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5th FPLC Seminar
Titisee, February 21–23, 1990

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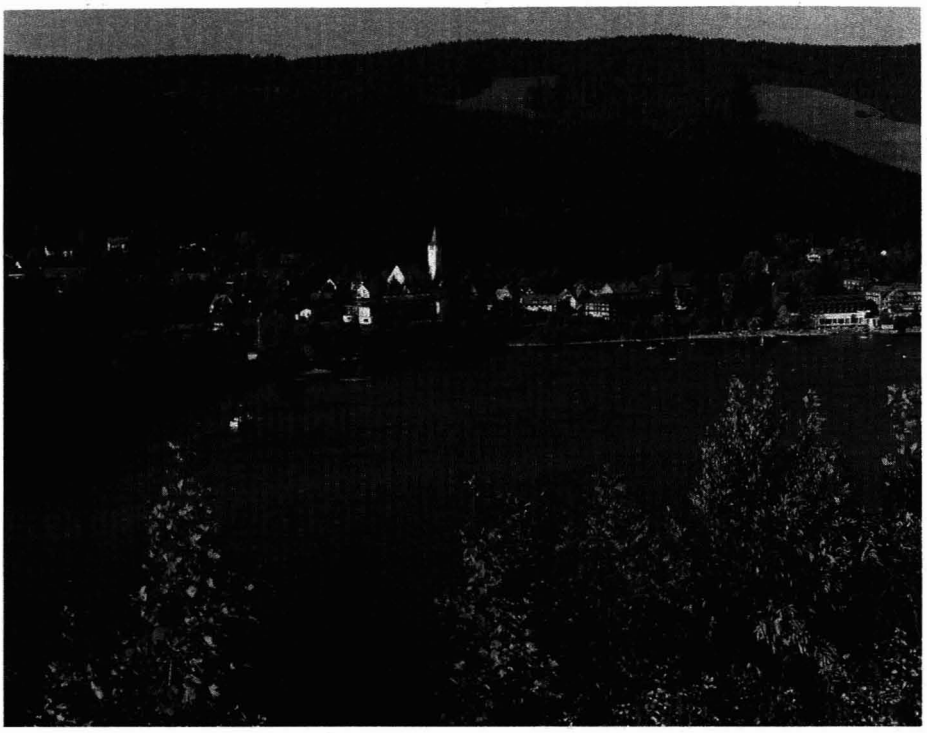

FIFTH FAST PROTEIN LIQUID CHROMATOGRAPHY SEMINAR

Titisee (F.R.G.), February 21–23, 1990

Guest Editors

RUDOLF DERNICK
(Hamburg, F.R.G.)

ULRICH STAUDER
(Freiburg, F.R.G.)

Participants of the seminar.

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FOREWORD

This issue contains the lectures presented at the *5th FPLC ® Seminar in Titisee* held from February 21st to 23rd, 1990. As the main topic of this seminar was the "purification of glycoproteins and membrane proteins", this issue contains a unique collection of methods and procedures for the purification of membrane proteins using fast protein liquid chromatography (FPLC).

The *FPLC Seminars in Titisee*, organized by Pharmacia LKB, have been held since 1984. Whereas the first seminars dealt mainly with water-soluble proteins and enzymes, this last one was dedicated exclusively to the purification of membrane-associated proteins. Because this substance class is much more difficult to handle than soluble proteins, the speakers presented not only chromatographic results but also methods for sample extraction and preparation.

The lectures, representing a broad range of different applications, provided a good understanding of the topic. In recent years, scientific interest in membrane proteins has increased rapidly. Key areas are the characterization of cell receptors for molecules of biological and clinical interest and their role in cell–cell recognition, in the regulation of the metabolism and in the origin and growth of tumour cells. This timeliness made for intensive and fruitful discussions. The beautiful view of the Titisee, with sunny weather without any snow this year, contributed to the pleasant atmosphere at the seminar.

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie, Universität Hamburg, Hamburg (F.R.G.)

RUDOLF DERNICK

Pharmacia LKB GmbH, Freiburg (F.R.G.)

ULRICH STAUDER

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CHROMSYMP. 1966

Application of high-performance liquid chromatography to the purification, disintegration and molecular mass determination of pyruvate dehydrogenase multi-enzyme complexes from different sources

EDITH MAAS, PASCALE ADAMI[a] and HANS BISSWANGER*
Physiologisch-chemisches Institut der Universität Tübingen, Hoppe-Seyler-Strasse 4, D-7400 Tübingen (F.R.G.)

ABSTRACT

The pyruvate dehydrogenase complex is associated with the inner mitochondrial membrane. A gentle and rapid purification procedure, especially for the very unstable pyruvate dehydrogenase complex from the extremely thermophilic organism *Thermus aquaticus*, is described. This procedure is based essentially on a combination of hydrophobic interaction and of adsorption chromatography by the rapid fast protein liquid chromatographic technique. Applying the same method, a relative molecular mass of $9.1 \cdot 10^6$ daltons was obtained by gel filtration on Superose 6 HR 10/30 for the pyruvate dehydrogenase complex from *T. aquaticus*. The same column served to resolve the pyruvate dehydrogenase complex into its enzyme components.

INTRODUCTION

The pyruvate dehydrogenase complex is the largest enzyme aggregate of the cell. It consists of three enzyme components, 60 α-chains and 60 β-chains of the pyruvate dehydrogenase (E1, E.C. 1.2.4.1), 60 chains of the dihydrolipoamide acetyltransferase (E2, E.C. 2.3.1.12) and 12 chains of the dihydrolipoamide dehydrogenase (E3, E.C. 1.8.1.4) [1,2]. Whereas in Gram-negative bacteria the enzyme complex is located in the cytoplasm, it is associated with the inner mitochondrial membrane in eukaryotes. Resolution of the enzyme complex from the membrane requires treatment with detergents such as Triton X-100 or digitonin [3,4]. Because of the association of the pyruvate dehydrogenase complex with membrane structures, hydrophobic-interaction chromatography is an appropriate purification method for this enzyme complex. In this paper, we describe a rapid and mild purification procedure based on hydrophobic-interaction and adsorption chromatography with elution gradients controlled by a fast protein liquid chromatographic (FPLC) system. This procedure is

[a] Present address: Laboratoire de Biochimie et Biologie Moléculaire, Université de Franche Comté, F-25030 Besançon, France.

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especially applicable to the isolation of the very unstable pyruvate dehydrogenase complex from the extremely thermophilic organism *Thermus aquaticus*. Gel filtration in the FPLC system was used to determine the molecular mass of the native pyruvate dehydrogenase complex from this organism. The same system was applied to separate the enzyme complex into its components. Because of the instability of the thermophilic enzyme, the bovine pyruvate dehydrogenase complex was taken for this experiment.

EXPERIMENTAL

Materials

Superose 6 HR 10/30, phenyl-Superose HR 5/5 and phenyl-Sepharose CL-4b were obtained from Pharmacia LKB (Freiburg, F.R.G.), the Bio-Gel HPHT hydroxyapatite column from Bio-Rad Labs. (Munich, F.R.G.), bovine serum albumin enzymes, cofactors and substrates for the enzyme tests from Boehringer (Mannheim, F.R.G.) and porcine thyroglobulin, Triton X-100 and the reagents for polyacrylamide gel electrophoresis (PAGE) from Serva (Heidelberg, F.R.G.). All other chemicals were obtained from Merck (Darmstadt, F.R.G.). The *Thermus aquaticus* strain AT62 (DSM No 674) was obtained from the Deutsche Sammlung für Mikroorganismen (Braunschweig, F.R.G.).

The method of Stanley and Perham [5] was used to purify the pyruvate dehydrogenase complex from bovine heart. The enzyme complex from *Bacillus subtilis* was purified according to Maas [6]. Column chromatography was performed with an FPLC system, (Pharmacia LKB) consisting of an LCC 500 controller, two P-500 pumps, a V-7 valve, a FRAC-100 fraction collector and a BT 3030 UV monitor, equipped with an 8-μl flow cell (Biotronic, Maintal, F.R.G.).

Cultivation of T. aquaticus and preparation of crude extracts

The bacteria were cultivated in medium M162 [7], supplemented with 0.4% sodium pyruvate as a carbon source. Fermentation was carried out in the same medium in an Intensor 20b 20-l fermenter (Giovanola Freres, Monthy, Switzerland) at 70°C with 0.2 volumes of air per fermenter unit volume per minute and stirring (1200 rpm) for 15 h. The cells were harvested by centrifugation. For the preparation of the crude extracts, aliquots of 7 g of wet cells were suspended in 14 ml of 50 m*M* potassium phosphate (pH 7.5)–0.1 m*M* EDTA–2 m*M* DTT–3 m*M* $MgCl_2$-5 m*M* β-mercapto-ethanol and sonicated for 4 min at 4°C in a B12 sonicator (Branson Ultrasonics, Danbury, CT, U.S.A.), adjusted to 50 W.

Polyacrylamide gel electrophoresis

PAGE in the presence of 0.1% sodium dodecyl sulphate (SDS) [8] employed a linear gradient of 10–20% (w/v) acrylamide and a ratio of acrylamide to N,N′-methylenebisacrylamide of 100:3.0. Staining of protein bands was performed with 0.2% (w/v) Coomassie Brilliant Blue R-250 in acetic acid–methanol–water (2:9:11, v/v/v) at 60°C for 10 min. For destaining the gels were treated for 30 min at 60°C with acetic–acid–methanol–water (1:3:6, v/v/v).

Enzyme assays and protein determination

The enzymatic activity of the pyruvate dehydrogenase complex was tested

following the reduction of NAD at 340 nm [9] at 37°C for the bovine enzyme and at 70°C for the thermophilic enzyme. The proteins were determined according to Lowry *et al.* [10].

