purification scheme was optimized for high-yield production of homogeneous p66/p51 RT using a combination of chromatographic matrices in the following order: Q-Sepharose, heparin-Sepharose, phenyl-Sepharose, S-Sepharose, Poly(A)-Sepharose and Q-Sepharose. The p66 polypeptide remained intact after the first chromatographic step on Q-Sepharose, where it was recovered in the non-adsorbed fraction. A high yield of p66/p51 RT was obtained when the time from application to elution of heparin-Sepharose in the second chromatographic step was prolonged. Phenyl-Sepharose was used in the next chromatographic step to separate the heterodimeric forms of RT from p66 RT on the basis of hydrophobicity. The chromatography on S-Sepharose resolved the major heterodimeric form, p66/p51, from other heterodimeric variants. Further purification was done by affinity chromatography on Poly(A)-Sepharose followed by anion-exchange chromatography on Q-Sepharose. Amounts of 25–35 mg of the pure heterodimer p66/p51 RT were recovered from 50 g of bacterial cells.

INTRODUCTION

Reverse transcriptase (RT) from the human immunodeficiency virus (HIV-1) contains RNA-dependent DNA polymerase and DNA-dependent DNA polymerase activities. RT catalyses the synthesis of first single-stranded and then double-stranded DNA from the viral RNA genome on infection of the cells by virus particles. HIV-1 RT has become a subject of intense study aimed at the three-dimensional structure and rational drug design (for a review, see ref. 1).

The RT molecule (p66) is a single polypeptide with a molecular mass of 66 000 (560 amino acids) composed of one N-terminal polymerase domain (p51) and one C-terminal RNAse H domain (p15). The RT molecule has a strong tendency to form a stable heterodimer from the p66/p66 dimer through proteolytic removal of one of the p15 domains.

RT has been expressed in bacterial and yeast cells from the *pol I* gene (encoding for protease, RT and endonuclease) or from the *RT* gene alone, and the purification of RT in the heterodimeric p66/p51 or the p66 form has been reported [2–12]. HIV-1 protease is reported to cleave p66 at the cleavage site Phe 440–Tyr441 [7], whereas bacterial proteases randomly cleave the p66 polypeptide at a susceptible region in the amino acid range 426–446 [1,13,14]. As a result, the carboxyl terminal end of so-called p51 varies and multiple forms of "p51" in association with

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p66 have been observed during the course of purification. The property of p66 to form aggregates with itself and with different degradation products of p66 and also with host cell proteins leads to a lower yield of the final product and increases the complexity of the purification.

Several groups have reported techniques to circumvent the problems caused by bacterial proteolytic processes during the preparation of a homogeneous p66/p51 RT. By gene technology procedures a fusion peptide, *e.g.*, a histidine tail [15,16] or an antigenic peptide [17], was attached to the N- or C-terminal end of the RT chain, which then permitted a rapid purification on a metal affinity or a monoclonal antibody matrix, respectively. Reconstitution of a heterodimer by mixing purified p66 and p51 expressed in separate systems [18–20] or cleaving the purified p66 with HIV-protease [21] are alternative ways which have also been tried to produce homogeneous p66/p51 RT.

The expression of *pol I* gene (encoding for protease, RT and endonuclease) and the mild purification and crystallization of the heterodimer p66/p51 RT have been reported previously [2,3] and reviewed [4]. For crystallization studies, a large amount of pure and homogeneous RT is required. As the yield of RT was fairly low when the *pol I* gene was expressed, we have improved the production of the pure and crystallizable RT material by altering the gene construct (*RT* gene only), bacterial strain and purification scheme.

When the earlier reported purification procedure [DEAE-Sepharose, heparin-Sepharose, S-Sepharose and Poly(A)-Sepharose in sequential order] was applied, the resulting RT was a mixture of p66 and different forms of heterodimers. To obtain a high yield of crystallizable heterodimer p66/p51 RT, the purification scheme was modified as follows: (i) instead of a rapid purification, the application and elution times in the second chromatographic step on heparin-Sepharose were prolonged to achieve optimum proteolytic degradation which resulted in an increase in the homogeneous heterodimeric form; (ii) hydrophobic interaction chromatography (phenyl-Sepharose) was introduced to separate undegraded p66 from the heterodimeric forms.

EXPERIMENTAL

Experiment

A fast protein liquid chromatographic (FPLC) system and a PhastSystem were obtained from Pharmacia (Uppsala, Sweden).

Materials

All chromatographic and electrophoretic gel media were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). The electrophoresis gels were PhastGel gel media. The chromatographic matrices were Q-Sepharose Fast Flow, heparin-Sepharose CL-6B and Poly-(A)-Sepharose 4B. HiLoad 26/10 S-Sepharose HP and HiLoad 16/10 phenyl-Sepharose HP prepacked columns were used.

Expression of RT in Escherichia coli

The RT cDNA was isolated by the polymerase chain reaction (PCR) technique from the plasmid pN10E15 [2]. The primers in the PCR were designed so that an NdeI site containing the start codon ATG was introduced at the 5′-terminus. A translation termination stop and BamHI site were introduced at the 3′-terminus of the fragment. The PCR fragment was blunt end ligated into the cloning vector bluescript KS+ at the EcoRV site. The new construct was called pRT.BS. The expression vector was constructed by isolation of the NdeI–BamHI fragment from pRT.BS and ligated to pET11 [22] (Novagene), which previously had been treated with NdeI and BamH1. Expression was performed in the *E. coli* strain BL21(DE3) which is deficient in *lon* and *ompT* protease-deficient. Transformed cells were grown to OD = 1.0 before induction with isopropyl-β-D-galactoside (IPTG) [22]. The cells were harvested 3 h after induction.

Analysis

Determination of protein concentration and analysis by electrophoresis were performed as described by Unge *et al.* [2] and Bhikhabhai *et al.* [3]. Western blotting was performed using antibodies against RT and detection was done using the ECL (Amersham, UK) Western blotting detection system. A Biotinylated SDS-

PAGE standard (Bio-Rad) was used as a molecular mass marker. Activity measurement of RT was done as described by Gronowitz *et al.* [23].

Purification procedure

All chromatographic experiments were performed using the FPLC System at 8°C. SDS-PAGE was used to detect RT in each chromatographic step. Purified heterodimer p66/p51 RT was used as the marker to identify fractions containing RT.

Lysis. Bacterial cells from a 10-l fermenter were harvester by centrifugation, washed twice with 20 m*M* Tris–HCl–100 m*M* NaCl–1 m*M* EDTA (pH 8.0) and then suspended in 150 ml of the lysis buffer (pH 8.0) [20 m*M* Tris–HCl–100 m*M* NaCl–1 m*M* EDTA–1 m*M* dithiothreitol (DTT)–1 m *M* $MgSO_4$] and treated with DNase and RNase. After disruption of the cells using a French pressure cell, the lysate was centrifuged at 23 000 *g* for 30 min. The supernatant was dialysed against buffer A (pH 8.0) (20 m*M* Tris–HCl–100 m*M* NaCl–1 m*M* EDTA–1 m*M* DTT).

Anion-exchange chromatography. After dialysis the solution was diluted with buffer A to 250 ml and applied to a pre-equilibrated Q-Sepharose fast flow column (120 × 50 mm I.D.; flow-rate 4 ml/min). The column was washed with 400 ml of buffer A and RT was recovered in the non-adsorbed fractions (pool I).

Affinity chromatography. The salt concentration of pool I was adjusted to 0.15 *M* NaCl and then applied over a period of about 15 h to the heparin-Sepharose column (70 × 50 mm I.D.; flow-rate 1 ml/min), which was pre-equilibrated with buffer A. The column was eluted with a linear gradient (1.5 bed volumes) of 0.10–0.35 *M* NaCl in buffer A. The RT was recovered in one broad peak (pool II).

Hydrophobic interaction chromatography. The ammonium sulphate concentration of pool II was adjusted to 1.0 *M* and then applied to a phenyl-Sepharose column (100 × 16 mm I.D.; flow-rate 2 ml/min), which had been pre-equilibrated with buffer B (pH 8.0) (1.0 *M* ammonium sulphate–20 m*M* Tris–HCl–1 m*M* DTT–1 m*M* EDTA). The column was eluted with a linear gradient (15 bed volumes) from 1.0 to 0.0 *M* ammonium sulphate buffer. RT eluted at 0.6 *M* ammonium sulphate (Fig. 3, first peak) and the fractions were pooled for further purification (pool III).