RESULTS AND DISCUSSION

Purification of the pyruvate dehydrogenase complex from T. aquaticus

As the pyruvate dehydrogenase complex from the extremely thermophilic Gram-positive bacterium *T. aquaticus* is very unstable, purification procedures described for the enzyme complex from other sources were not applicable to this organism [11]. Two reasons appeared to be responsible for this instability: (i) dissociation of subunits, especially during chromatography and (ii) destabilization of the native structure of the enzyme complex after detachment from the membrane. A purification procedure was developed based on the hydrophobic nature of the surface of this thermophilic pyruvate dehydrogenase complex in combination with the FPLC technique.

To detach the pyruvate dehydrogenase complex from the membrane fraction, Triton X-100 was added to the crude extract to give a final concentration of 20% (v/v). After stirring for 10 min and centrifuging for 30 min at 100 000 *g*, two layers were obtained. The upper layer, containing the detergent, was discarded. The activity of the pyruvate dehydrogenase complex remained in the lower, clear, yellow layer. This layer, which should be completely free of any traces of the detergent, was applied to a 16 × 2 cm I.D. phenyl-Sepharose CL-4B column, which was equilibrated with 1 *M* potassium phosphate (pH 7.0)–0.1 m*M* dithiothreitol (DTT)–0.1 m*M* EDTA. Under these conditions, the enzyme complex remained bound to the column and, after washing with 100 ml of the equilibration buffer, it was eluted with a 150-ml linear gradient from 1 to 10 m*M* potassium phosphate (pH 7.0)–0.1 m*M* DTT–0.1 m*M* EDTA, followed by a further 100-ml linear gradient from 10 to 1 m*M* potassium phosphate (pH 7.0)–0.1 m*M* DTT–0.1 m*M* EDTA. The gradients were controlled by the FPLC system. The elution profile is shown in Fig. 1. The enzyme activity was eluted as a single peak at the onset of the second gradient, corresponding to a concentration of 9 m*M* potassium phosphate. The active fractions were pooled and carefully layered on a cushion of the same volume of 35% (w/v) sucrose in 50 m*M* potassium phosphate (pH 7.0) in a centrifugate tube. It was centrifuged at 176 000 *g* and 4°C for 4 h. The clear yellow pellet, which contained the enzyme activity, was dissolved in a small volume of 25 m*M* potassium phosphate (pH 7.0).

An HPHT hydroxyapatite column, connected with the FPLC system, served as the final purification step. The column was equilibrated with 25 m*M* potassium phosphate (pH 7.0)–0.2 m*M* DTT. The enzyme solution was applied to the column in portions of 1 ml, each containing 1 mg of protein. Larger amounts of protein were prepared by repeated batchwise chromatography. The pyruvate dehydrogenase complex was eluted with a 10-ml linear gradient from 25 to 500 m*M* potassium phosphate (pH 7.0)–0.2 m*M* DTT. The enzyme complex was completely separated from other proteins (Fig. 2). PAGE of the enzyme preparation after this step established its purity (Fig. 3). The three major protein bands of this preparation observed on the gel (lane 3) represent (from the top) the E2 component and the α- and β-chain, respectively, of the E1 component. The E3 component could not be detected.

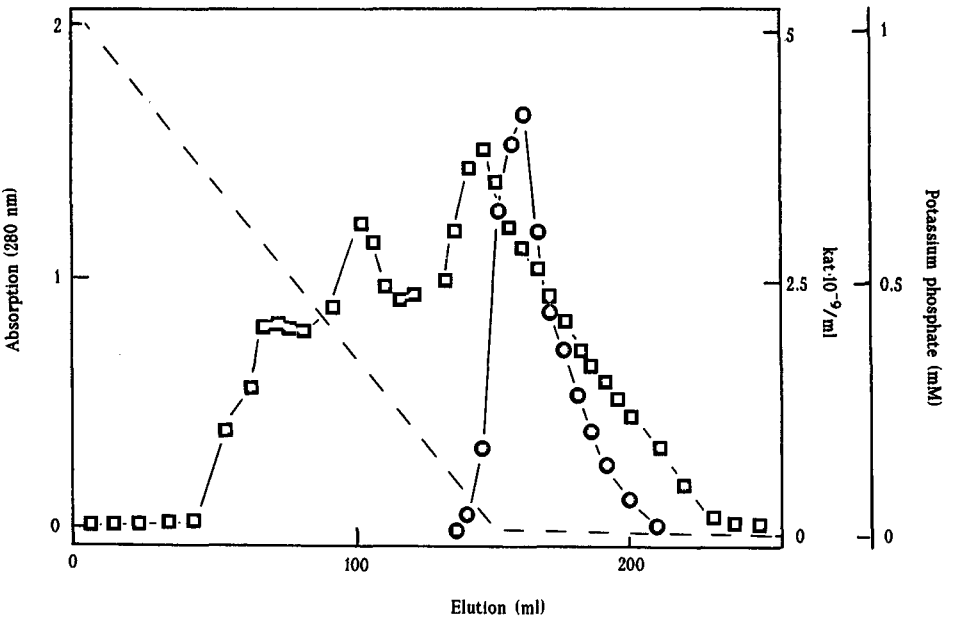

Fig. 1. Elution profile of the pyruvate dehydrogenase complex from *T. aquaticus* after chromatography on phenyl-Sepharose CL-4B. To 12 ml (23 mg of protein) of the Triton X-100 extract the same volume of 2 *M* potassium phosphate was added and it was applied to a 16 × 2 cm I.D. phenyl-Sepharose CL-4B column, equilibrated with 1 *M* potassium phosphate (pH 7.0)–0.1 m*M* DTT–0.1 m*M* EDTA. The column was washed with 100 ml of the equilibration buffer and eluted with a 150-ml linear gradient from 1 to 10 m*M* potassium phosphate (pH 7.0)–0.1 m*M* DTT, followed by a 100-ml linear gradient from 10 to 1 m*M* potassium phosphate (pH 7.0)–0.1 m*M* DTT–0.1 m*M* EDTA. The column was connected to the FPLC system. The flow-rate was 2 ml/min and fractions of 5 ml were collected. □ = Protein (absorption at 280 nm); ○ = activity of the pyruvate dehydrogenase complex; the dashed line indicates the gradient.

There is only a small amount of E3 chains bound to the native enzyme complex [2]. Further, owing to the weak binding of the E3 chains to the pyruvate dehydrogenase complex from *T. aquaticus*, parts of the chains were stripped off during ultracentrifugation [11]. The results of the purification procedure are summarized in Table I. Because of non-linearity, the values for the enzyme activity in the crude extract were underestimated. The yield and purification factor were therefore based on the Triton X-100 extraction.

Molecular mass determination of the pyruvate dehydrogenase complex from T. aquaticus

Special difficulties arise in the molecular mass determination of large enzyme aggregates by methods such as gel filtration and sucrose gradient centrifugation. Comparable large reference proteins with well established molecular masses are rare. Further, the experimental conditions, especially an extended stay in dilute solutions, favour dissociation and lead to underestimation. The structural heterogeneity reported for the pyruvate dehydrogenase complex from *Escherichia coli* [12] may be due to such dissociation processes. The FPLC technique can help to reduce the risk of dissociation.

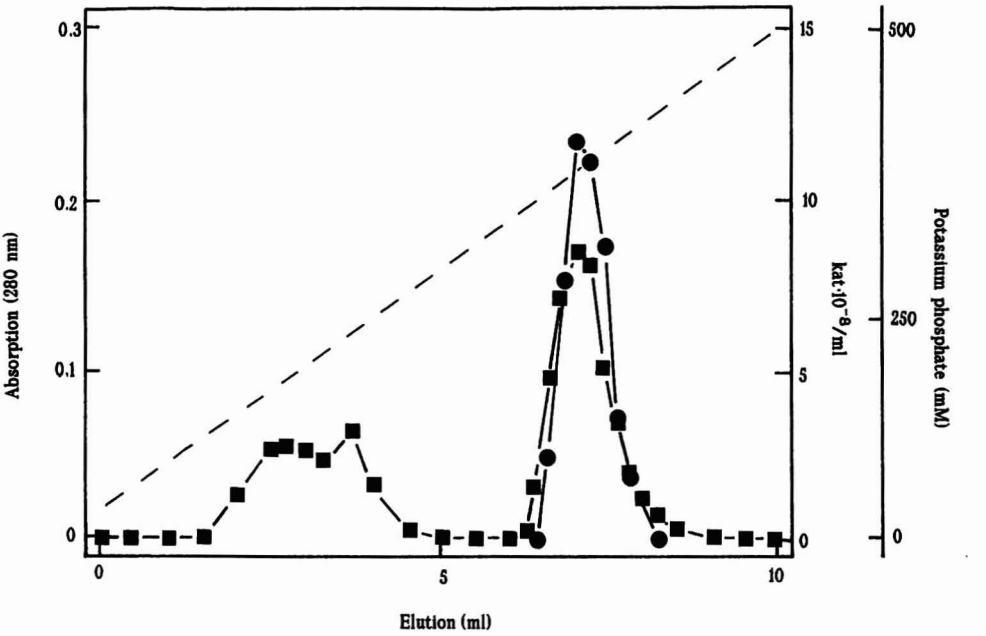

Fig. 2. Chromatogram of the pyruvate dehydrogenase complex from *T. aquaticus* on an HPHT hydroxyapatite column. The column (10 × 0.78 cm I.D.) was equilibrated with 25 m*M* potassium phosphate (pH 7.0)–0.2 m*M* DTT. Solutions of enzyme (1 mg/ml) were applied to the column and eluted with a 10-ml linear gradient from 25 to 100 m*M* potassium phosphate (pH 7.0)–0.2 m*M* DTT. The flow-rate was 0.1 ml per min and fractions of 0.2 ml were collected. ■ = Protein; ● = enzyme activity. The dashed line indicates the gradient.

To determine the molecular mass of the thermophilic pyruvate dehydrogenase complex from *T. aquaticus*, an FPLC Superose 6 HR 10/30 column was calibrated with different reference proteins in the range between $15 \cdot 10^4$ and $67 \cdot 10^4$ dalton and with the pyruvate dehydrogenase complex from bovine heart, having a molecular mass of $8.5 \cdot 10^6$ dalton (Fig. 4). A linear dependence of the retention volunes on the logarithm of the M_r (molecular mass) values was found for all reference proteins up to the very high value of the mammalian enzyme complex. This fact underlines the applicability of the method to large enzyme aggregates. The thermophilic pyruvate dehydrogenase complex was eluted prior to the mammalian complex, corresponding to a relative molecular mass of $9.1 \cdot 10^6$ dalton.

Resolution of the pyruvate dehydrogenase complex from bovine heart into its enzyme components

In the native pyruvate dehydrogenase complex the 60 chain of the E2 component form a stable core, which is surrounded by the α- and β-chains of the E1 component [13]. The contact between both enzyme components is very strong, and partially denaturating conditions, such as high pH and high ionic strength, are necessary to break these bonds [14]. Owing to these denaturating conditions, the reaction time must be kept short and, therefore, quantitative separation of the components cannot be achieved without a severe loss of enzymatic activity. The rate-determining step is the

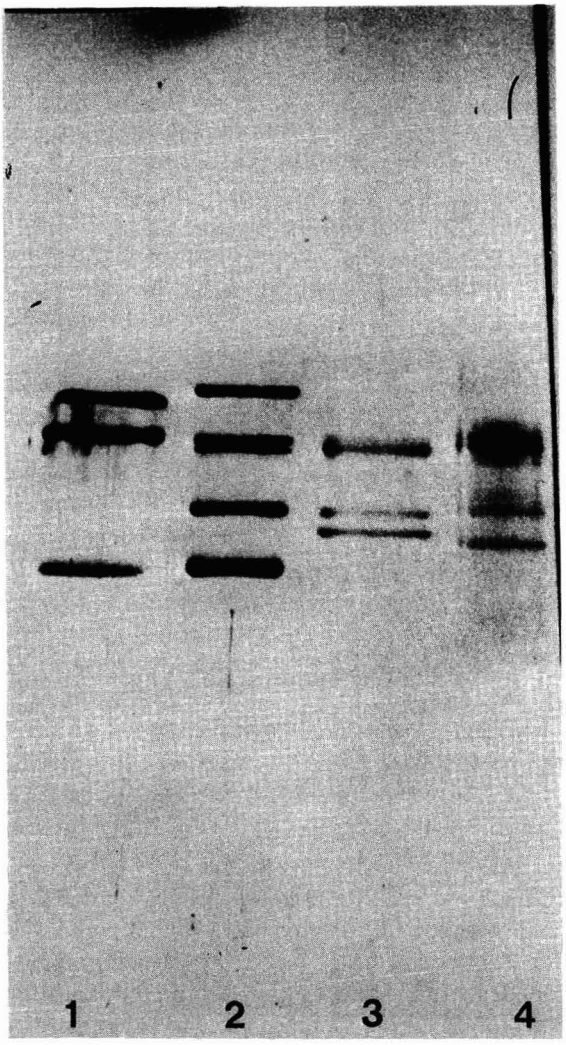

Fig. 3. SDS-PAGE of the pyruvate dehydrogenase complex from *T. aquaticus*. Purified pyruvate dehydrogenase complexes (2 μg of each) from *T. aquaticus* (lane 3), bovine heart (lane 2) and *Bacillus subtilis* lane 4) were applied to the gel. Lane 1 shows the reference proteins (from the top): bovine serum albumin, diaphorase from porcine heart and lactate dehydrogenase from porcine muscle (1 μg of each). The enzyme components of the pyruvate dehydrogenase complexes are as follows (from top to bottom): lane 2 = E2, E3, component X, E1α and E1β; lane 3 = E2, E1α, E1β; lane 4 = E2, E3, E1α, E1β.

chromatographic separation of the subunits. During the whole process, the enzyme components are in contact with the denaturating agents. Thus, a rapid method is required, which permits both the separation of the components and the removal of the denaturating agents in one fast step. Gel filtration in combination with the FPLC technique satisfies these conditions.

To the pyruvate dehydrogenase complex from bovine heart (30 mg/ml) in 50 m*M* potassium phosphate (pH 7.5)–1 m*M* DTT–0.1 m*M* EDTA, solid NaCl, 1 *M* DTT and

TABLE I

PURIFICATION OF THE PYRUVATE DEHYDROGENASE COMPLEX FROM *T. AQUATICUS*

Purification step	Volume (ml)	Protein (mg)	Specific activity (kat × 10^{-9} mg)	Total activity (kat × 10^{-9})	Yield (%)	Purification factor
Crude extract	28	420	0.20	78.3	89	0.62
Triton X-100 extraction	24	276	0.32	88.1	100	1.0
Phenyl-Sepharose Cl-4B	30	122	0.66	80.6	91.5	2.1
Ultracentrifugation	1	2.1	17.5	36.8	41.8	54.9
Hydroxyapatite	1.6	0.34	93.2	31.3	35.3	292

1 *M* glycine–NaOH (pH 9.5) were added to give final concentrations of 1 *M* NaCl, 10 m*M* DTT and 0.1 *M* glycine–NaOH (pH 9.0). After preincubation for 1 h at room temperature, aliquots of 0.1 ml were applied to a Superose 6 HR 10/30 column, equilibrated with 0.1 *M* glycine–NaOH (pH 9.0)–1 *M* NaCl–1 m*M* $MgCl_2$–2 m*M* DTT–0.1 m*M* EDTA. The same buffer was used to elute the enzyme components. Two well separated peaks were eluted (Fig. 5), the first containing the activity of the E2

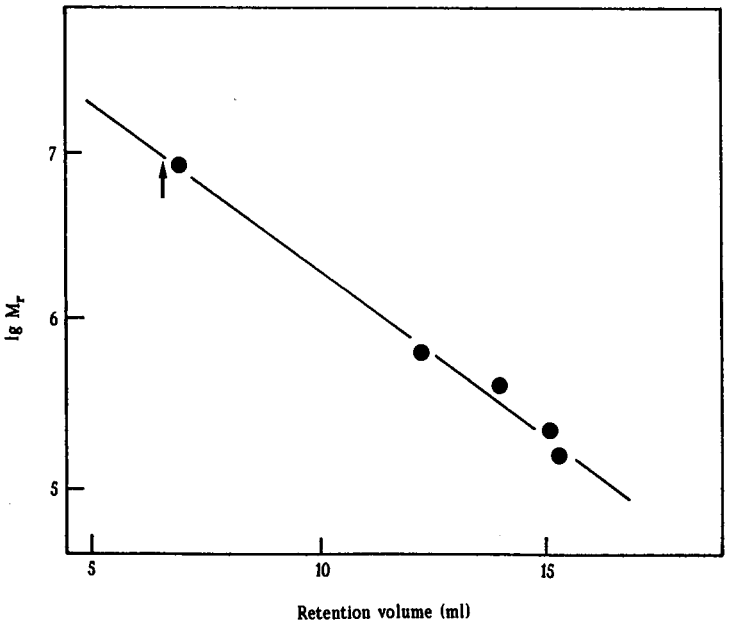

Fig. 4. Molecular mass determination of the pyruvate dehydrogenase complex from *T. aquaticus* on Superose 6. The column was calibrated with the reference proteins (from right to left): aldolase from rabbit muscle ($158 \cdot 10^3$ dalton), catalase from bovine liver ($24 \cdot 10^4$ dalton), ferritin from horse spleen ($45 \cdot 10^4$ dalton), porcine thyroglobulin ($67 \cdot 10^4$ dalton) and pyruvate dehydrogenase complex from bovine heart ($8.5 \cdot 10^6$ dalton). The arrow indicates the position of the pyruvate dehydrogenase complex from *T. aquaticus*. Equilibration and elution were performed with 50 m*M* morpholinopropanesulphonic acid–NaOH (pH 7.0)–150 m*M* NaCl, 0.4 mg of each protein was applied to the column and the flow-rate was 0.5 ml/min. Eluent was monitored at 280 nm and the respective retention volumes were determined.

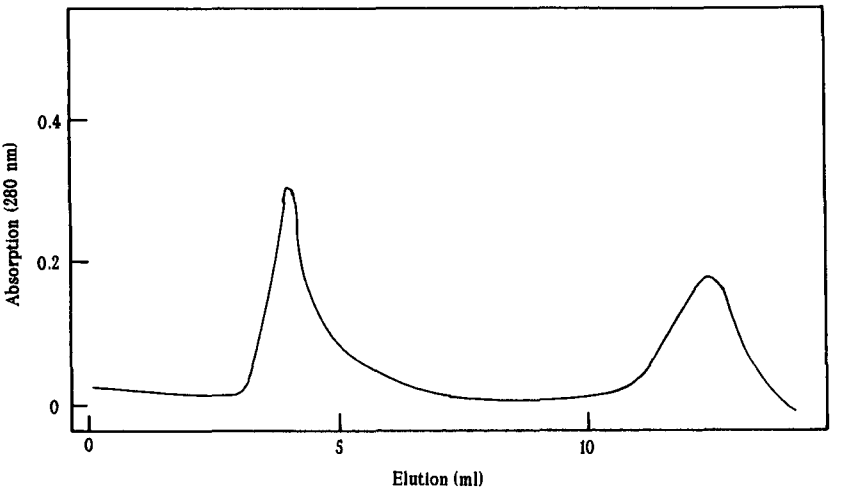

Fig. 5. Separation of the enzyme components of the pyruvate dehydrogenase complex from bovine heart by gel filtration on Superose 6 HR 10/30. The enzyme was pretreated as described in the text. Aliquots of 3 mg of protein in 0.1 ml of buffer were applied to the column, which was equilibrated with 0.1 *M* glycine–NaOH (pH 9.0)–1 *M* NaCl–1 m*M* $MgCl_2$–2 m*M* DTT–0.1 m*M* EDTA. The enzyme components were eluted with the same buffer at a flow-rate of 0.1–0.2 ml/min. The direction of the elution in the diagram is from right to left. The fractions of each peak were pooled and adjusted to pH 7.5 with 1 *M* KH_2PO_4 immediately after elution.

component and the second that of the E1 and E3 components. The pattern of the SDS PAGE, presented in Fig. 6, shows three protein bands for the first Superose peak. One major band corresponds to the E2 chain and a second is at the position of component X. The third band is a proteolytic fragment of the E2 chain, which is often observed in preparations of the mammalian pyruvate dehydrogenase complex [15]. The three protein bands on the gel, found in the second Superose peak, correspond to the E3, E1α and E1β chains, respectively.