Cation-exchange chromatography. Pool III was dialysed against buffer C (pH 6.5) (20 m*M* MES–1 m*M* DTT–1 m*M* EDTA) and applied to an S-Sepharose column (100 × 26 mm I.D.; flow-rate 2 ml/min) pre-equilibrated with buffer C. The column was eluted with a linear gradient (15 bed volumes) from 0.0 to 0.25 *M* NaCl in buffer C. The major peak containing the heterodimer RT eluted at about 0.18 *M* NaCl (Fig. 4A; pool IV).

Affinity chromatography on Poly(A)-Sepharose 4B. Pool IV was dialysed against buffer D (pH 8.0) (20 m*M* Tris–HCl–1 m*M* DTT–1 m*M* EDTA) and applied to a column of Poly(A)-Sepharose 4B (100 × 16 mm I.D.; flow-rate 0.8 ml/min) equilibrated with buffer D. Elution was carried out with a linear gradient (8 bed volumes) from 0.0 to 0.25 *M* NaCl in buffer D. RT was eluted in one broad peak (Fig. 5) and fractions were pooled (pool V).

Final purification on Q-Sepharose. Pool V was dialysed against buffer D containing 75 m*M* NaCl and applied to a Q-Sepharose column (100 × 10 mm I.D.) and RT was collected in the non-adsorbed fractions (pool VI).

Procedure for concentrating the RT. Pool VI was adjusted to 1.0 *M* with respect to ammonium sulphate concentration and applied to a phenyl-Sepharose column equilibrated with buffer B. RT was eluted with low ionic strength buffer D. For crystallization RT was further concentrated to about 15–18 mg/ml by ultrafiltration (Ultrafree-CL filters; Millipore).

RESULTS AND DISCUSSION

A pure and homogeneous p66/p51 in large amounts is a prerequisite for structural studies by X-ray crystallography. RT was expressed in fairly small amounts when the *pol I* gene (encoding for protease, RT and endonuclease) was expressed in bacteria [2, 3]. In this paper we report the expression of only the *p66* gene in a bacterial strain which is deficient in the *lon* protease and *ompT* outer membrane protease. In the lysate RT is present predominantly as p66 (Fig. 1, lane 1). During the early stages of purification, a considerable degradation of p66

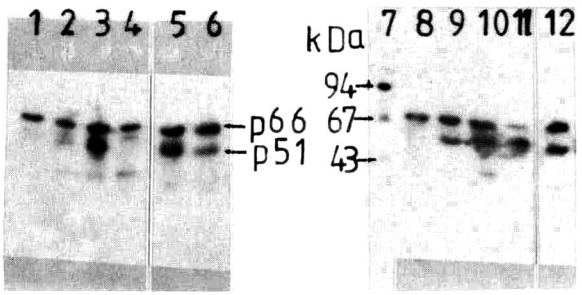


Fig. 1. Western blot analysis of fractions from different chromatographic steps for the purification of recombinant HIV-1 reverse transcriptase. Samples: lane 1 = bacterial lysate; 2 = the non-adsorbed fractions of Q-Sepharose, 3 = pooled fractions containing RT from heparin-Sepharose: 4 = the non-adsorbed fraction for heparin-Sepharose; 5 = the pooled fractions containing the heterodimeric form of RT from the phenyl-Sepharose step; 6 = pure heterodimer RT as marker; 7 = molecular mass markers; 8 = the pooled fractions containing p66 from phenyl-Sepharose; 9 = the major peak containing p66/p51 from S-Sepharose; 10 and 11 = minor peaks from S-Sepharose; 12 = pooled fractions from Poly(A)-Sepharose. kDa = kilodalton.

occurs through the proteolytic activity of bacterial proteases. We therefore optimized the purification process in order to obtain a maximum yield of the heterodimeric p66/p51 RT and to separate it from the unprocessed p66 RT. About 25–35 mg of pure and homogeneous p66/p51 RT, suitable for crystallization experiments, can now be purified from about 50 g of bacterial cells. Instead of rapid purification we extended the time of interaction between bacterial proteases and RT by slowing the purification process in the second chromatographic step. SDS-PAGE followed by silver staining was used to detect the microheterogeneities and other impurities during the purification. Western blotting experiments were done to detect the RT at different stages of purification (Fig. 1).

Purification of the heterodimer p66/p51 RT

The details of the purification are described under Experiment. The purification procedure is summarized in Table I. The purification scheme consists of chromatographic steps in the following order: Q-Sepharose, heparin-Sepharose, phenyl-Sepharose, S-Sepharose, Poly(A)-Sepharose and Q-Sepharose. The presence of protease inhibitors, phenylmethylsulphonyl fluoride (PMSF) and benzamidine, had negligible effect on the proteolytic activity of the bacterial proteases and were therefore excluded during the lysis of the cells. The lysed supernatant was dialysed and then applied to Q-Sepharose fast flow column. RT was obtained in the non-adsorbed fractions and 75% of the bacterial proteins were bound to Q-Sepharose. RT was detected mainly as p66 (Fig. 1, lane 2). In order to

TABLE I

PURIFICATION OF THE HETERODIMER p66/p51 OF HIV REVERSE TRANSCRIPTASE

The chromatographic matrix used in each step in the purification is given in italics. Volume, yield, concentration and percentage of protein recovered in each step are shown. Protein concentration was measured by UV absorbance (1 mg/ml solution of RT has an absorbance of 2.2 at 280 nm) except where indicated by the Bradford method.

Pool	Purification step	Volume (ml)	Total protein (mg)	Protein concentration (mg/ml)	% of total protein
	E. coli lysate	250	3400[a]	13.0	100
I	*Q-Sepharose* non-adsorbed fraction	590	940[a]	1.6	28
II	*Heparin-Sepharose* pool	280	370[a]	1.3	11
III	*Phenyl-Sepharose* peak 1	230	140	0.6	4
IV	*S-Sepharose* major peak	57	51	0.9	1.5
V	*Poly(A)-Sepharose* pool	66	46	0.7	1.4
VI	*Q-Sepharose* non-adsorbed fractions	86	43	0.5	1.3
	Phenyl-Sepharose	10	34	3.4	1.0
	Ultrafiltration	1.6	29	18.0	0.9

[a] Bradford method used.

recover RT in non-adsorbed fractions, the equilibration buffer for Q-Sepharose was optimized to pH 8.0 and to the conductivity equal to that of 0.1 *M* NaCl.

The non-adsorbed fractions were packed with heparin-Sepharose. The column was eluted with a linear gradient of NaCl and the majority of RT was eluted in one broad peak (Fig. 1, lane 3). SDS-PAGE of a number of fractions along the peak showed that the fractions contained both p66 and different forms of the heterodimer (Fig. 2, lanes 2–5). In the non-adsorbed fractions from heparin-Sepharose (Fig. 1, lane 4), Western blot analysis revealed the presence of p66 RT. This was not due to overloading of the column. The lack of affinity for the heparin by some of the p66 RT could instead be due to conformational differences in the RT.

Separation of the heterodimer from p66 fractions was achieved in the next chromatographic step on a phenyl-Sepharose high performance column (Fig. 3). The heterodimeric form eluted earlier than p66 (Fig. 2, lanes 6 and 7), indicating that the heterodimer is less hydrophobic than p66. The amount of the heterodimer material was compared with the p66 material and correlated with the time from the application to the elution from the heparin-Sepharose column.

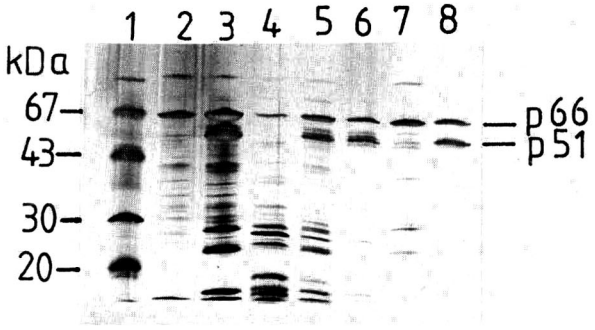


Fig. 2. Analysis of fractions from heparin-Sepharose and phenyl-Sepharose by SDS-PAGE (12.5%). Gel, PhastGel homogeneous 12.5. Samples: lane 1 = molecular mass marker; 2–5 = fractions from eluted peak from heparin-Sepharose (lane 2 = fraction from the start of the peak; 3 = top fraction of the peak; 4 = fraction from the end of the peak; 5 = pooled fractions); 6 = heterodimeric fraction from phenyl-Sepharose (Fig. 3, arrow 6); 7 = p66 fraction from phenyl-Sepharose (Fig. 3, arrow 7); 8 = pure heterodimer RT as marker.