CONCLUSION

Various kinds of information can be obtained from the application of the FPLC technique. The elution of the pyruvate dehydrogenase complex at very low ionic strength on phenyl-Sepharose is an indication of hydrophobic regions on the surface of this membrane-associated enzyme complex. This experiment also demonstrates the successful transfer of the conditions from an analytical FPLC phenyl-Superose HR 5/5 column, used for smaller amounts of crude extract (not shown), to the preparative phenyl-Sepharose column with nearly equal resolution.

The higher molecular mass obtained for the bacterial pyruvate dehydrogenase complex in comparison with the mammalian complex on Superose 6 is remarkable, as the mammalian enzyme complex possesses an additional component, X, with a so far unknown function, and two regulatory enzyme components [16,17]. A reason for the high molecular mass of the bacterial enzyme may be the avoidance of dissociation

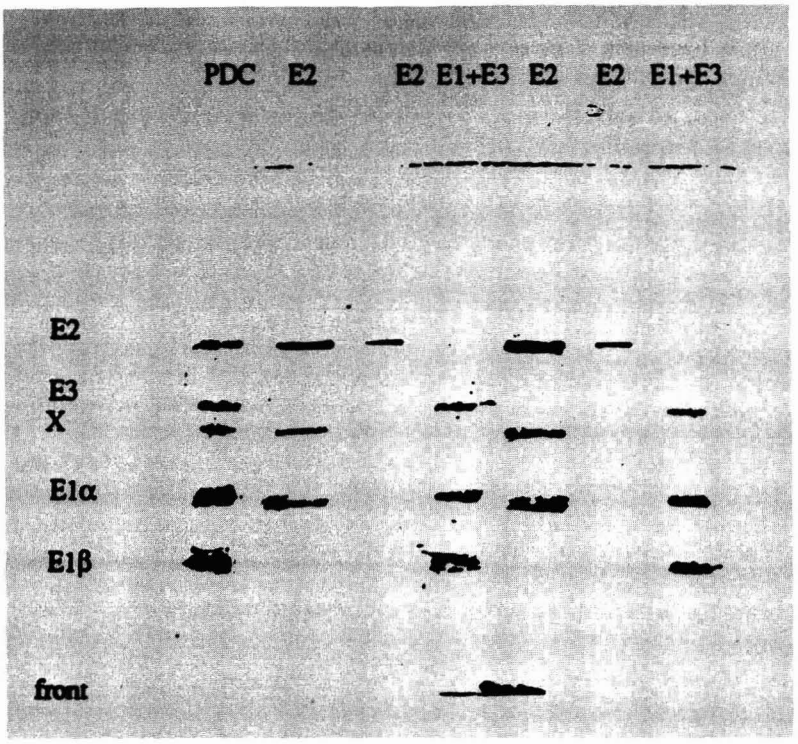

Fig. 6. SDS-PAGE of the enzyme components from the bovine pyruvate dehydrogenase complex. Samples of the purified pyruvate dehydrogenase complex from bovine heart (PDC), and of both of the peaks from the chromatogram shown in Fig. 5, each containing 5 μg of protein, and a further sample of peak 1, containing 0.5 μg of protein (lanes 3 and 6 from the left), were applied to the gel.

processes as a consequence of the fast chromatographic method, although structural differences between the two enzyme complexes cannot be completely excluded.

Separation of the E2 component from both the E1 and the E3 components by gel filtration on Superose 6 at high ionic strength and high pH is an appropriate technique for the dissociation of the native pyruvate dehydrogenase complex. As complex aggregation is mediated via the E2 core, the E1 and E3 components can be easily separated from one another by simple gel filtration without the need for denaturating agents.

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Chromatographic separation of four Ser/Thr–protein phosphatases from solubilized ciliary membranes of *Paramecium tetraurelia* by heparin–Sepharose

SUSANNE KLUMPP*
Pharmazeutisches Institut der Universität, Morgenstelle 8, D-7400 Tübingen (F.R.G.)
PHILIP COHEN
Department of Biochemistry, Medical Sciences Institute, The University, Dundee DD1 4HN (U.K.)
and
JOACHIM E. SCHULTZ
Pharmazeutisches Institut der Universität, Morgenstelle 8, D-7400 Tübingen (F.R.G.)

ABSTRACT

Chromatography of deoxycholate-solubilized proteins from *Paramecium* ciliary membranes on heparin–Sepharose resolved three peaks of protein phosphatase activities: one type 2A-like and a type 2C phosphatase in the flow-through fractions, another type 2A-like enzyme in the 0.1 *M* NaCl eluate and type 1 protein phosphatase in the 0.5 *M* NaCl eluate. The differential sensitivity of the two type 2A-like phosphatases to heparin and protamine further substantiated the existence of distinct isozymes. Once solubilized, none of these ciliary phosphatases required detergent to remain soluble. The molecular mass as determined by chromatography on Superose 6 was in the range 30 000–45 000 dalton for all four protein phosphatases.

INTRODUCTION

The protozoon *Paramecium* is used as a model organism to study signal reception and transduction at a unicellular level (for a review, see ref. 1). The availability of mutants with electrophysiologically characterized defects makes this ciliate a valuable tool for basic biochemical and pharmacological research on excitation-related processes [2,3].

Recently, regulation of voltage-dependent Ca-channels localized in the ciliary membrane of *Paramecium* by phosphorylation and dephosphorylation was suggested [4]. So far, cAMP-, cGMP- and Ca^{2+}-stimulated protein kinases and type 1, 2A-like and 2C protein phosphatases have been identified in the cilia from *Paramecium* [1,4,5].

This paper describes the resolution of the ciliary type 2A-like phosphatase activities by column chromatography. Heparin–Sepharose proved suitable for separating four protein phosphatases into three peaks of activity. The molecular mass of these enzymes was subsequently determined by fast protein liquid chromatography (FPLC) on Superose 6.

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EXPERIMENTAL

Cell culture and tissue preparation

Paramecium tetraurelia wild-type 51s was mass cultured axenically in 20-l bioreactors as described [5]. Cells at the early stationary phase (30 000/ml) were harvested in a cream separator. Cilia were removed from the cells by Ca-shock and purified by differential centrifugation [6]. The purity of the cilia was routinely checked by phase contrast microscopy.

Ciliary protein phosphatases were solubilized by adding 1 part of 2% deoxycholate [dissolved in 50 m*M* Tris–HCl (pH 7)] to 9 parts of a suspension of cilia (2 mg/ml) in 10 m*M* 3-(N-morpholino)propanesulphonic acid–Na (pH 7.5). After being vortex mixed and allowed to stand on ice for 30 min, the suspension was centrifuged for 1 h at 100 000 × *g*. The supernatant was used as a source of solubilized phosphatase activities and stored at −70°C.

Preparation of ^{32}P-labelled protein substrates

^{32}P-labelled phosphorylase *a* was prepared by phosphorylation with phosphorylase kinase [7], and casein by phosphorylation with cAMP-dependent protein kinase [8].

Protein phosphatase assays

Incubations (30 μl) contained 50 m*M* Tris–HCl (pH 7), 0.1 m*M* EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.6 mg/ml bovine serum albumin, ^{32}P-labelled substrate and enzyme. The substrate concentrations were 10 μ*M* for phosphorylase *a* or 1 μ*M* for phosphocasein. Caffeine (5 m*M*) was present when phosphorylase *a* was used as substrate [7], while assays for protein phosphatase 2C (using phosphocasein as a substrate) contained 20 m*M* magnesium acetate [8]. After 10 min at 30°C, the reactions were terminated and the solutions analysed as described [7,8]. Release of radioactivity was restricted to 30% of the input to ensure linear reaction rates. One unit of activity was defined as the amount which catalysed the release of 1 μmol phosphate/min.

Affinity chromatography on heparin–Sepharose

Solubilized phosphatases from ciliary membranes were applied to heparin–Sepharose. Up to 30 mg of protein in 50 ml could be successfully applied at once to a 10-ml column. The column was developed successively with buffer alone and with buffer containing 0.1 and 0.5 *M* NaCl. Further experimental details are given in the legend to Fig. 1.

Size-exclusion chromatography

Analyses were carried out on a Pharmacia (Uppsala, Sweden) automated FPLC system using a Superose 6 HR column (30 × 1.0 cm I.D.). The system was equipped with an LCC-500 gradient programmer, two P-500 dual piston pumps, an MV-7 automated injection valve, solvent mixer, prefilter, sample loop (0.5 ml), a UV-M monitor and a recorder.

RESULTS AND DISCUSSION

General purification problems

Membrane-bound mammalian protein phosphatase type 1 can be released by treatment with 1 *M* NaCl [9]. For solubilization of protein phosphatases localized in the ciliary membrane from *Paramecium*, however, detergent was required. At a protein concentration of up to 2 mg/ml, 0.2% deoxycholate released 90% of total particulate phosphatase activity into the supernatant. Although affinity chromatography is commonly used as one of the last steps in enzyme purification, in this work heparin–Sepharose (Pharmacia) was chosen at the beginning for the following reasons: (a) concentration and partial purification by precipitation of phosphatases with ammonium sulphate was impossible because, owing to the presence of detergent, a "floating pellet" was obtained; (b) chromatography on DEAE and Mono Q ion exchangers was precluded because of the anionic nature of deoxycholate; the anionic detergent could not be replaced by neutral detergents, such as Nonidet P40, Triton X-100 or Lubrol PX, without a substantial reduction in yields; (c) cation-exchange chromatography (Mono S) was unsuccessful as virtually all of the phosphatase activity was already recovered in the flow-through fractions; and (d) gel permeation chromatography was not practicable owing to the large starting volume of usually 50 ml.

Separation of Ser/Thr–protein phosphatases on heparin–Sepharose

Type 2A phosphatases from mammalian cells do not bind to heparin–Sepharose at 0.1 *M* NaCl whereas type 1 enzymes are retained and can be eluted with 0.5 *M* NaCl [10]. The same is true for the protein phosphatases from *Paramecium* cilia, cytosol and cell membranes [4]. By modifying this procedure we succeeded in further separating the protozoan type 2A-like phosphatase activities.

Solubilized ciliary phosphatases were applied to heparin–Sepharose in the absence of salt; 55% of the applied protein did not bind to the column material, 45% eluted with 0.1 *M* NaCl and only 5% was recovered in the high salt fraction (Fig. 1A). Phosphorylase phosphatase activity measured in the absence of divalent cations was found in the flow-through fractions (34% of total activity), the 0.1 *M* NaCl eluate (11%) and the 0.5 *M* NaCl eluate (59%, Fig. 1B). The dephosphorylation rate in any of these fractions was not affected by addition of up to 1 m*M* Ca^{2+}. The recovery of enzyme activity after correction for inhibition by salt was routinely 95–100%. In order to rule out that the binding capacity of heparin–Sepharose had been exceeded during the first column run, we rechromatographed the flow-through fractions on new material. All of the phosphatase activity was again recovered in the flow-through. Therefore, we conclude that the binding capacity during the first run was already sufficient.

The protein phosphatase activities in the flow-through and 0.1 *M* NaCl eluate were both classified as type 2A-like enzymes with regard to substrate specificity, divalent cation requirement and insensitivity toward inhibitor proteins I_1 and I_2 (for a phosphatase review, see ref. 9). However, these two type 2A-like phosphatases were clearly different in their responses to heparin and protamine (Fig. 2). The phosphatase activity in the flow-through was unaffected by protamine and only slightly activated by heparin. In contrast, the enzyme activity which was eluted by 0.1 *M* NaCl was potently

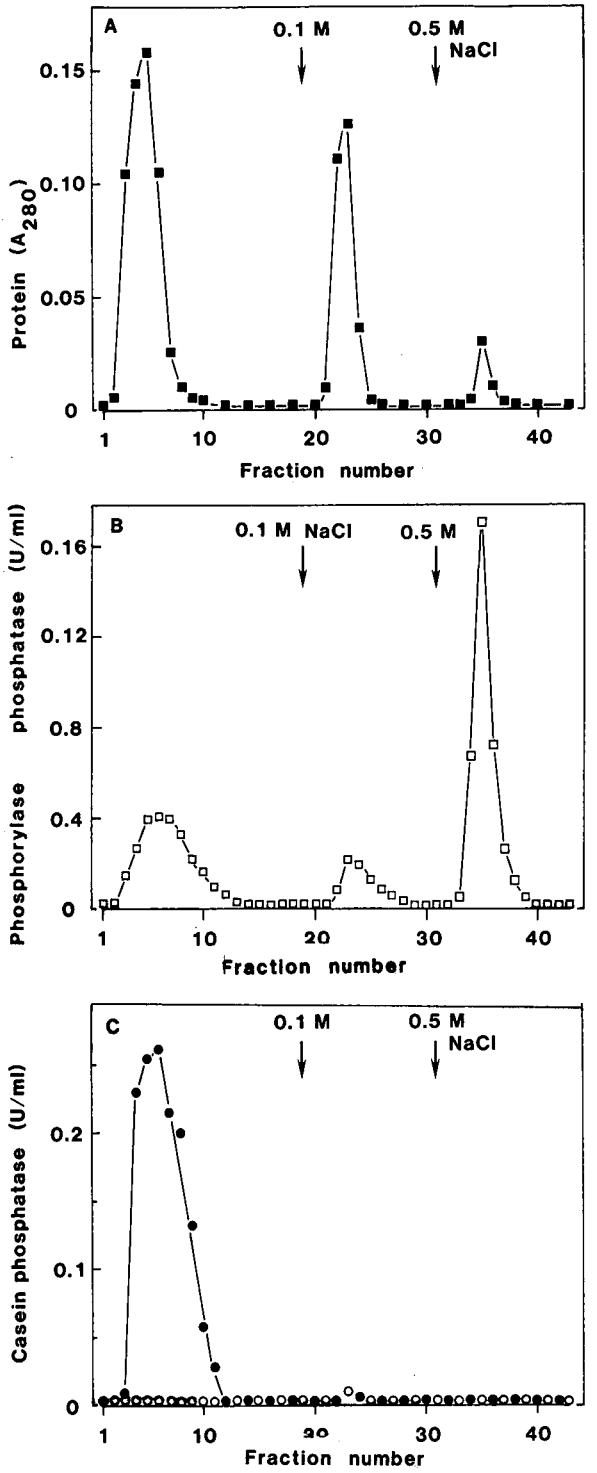

Fig. 1. Chromatography of ciliary protein phosphatases on heparin–Sepharose. The 100 000 *g* (1 h) supernatant fraction of 0.2% deoxycholate-treated cilia (6.7 mg, 10 ml) was applied to the column (4.8 × 1.6 cm I.D.) equilibrated in 20 m*M* Tris–HCl (pH 7)–0.1 m*M* EGTA–0.1% (v/v) 2-mercaptoethanol–0.1 mM phenylmethylsulphonyl fluoride–1 m*M* benzamidine–5% (v/v) glycerol. Arrows indicate the positions at which the column was eluted with equilibration buffer plus 0.1 or 0.5 *M* NaCl. The flow-rate was 3 ml/min and fractions of 3 ml were collected. (A) Absorbance at 280 nm; (B) phosphorylase phosphatase activity in the absence of divalent cations and in the presence of 0.1 m*M* EGTA; (C) casein phosphatase with (○) 0.1 m*M* EGTA or (●) 0.1 m*M* EGTA plus 10 m*M* Mg^{2+}.

inhibited by protamine and almost doubled by heparin (Fig. 2). This indicates that two distinct isozymic forms of phosphatase type 2A were efficiently and easily separated by this method.

The phosphatase activity in the 0.5 *M* NaCl eluate could be ascribed to a type 1 enzyme according to standard classification criteria, *e.g.*, inhibition by inhibitor proteins I_1 and I_2 and okadaic acid [4], inhibition by heparin and protamine (Fig. 2) and preferential dephosphorylation of the β-subunit of phosphorylase kinase [4]. As 0.5 *M* NaCl inhibited type 1 activity by about 50%, the salt concentration necessary for elution was reduced in subsequent experiments; 0.3 *M* NaCl was found to be sufficient for quantitative elution of this phosphatase type 1 activity from heparin–Sepharose (data not shown). None of the other protein phosphatases was affected by 0.3 *M* NaCl and inhibition of the type 1 enzyme was reduced to 20%. Hence dialysis of the fractions which resulted in complete removal of salt inhibition could be omitted in daily routine application. The heparin–Sepharose chromatography resulted in a 100-fold purification of the type 1 phosphatase. However, on silver-stained sodium dodecyl sulphate gels more than 20 discrete protein bands were clearly visible, indicating the need for further purification.

Using phosphocasein as a substrate in the presence of 10 m*M* Mg^{2+}, a single peak of casein phosphatase activity was detected in the flow-through fractions. Dephosphorylation of casein, in contrast to phosphorylase *a*, had an absolute requirement for Mg^{2+}, indicating that it was indeed catalysed by a type 2C protein

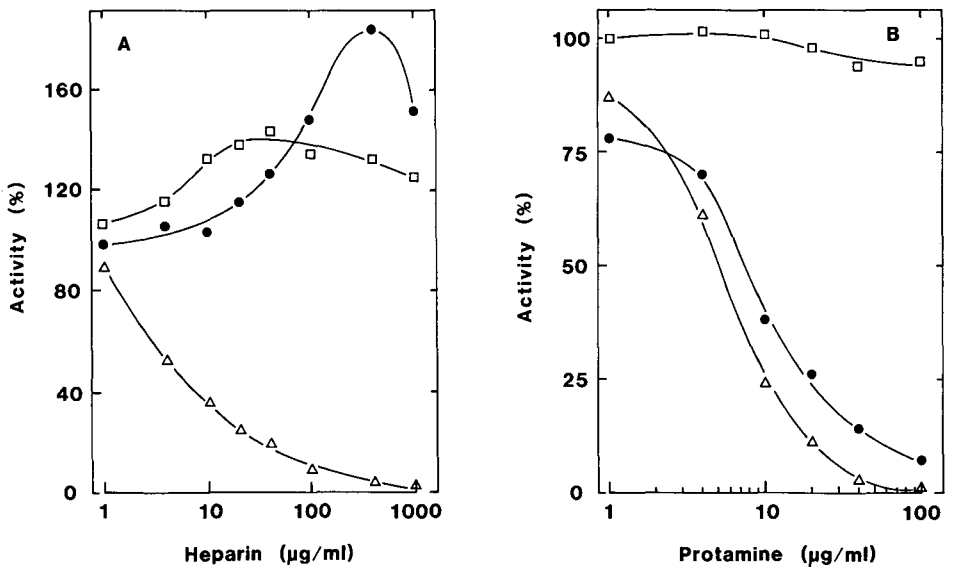

Fig. 2. Effect of (A) heparin and (B) protamine on phosphorylase phosphatase activities from the solubilized ciliary membranes of *Paramecium*. Flow-through fractions from (□) heparin–Sepharose, (●) 0.1 *M* NaCl and (△) 0.5 *M* NaCl eluates were assayed. Salt-containing fractions were dialysed prior to testing. Activities are expressed as a percentage of values obtained in the absence of heparin and protamine, respectively. Assays were carried out in the absence of Mg^{2+} to avoid interference from type 2C enzyme activity.