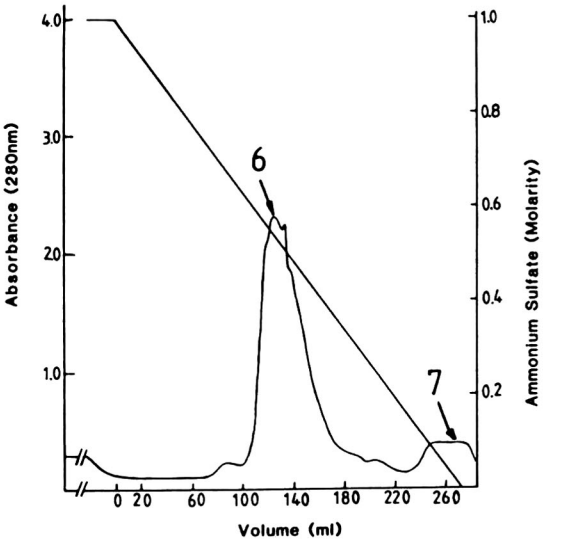
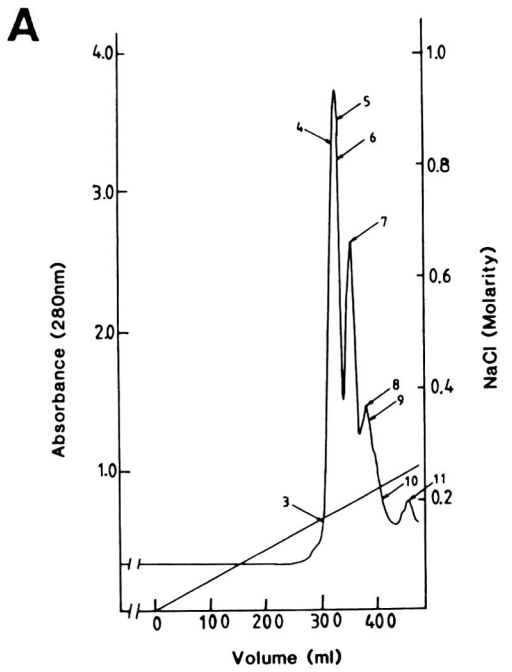


Fig. 3. Hydrophobic interaction chromatography using HiLoad 16/10 phenyl-Sepharose HP. Column, 100 × 16 mm I.D.; flow-rate, 2 ml/min; sample, pooled peak from the heparin-Sepharose column. Elution was performed with a linear gradient (300 ml) from 1.0 to 0.0 *M* ammonium sulphate in the buffer [20 m*M* Tris–HCl–1 m*M* EDTA–1 m*M* DTT (pH 8.0)].

When the heparin-Sepharose step was carried out rapidly within 3 h, the ratio of heterodimer peak to the p66 peak in the subsequent hydrophobic chromatography was estimated to be about 60:40, whereas prolonged application and elution (about 15 h or overnight) of the sample on the heparin-Sepharose column resulted in a ratio of about 80:20.

In the next purification step, on an S-Sepharose high performance column (Fig. 4A), SDS-PAGE (Fig. 4B) indicated that the major peak contained the heterodimer p66/p51 and the remaining peaks consisted of heterodimers where the size of "p51" differed. About 45% of RT was recovered as the exact heterodimeric p66/p51 RT. The other "p51s" were identified as being a fragment of RT by Western blot analysis (Fig. 1, lanes 10 and 11).

When either the lysate or the non-bound-fraction from the Q-Sepharose column was allowed to stand for about 15 h and then subjected to subsequent purification steps, an increase in the heterodimeric products was observed in the hyrophobic interaction chromatographic step. However, further purification of the heterodi-



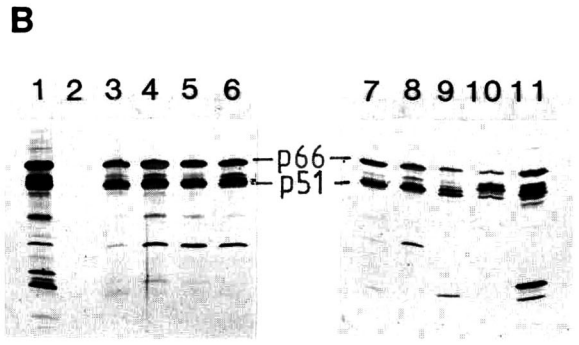


Fig. 4. (A) Cation-exchange chromatography using HiLoad 26/10 S-Sepharose HP. Sample, pooled fractions from phenyl-Sepharose; column, 100 × 26 mm I.D.; fraction volume, 10 ml. Elution was performed with a linear gradient (750 ml) of from 0.0 to 0.25 *M* NaCl in buffer [20 m*M* MES–1 m*M* EDTA–1 m*M* DTT (pH 6.5)]. The fractions indicated by arrows were analysed by SDS-PAGE. (B) Analysis of the fractions from S-Sepharose by SDS-PAGE (12.5%). Gel, PhastGel homogeneous 12.5. Samples: lane 1 = pooled fractions from phenyl-Sepharose; 2 = non-bound fractions of S-Sepharose; 3–11 = different fractions from the S-Sepharose step. The fractions are indicated by arrows 3–11 in the elution profile of S-Sepharose in (A).

meric peak fraction resulted in multiple peaks spread all over the chromatogram from the S-Sepharose column. Each peak fraction contained a heterodimer where the size of "p51" varied. The degree of the proteolytic degradation was judged by the chromatographic pattern on S-Sepharose. With the slow purification approach the heterodimer was recovered in only one major peak on S-Sepharose chromatography.

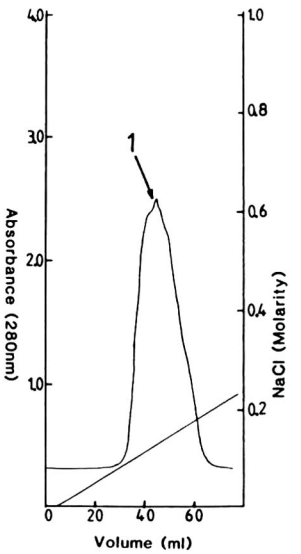


Fig. 5. Affinity chromatography of Poly(A)-Sepharose. Column, 100 × 16 mm I.D.; flow-rate, 0.8 ml/min; sample, pooled peak from S-Sepharose column. Elution was carried out with a linear gradient from 0.0 to 0.25 *M* NaCl in buffer [20 m*M* Tris–HCl–1 m*M* DTT–1 m*M* EDTA (pH 8.0)]. Total volume = 8 × bed volume. RT was eluted in one broad peak (Fig. 6, lane 1).

Further purification on Poly(A)-Sepharose was done mainly to separate the p66/p51 RT from small amounts of other DNA/RNA binding proteins which co-eluted with RT in all the preceding steps. The heterodimer p66/p51 RT was eluted as one peak (Figs. 5 and 6, lane 1).

In the last step, the RT material from the Poly(A)-Sepharose column was passed through a Q-Sepharose column (Fig. 7). About 95% of the RT did not bind to the Q-Sepharose (Fig. 6, lanes 3–6), whereas the remaining part of RT was bound to the matrix probably as another heterodimeric form (Fig. 6, lane 7). The non-adsorbed fraction from the Q-Sepharose column was first concentrated on the phenyl-Sepharose column and then further concentrated to 15–18 mg/ml by ultrafiltration. This heterodimeric p66/

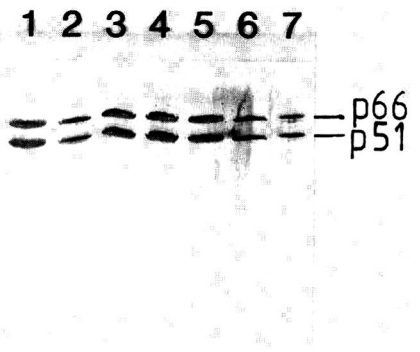


Fig. 6. Analysis of fractions from Poly(A)-Sepharose and Q-Sepharose step by SDS-PAGE (12.5%). Gel, PhastGel homogeneous 12.5. Samples: lane 1 = pooled fractions containing RT from Poly(A)-Sepharose; the fractions analysed in lanes 2–7 are indicated by arrows 2–7 in Fig. 7 (2–6 = non-bound fractions in the second Q-Sepharose step; 7 = bound fractions from Q-Sepharose).

p51 RT that has been processed from p66 by the bacterial proteases had an activity equal to that of RT material from the *pol I* polypeptide, processed by HIV-1 protease [2,3].

This pure and active RT was crystallized in a complex with $tRNA^{Lys3}$, the natural primer of HIV-1 RT. A three-dimensional crystallographic

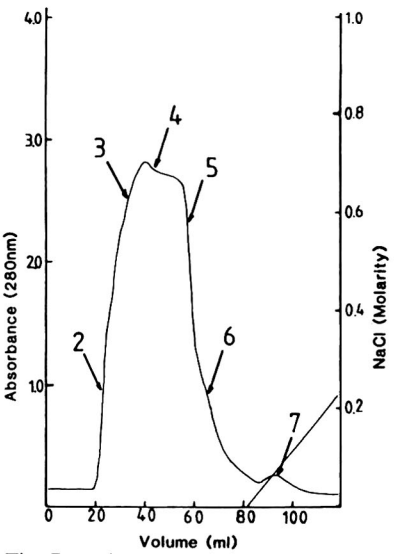


Fig. 7. Anion-exchange chromatography using Q-Sepharose. Sample, pooled fractions from Poly(A)-Sepharose; column, 100 × 10 mm I.D.; fraction volume, 10 ml. RT was recovered in the non-adsorbed fractions. The fractions indicated by arrows were analysed by SDS-PAGE (Fig. 6).

study is in progress using a synchrotron radiation X-ray source at the EMBL outstation in Hamburg. The presence crystals diffract to about 3.8 Å resolution using the X 31 beam in Hamburg. Crystal structure investigations are also in progress in other laboratories and crystals of the heterodimer complexed with a Fab fragment and double-stranded DNA diffracting to about 3.5 Å resolution have been reported [24]. Recently, the crystal structure to 3.5 Å resolution of the heterodimer in a complex with an inhibitor has been presented [25]. However, this structure is not detailed enough to allow for a proper positioning of the amino acid chains. Therefore, all efforts (such as that presented in this paper) which can result in a more homogeneous and thus a better heterodimer material are well justified.

CONCLUSIONS

We have shown that by using a combination of chromatographic techniques the homogeneous p66/p51 RT could be separated (i) from p66 by using hydrophobic interaction chromatography, (ii) from various heterodimeric forms of RT where the size of "p51" differed by cation-exchange chromatography on S-Sepharose and (iii) from various RT forms with different conformations by anion-exchange chromatography on Q-Sepharose. By slowing the purification process in the heparin-Sepharose step, an increase in the yield of the crystallizable heterodimer p66/p51 was achieved. With the present "slow" purification scheme the recovery yield is about 1% of the total protein or about 12% of the expressed p66. The activity of the heterodimer p66/p51, which is processed from p66 only by bacterial proteases, is equal to that of the heterodimer which is obtained by processing the *pol I* polypeptide by HIV-protease in the bacteria. This purification procedure has also been shown to be suitable for various mutants of RT produced in our laboratory.

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Preparation of DNA polymerase from *Bacillus caldotenax*

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ABSTRACT

A procedure with four chromatography steps was developed for the purification of DNA polymerase from *Bacillus caldotenax* by using fast protein liquid chromatography. The procedure was suitable for use with process-scale media. Elution profiles obtained from ion-exchange chromatography and triazine–dye affinity chromatography with fast protein liquid chromatography and process-scale media were similar. The enzyme showed stronger interaction, however, with phenyl-Sepharose FF in the scaled-up process than with the phenyl-Superose used in fast protein liquid chromatography. The surprising binding of the DNA polymerase to sulphonated ion-exchange media at pH 7.5 may be explained by the structure of the enzyme.

INTRODUCTION

DNA polymerase is an enzyme required for the replication and repair of DNA. Thermostable DNA polymerases are of particular interest because of their potential application in recombinant DNA techniques such as for the polymerase chain reaction (PCR) [1] and for DNA sequencing [2]. Thermophilic organisms from which DNA polymerase has been purified include *Thermus aquaticus* [3,4], *T. flavus* [5], *T. ruber* [6] and *T. thermophilus* [7–9]. The gene coding for DNA polymerase from *T. aquaticus* (*Taq* polymerase) was cloned and expressed in *E. coli* [10]. *Bacillus* spp. studied include *B. stearothermophilus* [11–13] and *B. caldovelox* [9].

DNA polymerase from *B. caldotenax* has been purified by two successive chromatography steps on DEAE-cellulose followed by separation on phosphocellulose and heparin-Sepharose [9]. Further purification was required to obtain homogenous enzyme by using sucrose gradient centrifugation. *B. caldotenax* is the most thermophilic of the *Bacillus* spp. and is capable of growth at 80°C [14].

The aim of the work described here was to use fast protein liquid chromatography (FPLC) to develop a facile purification procedure for DNA polymerase from *B. caldotenax* suitable to be scaled up to provide protein for characterisation and X-ray crystallography studies of the enzyme.

EXPERIMENTAL

Materials

Deoxynucleoside triphosphates (dNTPs) were from BCL, East Sussex, UK and tritiated thymidine triphosphate from Amersham, Aylesbury, UK. Activated calf thymus DNA, dithiothreitol (DTT), phenylmethanesulphonyl fluoride (PMSF), Nonidet P40, polyoxyethylene sorbitan (Tween 20) and trichloroacetic acid (TCA) were from Sigma, Poole, UK. 3MM chromatography paper was from Whatman Lab Sales, Maidstone, UK. Optiscint Hisafe, Q-Sepharose FF, S-Sepharose FF, phenyl-Sepharose FF and M_r marker proteins were from Pharmacia–LKB, Uppsala, Sweden. Blue-Tris-acryl M was from Life Science Labs., Luton,

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UK. All other reagents were obtained from BDH, Poole, UK.

Equipment

The FPLC system supplied by Pharmacia–LKB, consisted of an LCC-500 Plus liquid chromatography controller, two P500 pumps, a Frac 100 fraction collector, a single path monitor UV-1 optical unit and a Rec 482 recorder. Samples were injected either from a 1-ml sample loop or from a 10-ml Superloop from Pharmacia-LKB. Chromatography columns used in FPLC were obtained prepacked and an HR 5/5 column was packed with Blue-Trisacryl M, according to the manufacturers instructions. Adjustable capacity columns were obtained for the scaled-up process from Amicon, Stonehouse, Gloucestershire, UK, and a 101U pump was supplied by Watson Marlow, Smith and Nephew Pharmaceuticals, Falmouth, UK. A single-path monitor UV-1 optical unit, Rec 482 recorder and a Frac 100 fraction collector were also used.

Measurement of DNA polymerase activity

A 50-μl portion of assay mixture (50 m*M* Tris–HCl buffer, pH 7.5, containing 70 m*M* $MgCl_2$, 1 m*M* DTT, 100 μM dATP, 100 μM dCTP, 100 μM dGTP, 25 μM dTTP, 6.25 μg activated calf thymus DNA and 0.5 μCi [^{3}H]dTTP, of specific radioactivity 43 Ci mmol^{-1}). The reaction was stopped by placing the reaction mixture on ice and 50 μl were transferred to a strip (3 cm × 1 cm) of 3MM chromatography paper. DNA was precipitated by a wash in 10% (w/v) TCA for 1 h followed by two further washes for 30 min each in 5% (w/v) TCA and then a final wash in ethanol for 30 min. The chromatography paper strips were dried in air, placed in 4 ml of Optiscint Hi-Safe Scintillant and incorporated radioactivity counted in an LKB Wallac 1215 Rackbeta counter. The definition of one unit of DNA polymerase is that required to catalyse the incorporation of 10 nmol dNTP into acid-insoluble radioactivity in 30 min at 70°C.

Measurement of protein concentration

Protein concentrations were determined by the Folin method of Lowry *et al.* with bovine serum albumin as the standard [15]. The protein content of column eluates was also monitored by A_{280}.