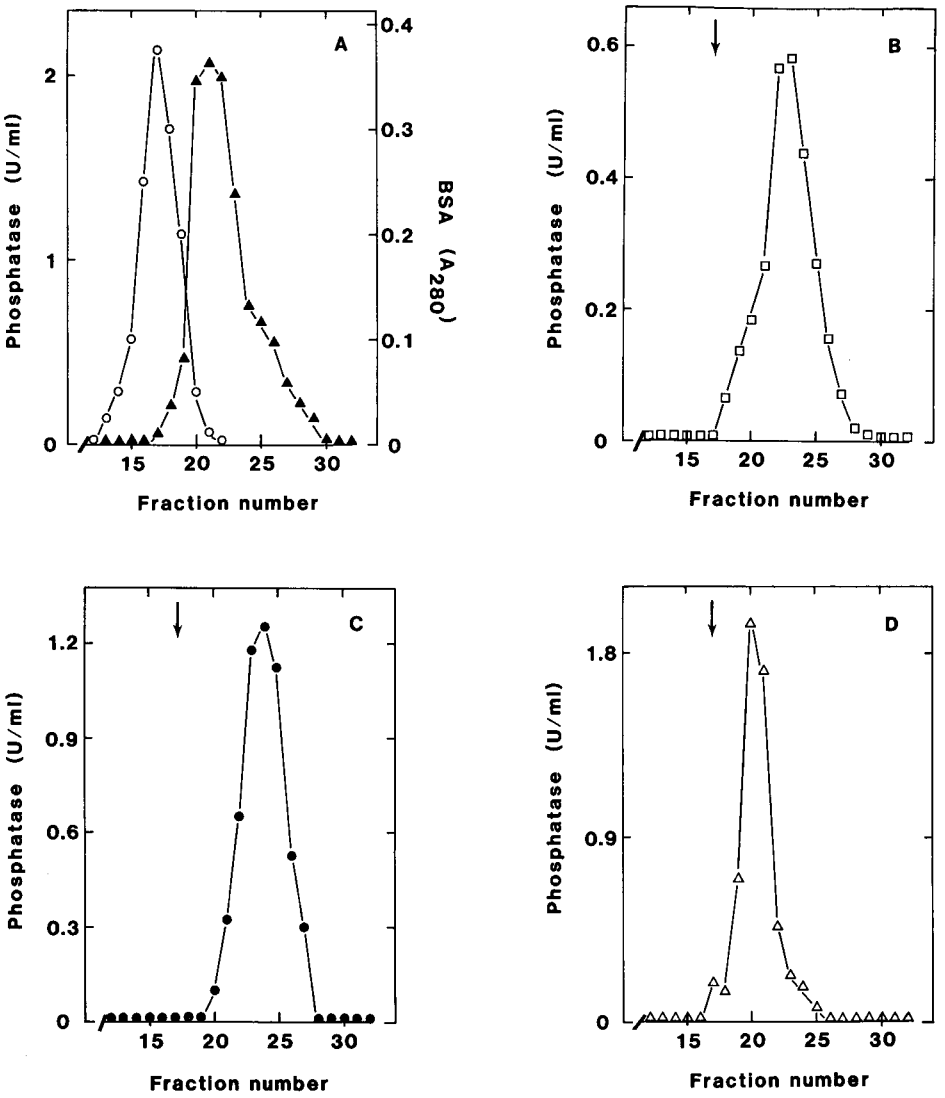

Fig. 3. Gel filtration of ciliary protein phosphatases on Superose 6. The column (23 ml) was equilibrated and run with 20 m*M* Tris–HCl (pH 7)–0.1 m*M* EGTA–0.1% (v/v) 2-mercaptoethanol–0.1 m*M* phenylmethylsulphonyl fluoride–1 m*M* benzamidine at a flow-rate of 0.3 ml/min. Fractions of 0.5 ml were collected. Sample input volume, 0.5 ml. (A) Chromatography of (○) bovine serum albumin (66 000 dalton) and (▲) catalytic subunit of protein phosphatase type 1 isolated from rabbit liver (37 000). The marker proteins were detected by absorbance at 280 nm and phosphorylase phosphatase activity, respectively. The arrows in B–D denote the position of bovine serum albumin. In B–D fractions from deoxycholate-solubilized ciliary phosphatases after chromatography on heparin–Sepharose (see Fig. 1) were applied to Superose 6. (B) Heparin–Sepharose flow-through; (C) heparin–Sepharose 0.1 *M* NaCl eluate; (D) heparin–Sepharose 0.5 *M* NaCl eluate.

phosphatase. No type 2C activity was detectable in either the 0.1 or 0.5 *M* NaCl eluates (Fig. 1C).

Gel filtration of heparin–Sepharose fractions on Superose 6

The molecular mass of the free catalytic subunits from mammalian protein phosphatases types 1 and 2A are almost identical, *i.e.*, 35 000 and 37 000 dalton, respectively [9]. The size of the holoenzymes, in contrast, is much larger (> 150 000 dalton) [9].

To determine the molecular mass of solubilized ciliary phosphorylase phosphatases from *Paramecium*, aliquots of those fractions with the highest activity from heparin–Sepharose flow-through and 0.1 and 0.5 *M* NaCl eluates were directly applied to Superose 6, respectively. For calibration, bovine serum albumin was run on the same column under otherwise identical conditions (Fig. 3A). Further, to compare directly the chromatographic behaviour of the protozoan enzymes with those from mammalia n tissue, the catalytic subunit of phosphatase type 1 from rabbit liver was also passed through the same column (Fig. 3A).

All phosphorylase phosphatases originating from *Paramecium* cilia chromatographed on Superose 6 at positions corresponding to molecular masses of 30 000–45 000 dalton (Fig. 3B–D). The type 2A-like phosphatase activity from heparin–Sepharose flow–through reproducibly revealed a pronounced shoulder on the ascending part of its elution profile on Superose 6 (Fig. 3B). This effect may be due to micelle formation, as deoxycholate used for solubilization of membraneous phosphatases is not retained by heparin–Sepharose and was, therefore, applied to Superose 6 concomitantly with the phosphatase present in the heparin–Sepharose flowthrough fractions. The other type 2A-like phosphatase present in the 0.1 *M* NaCl eluate from heparin–Sepharose no longer contained any deoxycholate and chromatographed on Superose 6 as a sharp and symmetrical peak (Fig. 3C). *Paramecium* type 1 phosphatase present in the heparin–Sepharose 0.5 *M* NaCl fractions eluted at the same position on Superose as did the catalytic subunit from rabbit liver type 1 phosphatse (compare Fig. 3A and D).

So far, heparin–Sepharose has been established for the separation of mammalian types 1 and 2A protein phosphatases. It is commonly used in the presence of 0.1 *M* NaCl [11,12]. As is obvious from the data presented here, running heparin–Sepharose in the absence of NaCl prior to salt step elution results in the separation of two phosphatases that otherwise would have coeluted in the 0.1 *M* NaCl flow-through. Therefore, this procedure seems to be very valuable for the efficient and rapid separations of closely related Ser/Thr–protein phosphatases. Further studies with phosphatases from sources other than *Paramecium* cilia will be necessary in order to clarify whether subtypes of 2A-like phosphatase are ubiquituous in nature.

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Isolation and purity determination of a glycoprotein elicitor from wheat stem rust by medium-pressure liquid chromatography

B. BEISSMANN* and H. J. REISENER
RWTH Aachen, Institut für Biologie III, Worringerweg, D-5100 Aachen (F.R.G.)

ABSTRACT

Previously the isolation of a single glycoprotein (pgt-elicitor) from cell walls of the phytopathogenic fungus *Puccinia graminis* f.sp. *tritici* Erics. & Henn., which elicits defence reactions in wheat leaves was described. The apparent molecular mass of this compound, isolated via concanavalin A affinity chromatography and anion-exchange chromatography, was 67 000 as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. A scaled-up procedure for purifying larger amounts of fungal elicitor based on anion-exchange fast protein liquid chromatography (FPLC) with Q-Sepharose Fast Flow medium has now been developed. The yield of pure elicitor was further increased by the use of the volatile buffer ammonium carbonate for eluting the bound glycoproteins. Analytical size-exclusion FPLC supplemented gel electrophoretic results by quantifying small amounts of inactive components contaminating the most active elicitor fraction. Further, purity determination by FPLC provided evidence of well defined complex formation leading to co-migration of elicitor-associated glycoproteins during anion-exchange chromatography. The glycoprotein complexes were separated during size-exclusion FPLC at lowered pH (100 m*M* acetic acid). Thus, size exclusion FPLC resulted in final purification of the pgt-elicitor.