Bacterial cell culture

B. caldotenax [14] obtained from Dr. R.J. Sharp (Division of Biotechnology, PHLS Centre for Applied Microbiology and Research) was grown in a 25-l New Brunswick fermenter in double-strength Lüria broth (30 g l^{-1} tryptone, 10 g l^{-1} yeast extract and 10 g l^{-1} NaCl). The temperature was maintained at 60°C and the pH maintained at 7.2 ± 0.1 by the addition of NaOH or HCl. Cells were harvested after 4 h, while still in the exponential phase of growth (A_{600} 7.2), quick frozen and stored at −20°C. The culture produced 230 g of cell paste.

Lysis of cells

Cells were thawed at 4°C in 50 m*M* Tris–HCl buffer, pH 7.5 containing 1 m*M* EDTA, 1 m*M* DTT, 0.2 m*M* PMSF, 10% (v/v) glycerol, 0.01% (v/v) Nonidet P40 and 0.01% (v/v) Tween 20 (buffer A). The cell suspension was disrupted with an MSE Soniprep 150 at a frequency of 18 MHz for periods of 30 s followed by cooling periods of 1 min in an iced-water chamber. Cell debris was removed by contrifugation for 45 min at 13 000 *g* and at 4°C.

Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed under denaturing conditions in a Pharmacia–LKB PhastGel apparatus with PhastGel Gradient 10–15 gels and SDS buffer strips as described by the manufacturer. The protein bands were visualised with silver stain as described by Pharmacia–LKB. Alternatively, slab gels containing 12.5% (w/v) acrylamide were run in an LKB vertical electrophoresis unit under denaturing conditions [16], and the protein bands stained with Coomassie Brilliant Blue R-350.

PREPARATION OF DNA POLYMERASE

Development of a purification procedure by using FPLC

An FPLC system (Pharmacia–LKB) was used

to determine optimum conditions for purification of the enzyme. Buffers were prepared with Milli-Q water (Millipore) and filtered through a 0.22-μm pore-size filtration membrane. Mono Q, phenyl-Superose and Mono S have an average particle size of 10-μm and Blue-Trisacryl M has a particle size of 40 to 80 μm. Chromatographic media were equilibrated, cleaned and stored according to the manufacturers instructions. All separations with FPLC were conducted at ambient temperature (18°C to 25°C). DNA polymerase activity was determined in each of the fractions collected.

Cell-free extract from 2 g of cell paste was applied to an 8-ml Mono Q HR 10/10 column (10 cm × 1 cm I.D.), equilibrated with buffer A at a linear flow-rate of 300 cm h^{-1}. Protein was eluted with a 160-ml linear gradient of 0 to 1 *M* NaCl in buffer A and fractions of 8 ml were collected. Ammonium sulphate was added to the Mono Q eluate to a final concentration of 1 *M*. A 1-ml phenyl-Superose HR 5/5 column (5 cm × 0.5 cm I.D.) was equilibrated with buffer A, containing 1 *M* ammonium sulphate, at a linear flow-rate of 150 cm h^{-1}. A 1-ml portion of sample was applied to the column and protein was eluted with a 20-ml linear gradient of equilibration buffer to buffer A containing no ammonium sulphate and fractions of 1 ml were collected. Active fractions were pooled and dialysed exhaustively against buffer A. A 1-ml HR 5/5 column (5 cm × 0.5 cm I.D.) was packed with Blue-Trisacryl M according to the manufacturers instructions. The column was equilibrated with buffer A and 1 ml of the phenyl-Superose eluate was applied at a linear flow-rate of 300 cm h^{-1}. Protein was eluted with a 20-ml linear gradient of 0 to 1 *M* NaCl in buffer A and fractions of 1 ml were collected. Active fractions were combined and dialysed exhaustively against buffer A. A 1-ml Mono S HR 5/5 column (5 cm × 0.5 cm I.D.) was equilibrated with buffer A and 1 ml of the Blue-Trisacryl eluate was applied to the column at linear flow-rate of 300 cm h^{-1}. Protein was eluted with a 20-ml linear gradient of 0 to 1 *M* NaCl in buffer A and fractions of 1 ml were collected. Active fractions were combined based upon activity and electrophoretic homogeneity and dialysed exhaustively against buffer A.

Scale-up of the purification procedure

The purification scheme developed with FPLC media was scaled-up by a factor of about 25 fold. The FPLC media were replaced with Q-Sepharose FF, phenyl-Sepharose FF and S-Sepharose FF. The particle size of each matrix is 45–165 μm. The matrix for the triazine-dye affinity chromatography step was the same as for FPLC. DNA polymerase activity was determined in each of the fractions collected.

Cell-free extract from 50 g of cell paste was applied to a 200-ml Q-Sepharose FF column (12.5 cm × 4.5 cm I.D.). The column was equilibrated with buffer A at a linear flow-rate of 60 cm h^{-1}. The protein was eluted with a 4-l gradient of 0 to 1 *M* NaCl in buffer A and fractions of 95 ml were collected. Active fractions were combined and ammonium sulphate added to a final concentration of 1 *M*. The eluate was applied to a 25-ml phenyl-Sepharose column (12 cm × 1.6 cm I.D.) equilibrated with buffer A containing 1 *M* ammonium sulphate, at a linear flow-rate of 25 cm h^{-1}. The protein was eluted in a 500-ml linear gradient of 1 *M* to 0 *M* ammonium sulphate in buffer A followed by 100 ml buffer A. Fractions of 10 ml were collected and active enzyme fractions were combined. The active fractions were applied to a 25-ml Blue-Trisacryl M column (12 cm × 1.6 cm I.D.) equilibrated with buffer A at a linear flow-rate of 25 cm h^{-1}. The protein was eluted with a 500-ml linear gradient of 0 to 1 *M* NaCl in buffer A. Fractions of 10 ml were collected, active enzyme fractions were combined and dialysed exhaustively against buffer A. The dialysed active fractions were applied to a 25-ml S-Sepharose FF column (12 cm × 1.6 cm I.D.), equilibrated with buffer A at a linear flow-rate of 25 cm h^{-1}. Protein was eluted in a 500-ml linear gradient of 0 to 1 *M* NaCl in buffer A and fractions of 10 ml were collected. Fractions containing DNA polymerase were combined based on activity and electrophoretic homogeneity and dialysed exhaustively against buffer A.

RESULTS AND DISCUSSION

Elution profiles for DNA polymerase purification on FPLC and on process-scale media are shown in Figs. 1 and 2 and a summary of each

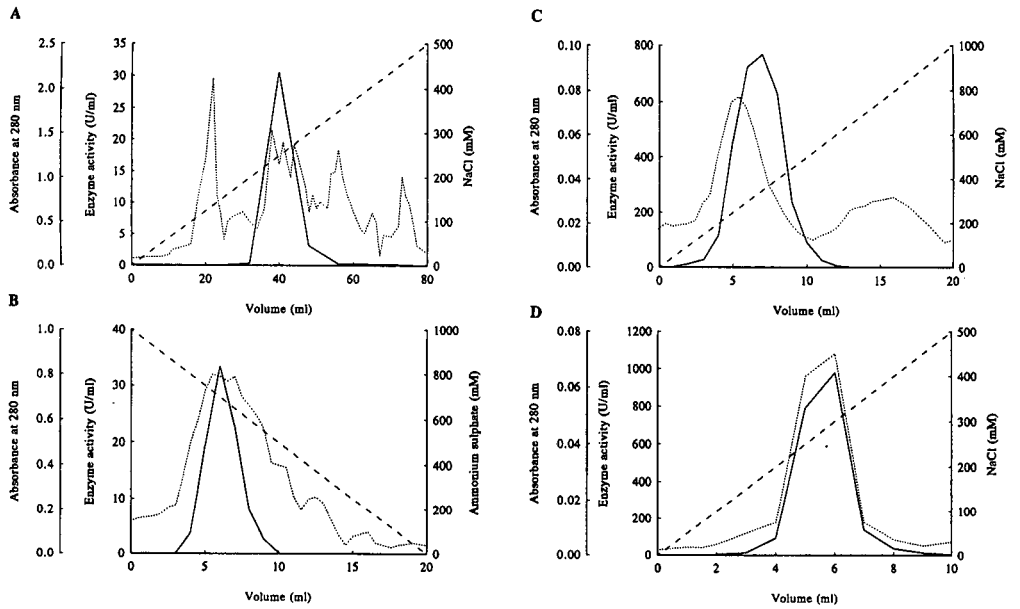

Fig. 1. Chromatography elution profiles for FPLC. The elution profiles of DNA polymerase (——), A_{280} (· · ·) and the salt gradient (– – –) are shown for (A) anion-exchange chromatography on Mono Q, (B) hydrophobic interaction chromatography on phenyl-Superose, (C) triazine-dye affinity chromatography on Blue-Trisacryl M and (D) cation-exchange chromatography on Mono S. The salt gradients shown in the chromatograms for Mono Q and Mono S represent half of the total volumes used in the gradients; the gradients were actually extended from 0 to 1000 m*M* NaCl.

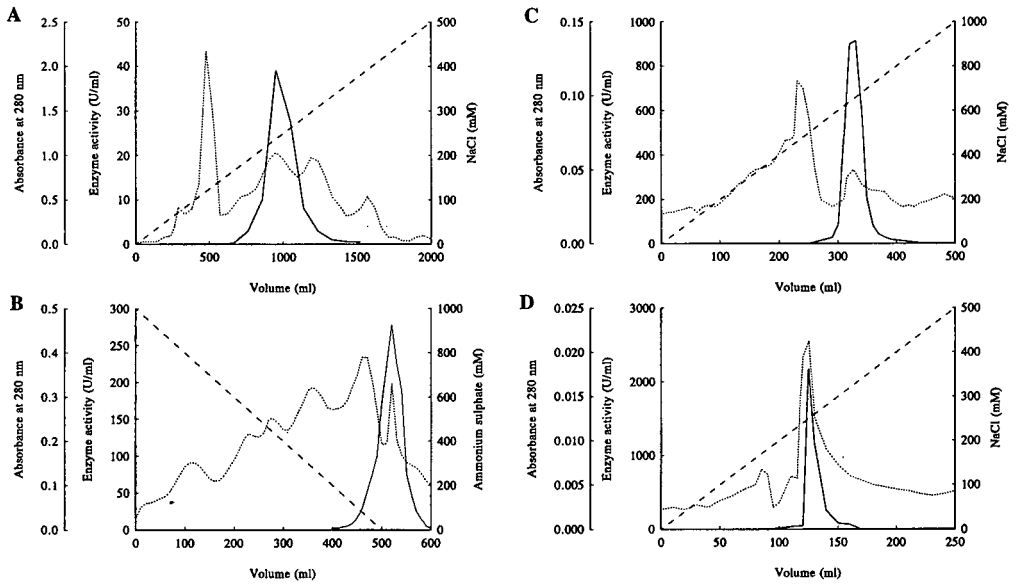

Fig. 2. Chromatography elution profiles for the scaled-up process. The elution profiles of DNA polymerase (——), A_{280} (· · ·) and the salt gradient (– – –) are shown for (A) anion-exchange chromatography with Q-Sepharose FF, (B) hydrophobic interaction chromatography with phenyl-Sepharose FF, (C) triazine-dye affinity chromatography with Blue-Trisacryl M and (D) cation-exchange chromatography with S-Sepharose FF. The salt gradients shown in the chromatograms for Q-Sepharose and S-Sepharose represent half of the total volumes used in the gradients; the gradients were actually extended to 1000 m*M* NaCl.

purification procedure is shown in Tables I and II. In both cases homogenous enzyme of specific activity about 1950 U mg^{-1} (Tables I and II) was obtained after four chromatography steps as shown by SDS-PAGE (Fig. 3). The elution profiles for anionic and catonic exchange are similar with both FPLC media and process-scale media. The enzyme eluted from Mono Q in about 220 m*M* NaCl and from Q-Sepharose in about 240 m*M* NaCl. Enzyme eluted from Mono S and S-Sepharose FF in about 250 m*M* NaCl. The elution profiles obtained after hydrophobic interaction chromatography on phenyl-Superose and phenyl-Sepharose FF are, however, different. The enzyme eluted in about 650 m*M* ammonium sulphate on FPLC and yet at the end of the ammonium sulphate gradient in the scaled-up process. An advantage of this delayed elution was that dialysis of the eluate was not required. Blue-Trisacryl M was used in both procedures for triazine-dye affinity chromatography. The enzyme eluted in about 650 m*M* NaCl on the larger scale compared to about 350 m*M* during FPLC. The difference may be due to the linear flow-rate of 25 cm h^{-1} at which the scaled-up process was run compared with 300 cm h^{-1} on FPLC.

Chromatography was carried out under identical conditions for both cation-exchange and anion-exchange chromatography. The p*I* of 4.9 [17], indicates that the enzyme has a negative surface charge and should only bind to the anion-exchange matrices Mono Q and Q-Sepharose FF. The surface charge of the protein is likely to be negative at pH 7.5 and true cation exchange cannot take place. The enzyme therefore binds to Mono S and S-Sepharose FF by some mechanism other than by cation exchange. A possible mechanism is that a positively charged DNA-binding site of the enzyme inter-

TABLE I

DNA POLYMERASE PURIFICATION ON FPLC MEDIA

Step	Volume (ml)	Protein (mg)	Enzyme (Units)	Specific activity (U mg^{-1})
Cell-free extract	10	90	42[a]	0.5
Mono Q eluate	8	18	245[a]	14
Phenyl-Superose eluate	5	4	1046[a]	262
Blue-Trisacryl M eluate	5	2	2989	1495
Mono S eluate	2	0.9	1772	1970

[a] Apparent DNA polymerase activity is low due to nucleolytic enzyme activity.

TABLE II

DNA POLYMERASE PURIFICATION ON PROCESS-SCALE MEDIA

Step	Volume (ml)	Protein (mg)	Enzyme (U)	Specific activity (U mg^{-1})
Cell-free extract	250	2250	1 056[a]	0.5
Q-Sepharose FF eluate	190	285	6 338[a]	22
Phenyl-Sepharose FF eluate	50	36	10 878[a]	302
Blue-Trisacryl M eluate	50	18	31 047	1725
S-Sepharose FF eluate	20	10	19 136	1914

[a] Apparent DNA polymerase activity is low due to nucleolytic enzyme activity.

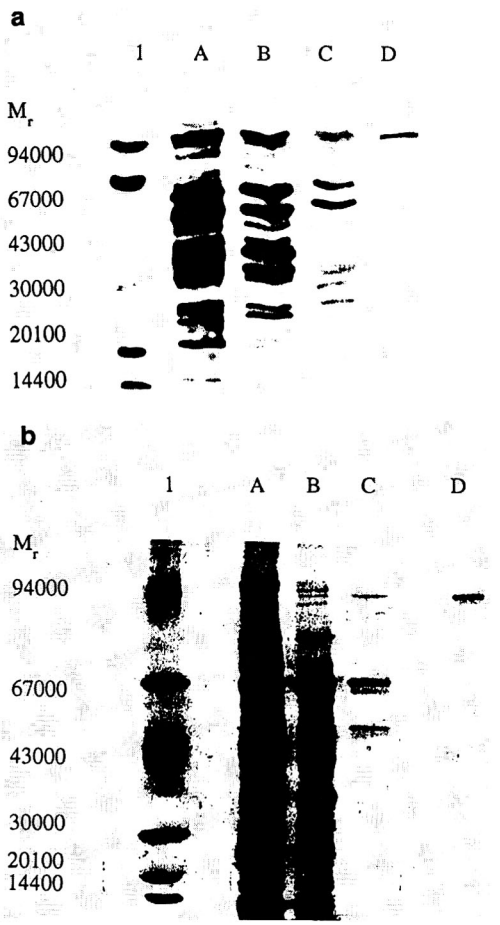

Fig. 3. SDS-PAGE of eluates from FPLC. (a) Mono Q (A), phenyl-Superose (B), Blue-Trisacryl M (C) and Mono S (D) eluates were separated on PhastGel Gradient 10–15 gels and with SDS buffer strips (Pharmacia-LKB). (b) Q-Sepharose FF (A), phenyl-Sepharose FF (B), Blue-Trisacryl M (C) and S-Sepharose FF (D) eluates were separated on a 12.5% (w/v) acrylamide gel in the presence of SDS. The following standards (1) were used: phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100) and α-lactalbumin (M_r 14 400).

acts with the charged sulphonate groups of Mono S and S-Sepharose FF. X-ray crystallography studies of the Klenow fragment of *E. coli* DNA polymerase I [18] have shown that a 400 amino-acid polypeptide forms a deep cleft and is thought to accommodate double-stranded DNA. The enzyme has a negative surface charge and is acidic, but a positive charge was detected in the cleft at physiological pH [19]. The enzyme from *B. caldotenax* is also acidic [17] and may have a similar cleft involved in binding to DNA. This putative positively-charged cleft may be shown by X-ray crystallography studies currently in progress for the enzyme from *B. caldotenax*.