INTRODUCTION

Active disease resistance in plants involves inducible defence mechanisms, such as accumulation of chemical (phytoalexin antibiotics) [1,2] and structural barriers (lignin-like material or callose) [3] or increasing activities of cell wall-degrading enzymes (chitinase and β-1–3-glucanase) [4], which block infection by a potential pathogen. Defence reactions as induced by pathogen invasion can be simulated by molecules termed elicitors. A wide range of molecules of fungal or bacterial origin such as fatty acids, polysaccharides, glycoproteins and enzymes [5] have been described as elicitors in different host–parasite interactions.

Wheat plants highly resistant to the attack from the fungal pathogen *Puccinia graminis* f.sp. *tritici* Erics & Henn., which causes wheat stem rust disease, are characterized by the so-called hypersensitivity reaction. This reaction consists in rapid lignification and subsequent cell death in infected leaf tissue, thus preventing further fungal growth [6,7]. Apparently, phytoalexins are not involved in the hypersensitivity reaction of wheat plants.

A glycoprotein fraction isolated from germ tubes of *Puccinia graminis* f. sp. *tritici-uredospores* elicits symptoms in wheat leaves similar to those observed after rust

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infection [8–10]. Fractionation of this material by affinity chromatography using concanavalin A (Con A)-Sepharose, followed by anion-exchange fast protein liquid chromatography (FPLC), yielded a highly pure Con A binding glycoprotein elicitor (pgt-elicitor) with a relative molecular mass of about 67 000 dalton [11]. The active site of the elicitor consists of carbohydrate structures [11] and is mainly composed of galactose (50%) and mannose (47%). The inactive peptide portion represents 7% of the glycoprotein only. Purification of larger amounts of this glycoprotein has been a prerequisite for the evaluation of structural requirements for elicitor activity in more detail. The aim of this work was therefore to scale up the purification of the pgt-elicitor. In addition, the advantages and applications of size-exclusion FPLC *versus* sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) for purity determination of glycoproteins are discussed.

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade or higher quality, if not stated otherwise.

Nitrocellulose membranes of 0.45 μm pore size were purchased from Schleicher & Schüll (Dassel, F.R.G.). Acetic anhydride, betaine, Con A, fish gelatin, methyl α-D-mannopyranoside, protein standards, sodium dodecyl sulfate, taurine and trifluoroacetic acid were supplied by Sigma (F.R.G.), anthrone, acetic acid, ammonium carbonate, bisacrylamide, ethanol, glutardialdehyde, glycerol, hydrochloric acid, phosphoric acid, polyacrylamide, silver nitrate, sodium carbonate, sodium chloride and tris(hydroxymethyl)aminomethane (Tris) from Merck (Darmstadt, F.R.G.) and Ammonium peroxodisulphate and horseradish peroxidase from Serva (Heidelberg, F.R.G.).

The FPLC equipment consisted of a GP-250 gradient programmer, two P 500 pumps, a Uvicord II Model 2238, a Model 2210 two-channel recorder, and a Frac-100 fraction collector (Pharmacia–LKB, Uppsala, Sweden). All chromatographic media including prepacked columns of Mono Q HR 5/5, Superose 12 HR 10/30 and the PD 10 desalting column and laboratory-filled columns of Q-Sepharose Fast Flow HR 10/10 and Con A-Sepharose 4 B were obtained from Pharmacia–LKB.

Extraction of glycoproteins

Elicitor-active glycoproteins were solubilized from germ tube walls of *Puccinia graminis* using a published method [8,9]. The protein concentration of this crude elicitor preparation (CEP) was determined according to Bradford [12]; the carbohydrate content was determined by the anthrone method [13].

Assays for elicitor activity

Samples to be tested for elicitor activity (50 μl) were injected into the intercellular spaces of five wheat primary leaves using a hypodermic syringe [14]. Growth conditions for wheat have been described previously [14]. Infiltrated areas of the primary leaves were cut off 24 h later, frozen in liquid nitrogen and tested for phenylalanine–ammonia–lyase (PAL) activity [15]. Dose–response curves were measured to determine the specific elicitor activity, which was defined as the specific

enzyme activity induced per gram of elicitor carbohydrates (μkat kg^{-1} protein g^{-1} carbohydrate) [11]. The extent of lignification was assessed microscopically by observing yellow autofluorescence [11] or positive phloroglucinol staining [16] of affected cells.

Affinity chromatography with Con A Sepharose

A column of immobilized Con A (Con A-Sepharose 4 B, 5 × 1 cm I.D.) was equilibrated with 20 m*M* Tris–HCl (pH 7.4) containing 0.5 *M* NaCl and 1 m*M* each of $MgCl_2$, $MnCl_2$ and $CaCl_2$ (starting buffer). The CEP (3–5 mg glucose equivalents), dissolved in 3 ml of the same buffer, was applied to the column. Con A-Sepharose was eluted successively with 50 ml of starting buffer, then with 0.2 *M* methyl α-D-mannopyranoside in starting buffer. The absorbance was recorded at 280 nm. Fractions of 2 ml were pooled. All buffers contained 0.1% *n*-butanol to prevent microbial contamination. Con A-binding glycoproteins were identified in a fast dot blot assay. Samples of 10 μl were dotted on a nitrocellulose sheet and stained as described [11].

Gel electrophoresis, blotting and staining procedures

Glycoproteins were separated by SDS-PAGE using 16-cm 12.5% polyacrylamide gels and the Laemmli buffer system [17]. For lectin staining, proteins were electroblotted to nitrocellulose [18] using a transblot cell (Bio-Rad Labs., Munich, F.R.G.). The transfer buffer contained 25 m*M* Tris and 192 m*M* glycine (pH 8.3) with 20% methanol (v/v), and transfer was carried out at 400 mA for 2 h with cooling to 4°C. Blots were first incubated in Tris-buffered saline [50 m*M* Tris–HCl–200 m*M* NaCl (pH 7.9)] supplemented with 0.45% of fish gelatin. Con A-binding glycoproteins were detected by Con A–peroxidase staining as described [19].

For more sensitive detection, glycoprotein samples were run in 8–25% gradient gels using a PhastGel separation system (Pharmacia–LKB). Proteins were stained with silver [20].

Gas chromatographic determination of carbohydrate content

Monosaccharides of the carbohydrate part were released by acid hydrolysis of the pgt-elicitor. Alditol acetate derivatives of the products of hydrolysis were prepared by the method of Jones and Albersheim [21] and separated by gas chromatography as described [11].

Anion-exchange FPLC with Mono Q

The separation of glycoproteins was carried out by FPLC with a prepacked Mono Q HR 5/5 anion-exchange column. Elution was performed at a flow-rate of 1 ml min^{-1} using two buffers. Buffer A contained 25 m*M* Tris–HCl (pH 8.5) with 4% (w/v) of betaine or tautine to minimize ionic interactions of molecules [22]. Buffer B contained 1 *M* NaCl in the same buffer as A. Eight minutes after injection of the sample, the ratio of buffer B to A was increased with a linear gradient to 17% in 16 min. Strongly binding (glyco)proteins were finally eluted with 100% buffer B. The fractions were desalted on prepacked PD 10 gel filtration columns.

Anion-exchange FPLC with Q-Sepharose Fast Flow

Crude elicitor preparation (50 mg in 50 ml) was injected onto a laboratory-packed Q-Sepharose Fast Flow HR 10/10 column equilibrated with 20 m*M* $(NH_4)_2CO_3$ (pH 9.1). The column was washed for 60 min at a flow-rate of 1 ml min^{-1} and eluted with a 220-min gradient from 20 to 200 m*M* $(NH_4)_2CO_3$. Fractions were collected from different runs. Desalting could easily be achieved by lyophilizing the volatile buffer components.

In order to regenerate the Q-Sepharose Fast Flow gel, the column was washed with 50 ml of distilled water (flow-rate 1 ml min^{-1}) followed by a 10-ml wash with 2 *M* NaOH (flow-rate 0.5 ml min^{-1}) and a 10 ml wash with 1 *M* NaCl (flow-rate 0.5 ml min^{-1}). Finally, the column was re-equilibrated with 10 m*M* $(NH_4)_2CO_3$.

Size-exclusion FPLC

Fractionation of glycoproteins by size-exclusion FPLC was conducted on Superose 12 HR 10/30. The column, equilibrated with 100 m*M* acetic acid, was calibrated with the standard proteins aldolase (molecular weight, M_r 146 000), bovine serum albumin (BSA) (M_r 66 000) and carbonic anhydrase (M_r 29 000).

Samples of 200 μl containing up to 60 μg of glycoprotein were applied to the column per run. Size-exclusion FPLC was performed at a flow-rate of 0.3 ml min^{-1}.

RESULTS AND DISCUSSION

Affinity chromatography

Many proteins of the crude cell wall elicitor preparation (CEP) eluted unbound from the Con A-Sepharose column as indicated by an increased absorbance at 280 nm, but this material was inactive in bioassays for elicitor activity. Active molecules were displaced by 0.2 *M* methyl α-D-mannopyranoside in Con A buffer. Negative staining of the unbound material in a spot dot test after Con A–peroxidase overlay and chloronaphthol–hydrogen peroxide addition confirmed the binding activity of the column-immobilized lectin. Gel electrophoresis under denaturing conditions of the Con A binding fraction and staining revealed several glycoprotein bands ranging in molecular weight from 20 000 to 95 000 dalton [11].

For further purification, fractions were desalted by extensive dialysis against distilled water or repeated gel filtration (up to three times) on PD 10 desalting columns. Gas chromatographic carbohydrate determination showed that a single gel filtration step was ineffective for complete removal of methylmannosides. Difficulties in separating phytotoxic glycopeptides and methylmannosides were also described by Lazarovits *et al.* [23]. We assume that aggregation between methyl α-D-mannopyranoside and the glycoprotein elicitor interferes with the separation.