The procedure developed by using FPLC was scaled-up further [17] to provide protein for X-ray crystallography studies and for enzyme characterisation.

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Purification of antifungal lipopeptides by reversed-phase high-performance liquid chromatography

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ABSTRACT

A rapid procedure for the purification of antifungal lipopeptides from *Bacillus subtilis*, a potential agent for biocontrol of plant diseases, was tested. It consists of a solid-phase extraction on C_{18} gel followed by reversed-phase chromatography using a biocompatible PepRPC HR 5/5 column with a pharmacia fast protein liquid chromatographic system. This is a very effective method for isolating and fractionating iturin A and surfactin, two lipopeptides of different nature, co-produced by *Bacillus subtilis* strain S499. The presence of homologous lipopeptides was easily detected.

INTRODUCTION

Several strains of *Bacillus subtilis* produce various lipopeptides with antifungal activity. About ten lipopeptides have been isolated and their structures have been determined [1,2]. They are cyclic lipopeptides containing seven residues of D- and L-α-amino acids and one residue of a β-amino fatty acid or β-hydroxy fatty acid. Most of them were identified as mixtures of homologous series that differ in the length of the lipidic chain. This is the case with iturin A [3,4], bacyllomicin L, D and F [5–7] and mycosubtilin [8]. More recently, it was found that surfactin, a surfactant lipopeptide, was a mixture of two variants that differ in the terminal amino acid residue [9]. This kind of lipopeptide does not exhibit an antifungal activity but it enhances the action of iturin A if they are present in a mixture [10].

It is therefore of obvious interest to acquire an effective separation method in order to identify completely the composition of lipopeptides produced by a strain of *Bacillus subtilis*. Further, pure lipopeptides are necessary for the study of their structure–function relationship, which is still unclear.

Generally, the purification of these molecules requires three or more steps, including precipi-

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tation, extraction and chromatographic techniques. We describe in this paper a rapid and efficient fast protein liquid chromatographic (FPLC) technique for the purification of all lipopeptides produced by *Bacillus subtilis* strain S499.

EXPERIMENTAL

Strain and culture conditions

Lipopeptides were produced from a culture medium of *Bacillus subtilis* strain S499 isolated by Delcambe (1956). The cells were grown in a 20-l fermenter (Biolafitte, Poissy, France) at 30°C for 72 h with the medium described previously [11]. The stirrer speed was 300 rpm and the aeration rate was 0.3 vvm (*i.e.*, volumetric air flow per minute per working volume). The pH was maintained at 7 during the culture.

Isolation of lipopeptides

After 72 h of growth, the culture medium was centrifuged at 11 000 *g* for 25 min to remove the cells. The supernatant (500 mg of dry material) was applied to a Bond Elut C_{18} cartridge (5 g per 20 ml) from Analytichem International (Harbour City, CA, USA). The cartridge, which retained lipopeptides, was rinsed successively with 20 ml of water and 40 ml of 50% aqueous methanol and finally the lipopeptides were eluted from the cartridge with 20 ml of methanol. The eluate was evaporated and the residue was dissolved in 0.5 ml of methanol. The solution was analysed by FPLC as described below.

Antagonistic test

The antifungal activity of substances eluted from the Bond Elut C_{18} cartridge was evaluated by a classical antibiotic test using the agar diffusion method in Petri dishes. Each eluate was evaporated and the residue was lyophilized. The dry material was dissolved in 0.1 *M* $NaHCO_3$ to give a 5 mg/ml solution. Aliquots of 5 μl were placed in small open-ended cylinders placed on the surface of an agar plate pre-seeded with various phytophathogenic moulds: *Fusarium graminirum*, *Fusarium oxysporum*, *Rhizopus sp.*, grown on potato dextrose agar, and *Botrytis cinerea*, grown on Malt agar (both from Merck, Darmstadt, Germany). The plate was incubated at 37°C. Antifungal activity was detected by the presence of clear zones where fungi growth was inhibited. The minimum inhibitory concentration (MIC) was determined by decreasing the amount of substance tested.

FPLC purification

Instrumentation. A Pharmacia (Uppsala, Sweden) FPLC system, consisting of two Model P-500 high-precision pumps, a Model 3500 pump, a flow equalizer, an LCC-500 liquid controller, an MV-7A motor valve, a FRAC-100 fraction collector and a REC-482 recorder, was used throughout.

Chemicals. Acetonitrile was of HPLC grade from Alltech (Deerfield, IL, USA) and trifluoroacetic acid (TFA) was from Merck. Water was of Milli-Ro quality. All other reagents were of analytical-reagent grade.

Reversed-phase chromatography. The separation was performed on a PepRPC HR5/5 C_2/C_{18} column (Pharmacia). Lipopeptides were detected at 214 nm with a Waters Model 484 multiwavelength detector. The mobile phase components were (A) 0.1% TFA in Water and (B) 0.1% TFA in Water–Acetonitrile (30:70, v/v).

Thin-layer chromatography (TLC). TLC was performed on silica gel 60 plates (Merck), which were developed with chloroform–methanol–water (65:25:4, v/v/v). Lipopeptides were detected with a water spray, ninhydrin [12] and chlorination [13].

Amino acid analysis. The lipopeptide samples were hydrolysed with 6 *M* HCl at 110°C for 24 h. Water-soluble amino acids were reacted with phenyl isothiocyanate (PITC) to give phenylthiohydantoin (PTH) derivatives. The amino acid derivatives were analysed by HPLC (Pharmacia) using a PICO-TAG column (30 × 3.9 mm I.D.) (Waters–Millipore) and detected at 254 nm. The separation conditions used were those given in the Waters–Millipore manual.

RESULTS

The lipopeptides from the *Bacillus subtilis* strain S499 were isolated and purified according to the scheme shown in Fig. 1. Cells were

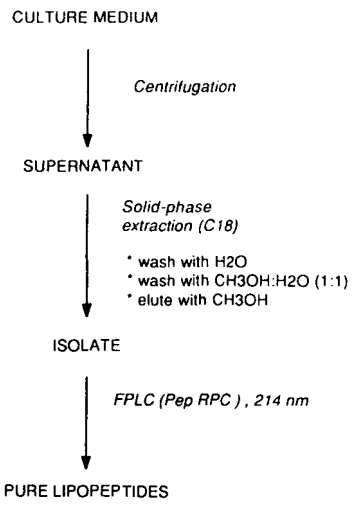

Fig. 1. Scheme of purification procedure.

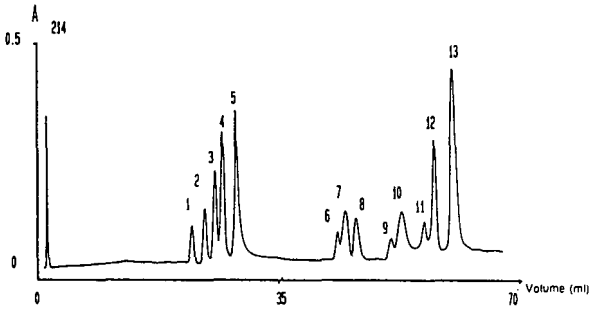

Fig. 2. FPLC separation of lipopeptides isolated from the Bond Elut C_{18} cartridge. Peaks: 1–5 = iturin A; 6–8 = unknown molecules; 9–13 = surfactin. Column, PepRPC HR 5/5 C_2/C_{18}; mobile phase, (A) 0.1% TFA in water and (B) 0.1% TFA in water–acetonitrile (30:70, v/v); gradient, 0% B in 5 ml, 0–45% B in 20 ml, 45–60% B in 11 ml, 60–80% B in 9 ml, 80–100% B in 15 ml, 100% B in 5 ml; flow-rate, 0.8 ml/min; detection, 214 nm.

removed by centrifugation and supernatant (500 mg of dry material) was passed through the C_{18} cartridge. The cartridge was then washed successively with 20 ml of water, 20 ml of 25–75% aqueous methanol and 20 ml of methanol. The elution of lipopeptides was monitored by TLC on silica gel as described above. They were detected as water spray-positive (white spot), ninhydrin-negative and chlorination-positive. The lipopeptides began to elute when the cartridge was washed with aqueous methanol of concentration >50% and were completely eluted with methanol. Antifungal activity against various phytopathogenic moulds was only detected in the fraction eluted with methanol. The MIC values for the supernatant and the isolate were determined and were of 500 and 50 μg/ml, respectively, on average.

A chromatogram of lipopeptides eluted from the Bond Elut C_{18} cartridge is presented in Fig. 2. The peaks were analysed by TLC on silica gel and their amino acid compositions were determined by HPLC after acid hydrolysis. Table I gives the results of these analyses. Each peak gave a single spot in TLC. According to the R_F values and α-amino acid composition, two groups of known lipopeptides, iturin A (peaks 1–5) and surfactin (peaks 9–13), were identified. A third group of unknown molecules (peaks 6–8) was also separated.

DISCUSSION

The present lipopeptide purification procedure includes an extraction step exploiting a solid-phase extraction technique and a chromatographic step involving reversed-phase chromatography with an FPLC system. The Bond Elut C_{18} phase is very efficient at adsorbing lipopeptides, owing to the important hydrophobic part of these molecules. Hence the majority of impurities such as inorganic materials and polar contaminants are easily removed by washing the Bond Elut C_{18} cartridge successively with 50% aqueous methanol. Lipopeptides are completely retained using less than 50% aqueous methanol as a washing agent. The TLC system used to monitor the elution of lipopeptides ensures a distinction between cyclic lipopeptides and other substances. Methanol is required to elute the lipopeptides completely. The antagonistic tests showed that the isolate is ten times more active than the crude supernatant by comparison of the MIC values. This illustrates the effectiveness of this clean-up step.

The solid-phase extraction on C_{18} shows some advantages over classical extraction by acid precipitation followed by extraction with methanol [4] or chloroform–methanol (2:1) [3]. It is rapid and no reactions such as hydrolysis or esterification of the lipopeptide functional groups were

TABLE I

AMINO ACID COMPOSITION AND R_F VALUES OF DIFFERENT FRACTIONS FROM FPLC SEPARATION

Peak	Amino acid composition										R_F [a]
	Ala	Val	Leu	Ileu	Ser	Tyr	Asx	Glx	Arg	Pro	
1	–	–	–	–	1	1	3	1	–	1	0.32
2	–	–	–	–	1	1	3	1	–	1	0.32
3	–	–	–	–	1	1	3	1	–	1	0.32
4	–	–	–	–	1	1	3	1	–	1	0.32
5	–	–	–	–	1	1	3	1	–	1	0.32
9	–	1	4	–	–	–	1	1	–	–	0.64
10	–	1	4	–	–	–	1	1	–	–	0.64
11	–	1	4	–	–	–	1	1	–	–	0.64
12	–	1	4	–	–	–	1	1	–	–	0.64
13	–	1	4	–	–	–	1	1	–	–	0.64

[a] TLC was carried out on silica gel G-60 with chloroform–methanol–water (65:25:4, v/v/v).

observed. Such reactions are possible in acidic conditions or during prolonged extraction with solvents [14].

The lipopeptides isolated from the Bond Elut C_{18} cartridge were well separated on the PepRPC C_2/C_{18} biocompatible reversed-phase column under the conditions given in Fig. 2. Two groups of lipopeptides were identified as iturin A and surfactin according to their R_F values on silica gel in chloroform–methanol–water (65:24:5, v/v/v), which were 0.32 and 0.65 respectively [14,15]. The α-amino acid composition of peaks 1–5 and 9–13 (Fig. 2) conform to the known heptapeptide moieties of iturin A and surfactin, as shown in Fig. 3, confirming the identification by the R_F values. Moreover, no minor components were detected with each peak, which indicates the high purity of the molecules.

In fact, *Bacillus subtilis* strain S499 has been shown to produce a high yield of iturin A and surfactin [14], but no details were given about their constituents. Although those lipopeptides are often produced together [16], they were purified by different chromatographic techniques. Chromatography on silicic acid has been used to purify iturin [3,4] but it does not allow the separation of homologous series. On the other hand, preparative HPLC using a C_{18} column enabled six homologues of iturin with different lengths of the side-chain to be separated [4]. Other chromatographic techniques such as ion-exchange [15] or gel permeation chromatography [17] have been used for surfactin purification. Our results show that iturin A and surfactin may be purified in one chromatographic step and both are constituted by a mixture of five homologous lipopeptides as the amino acid residues of the peptidic parts are identical. Confirmation should be obtained by lipidic chain analysis of each component. A third group of molecules also appeared in the chromatogram. They gave a white spot like iturin A and surfactin after a water spray on the TLC plate, with an R_F value of 0.11. A complete study of these molecules is in progress.

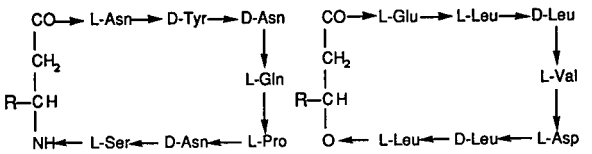

Fig. 3. Structures of iturin A and surfactin. R = Aliphatic chains.

In conclusion, it is possible to purify simultaneously iturin A and surfactin including their homologues using a PepRPC column with an FPLC system after crude clean-up on a Bond Elut C_{18} cartridge. This purification procedure is rapid and efficient and provide molecules with a high degree of purity. The preparation of a large

amount of pure lipopeptides is easy by means of a direct scale-up with larger columns. In addition, the biocompatibility of this method allows the structure and function of lipopeptides to be maintained, which is essential for fundamental studies of these molecules. Such a method will be suitable for the identification and purification of other lipopeptides produced by different strains of *Bacillus subtilis*.

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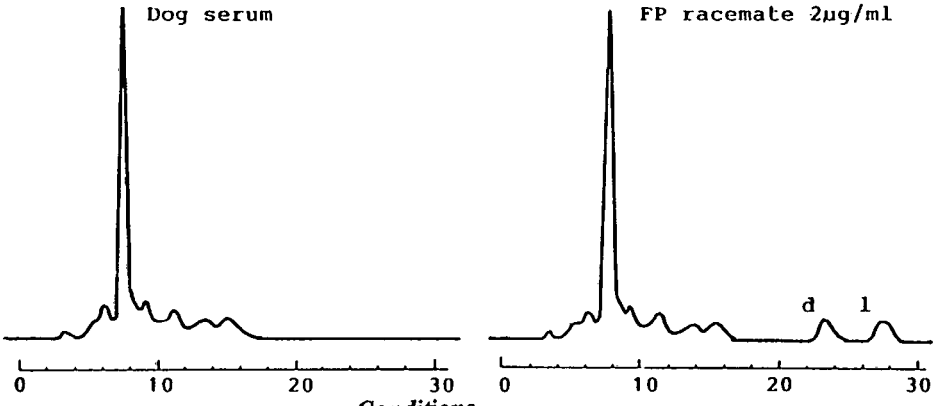
Ovomucoid Bonded Column for Direct Chiral Separation

ULTRON ES-OVM

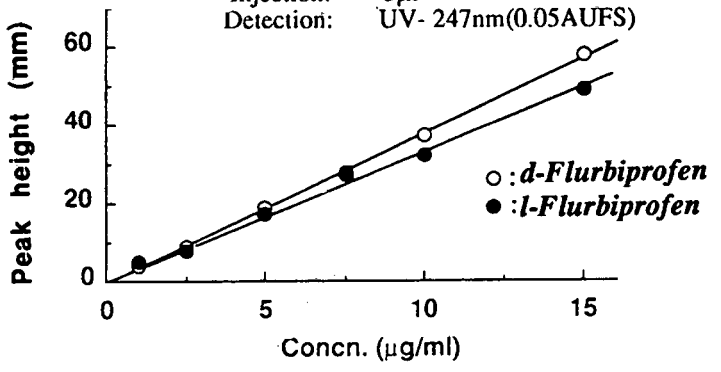
Narrow-Bore Column (2.0 I.D. x 150 mm) for Trace Analyses
Analytical Column (4.6 I.D., 6.0 I.D. x 150 mm) for Regular Analyses
Semi-Preparative Column (20.0 I.D. x 250 mm) for Preparative Separation

Analysis of Trace FLURBIPROFEN in Metabolite with NARROW-BORE COLUMN



Conditions

Column: ULTRON ES-OVM(2.0I.D. x 150mm)
Mobile Phase: 20mMPhosphate Buffer(pH=3.0)/CH3CN =100/15
Flow Rate: 0.1ml/min
Temperature: 25°C
Injection: 5µl
Detection: UV- 247nm(0.05AUFS)



Calibration Curve for Each Enantiomer of Flurbiprofen

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