Affinity chromatography followed by desalting procedures resulted in a considerable loss of glycoproteins, amounting to more than 50% of the starting material.

Anion-exchange FPLC with Mono Q

Con A binding glycoproteins were further separated by anion-exchange FPLC on a Mono Q HR 5/5 column. Elution of bound material with NaCl resulted in four main peaks, which were sharply baseline separated (Fig. 1). Peaks C and D both contained molecules with high elicitor activity, whereas the unbound material was inactive and the material of peaks A and B exhibited only low activity.

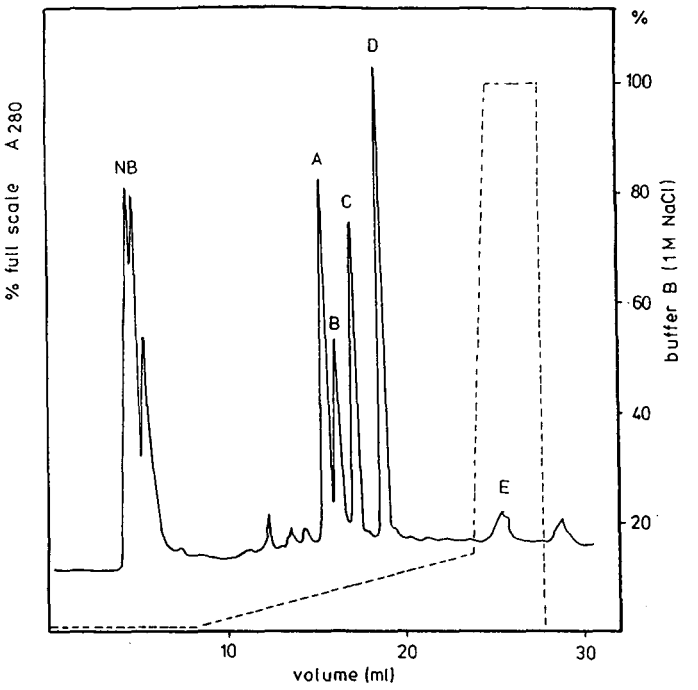

Fig. 1. Anion-exchange FPLC of the CEP isolated from germ tube walls of *Puccinia graminis* uredosporelings. 6 mg of CEP in 10 ml of Tris–HCl buffer (pH 8.5) containing 4% of betaine were applied to a Mono Q HR 5/5 column. Elution was performed at a flow-rate of 1 ml min^{-1} with a linear gradient of 0–17 m*M* NaCl. Peak C exhibited very high elicitor activity and was referred to as pgt-elicitor.

SDS-PAGE showed the most active fraction C to consist of a single glycoprotein with a molecular weight of 67 000. This glycoprotein also appeared in fraction D and to a minor extent in fractions A and B. The 67-kilodalton glycoprotein was the only active part of all fractions, as proved by re-extraction from polyacrylamide gel [11]. Fractions A and B additionally contained at least four low-molecular-weight and one high-molecular-weight glycoprotein. We also detected some faintly stained indistinct bands of low-molecular-weight components in fractions C and D. This effect was not observed in previous studies [11] and may be due to a slightly changed quality of elicitor preparation, as indicated by the fact that now all binding (glyco)proteins eluted at lower NaCl concentration.

The finding that all fractions contained the active glycoprotein, although the peaks were baseline separated, suggested that glycopeptide interaction led to stable complexes co-migrating during anion-exchange chromatography. However, there is no simple subunit relationship, indicating a more complex association of different glycoproteins. The assumption of complex formation is confirmed by the finding of smaller elicitor-active glycoproteins only after gel filtration under denaturing conditions as described previously [11]. In the absence of SDS, the active glycoproteins formed aggregates larger than 130 000 dalton [11]. Complex formation of glycopeptides was also detected on fractionating phytotoxic glycopeptides of *Stemphylium botryosum* [24].

CEP was directly subjected to FPLC-associated Mono Q anion-exchange chromatography. The elution profile and the distribution and gel electrophoretic behaviour of the active fractions showed no difference to the anion-exchange FPLC of prepurified glycoproteins (Fig. 1). However, after performing 3–5 runs each with 6 mg of CEP dissolved in 10 ml of separation buffer, the back-pressure of the column increased considerably and the elution quality decreased, yielding broader peaks. Neither washing with salts, acids and bases as described [25] nor changing prefilters resulted in regeneration of the column. The separation capacity could only be restored by removal of the Mono Q gel, incubating it in 50% (v/v) methanolic 1 *M* hydrochloric acid for 1 h at room temperature and finally refilling the column. The procedure is time consuming and may shorten the lifetime of the column.

Semi-preparative anion-exchange FPLC

The aim of isolating larger amounts of glycoprotein elicitor was achieved by performing anion-exchange FPLC with Q-Sepharose Fast Flow medium packed in an HR 10/10 column. Without any kind of prepurification the chromatographic separation of CEP on this column led to an elution profile comparable to that recorded from FPLC with Mono Q (Fig. 2). Fraction C again contained the most active material, as shown by measuring dose–response curves of PAL-inducing activity. Interestingly, after SDS-PAGE all active fractions showed the same glycoprotein pattern, with the exception of fraction C, which contained large amounts of the pgt-elicitor together with a small portion of smaller glycopeptides (Fig. 3). The yield of

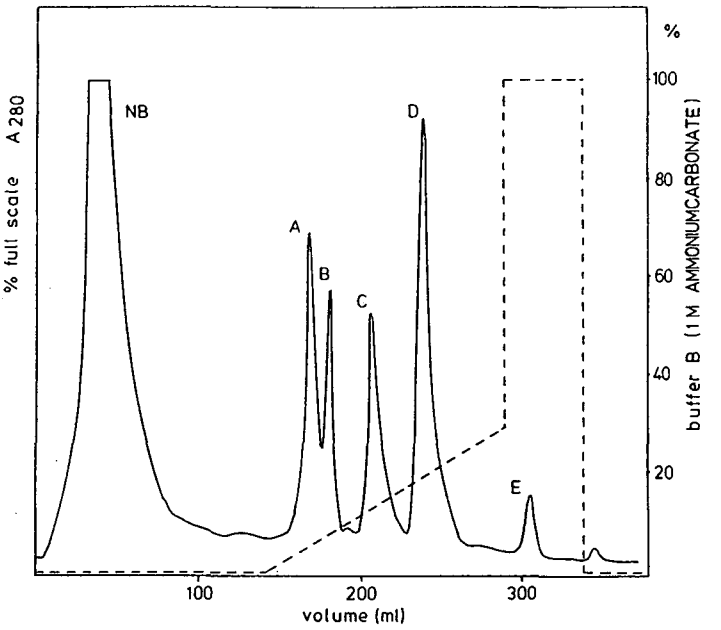

Fig. 2. Anion-exchange FPLC of CEP isolated from mycelial walls of *Puccinia graminis* uredosporelings. 12 mg of CEP in 20 ml of $(NH_4)_2CO_3$ buffer were applied to a Q-Sepharose Fast Flow HR 10/10 column. Elution was performed at a flow-rate of 1 ml min^{-1} with a linear gradient of 20–200 m*M* $(NH_4)_2CO_3$.

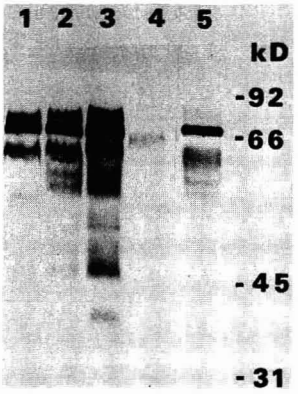

Fig. 3. Electroblot of glycoproteins from 12.5% SDS-PAGE of elicitor-active fractions (A, B, C, D) obtained by anion-exchange FPLC on a Mono Q HR 5/5 column (Fig. 2). Glycoprotein bands were stained with Con A–peroxidase. 0.5 μg glucose equivalents of each fraction were applied to the gel. Lanes: 1 = fraction D; 2 = fraction A; 3 = fraction B; 4 = fraction E; 5 = fraction C. kD = kilodalton.

highly active elicitor fraction obtained by the scaled-up procedure was further increased by using $(NH_4)_2CO_3$ as eluent. Without any loss of active material due to desalting procedures, the volatile buffer compound was removed by freeze-drying. More than 1.5 mg of purified pgt-elicitor were obtained by purifying CEP containing 18 mg glucose equivalents.

Mono Q versus *Q-Sepharose FPLC*

Rechromatography of fraction C separated by Q-Sepharose Fast Flow medium on the Mono Q column resulted in a large peak corresponding to the pgt-elicitor and some very small peaks corresponding to fractions A, B and D (data not shown). Material taken from the sample of fraction C after FPLC with the Mono Q column was reapplied to the Monio Q column. During the following separation only one peak, C, was recorded. Obviously, FPLC on the Mono Q column yielded elicitor of higher purity than FPLC on a Q-Sepharose Fast Flow column.

Separation of the carbohydrate content by gas chromatography pointed to an additional advantage of the new semi-preparative purification method. The monosaccharide composition of the most active fraction C consisted of 22% mannose, 77% galactose and traces of N-acetylglucosamine, but less than 1% of glucose was detected. Thus, it became evident that the 3% of glucose found in fraction C after the two-step purification [11] was a contaminant from polyglucose separation matrices, *e.g.*, the Con A Sepharose or the PD 10 desalting columns. The enrichment of galactose content from 47% to 77% achieved by Q-Sepharose Fast Flow-FPLC indicates another difference in the purification.

Complex formation of the pgt-elicitor

CEP was separated by size-exclusion FPLC with Superose 12 into seven well resolved peaks corresponding to molecular masses from 300 to 1 kilodalton (Fig. 4). The amount of each eluting (glyco)protein could be calculated from the peak area and

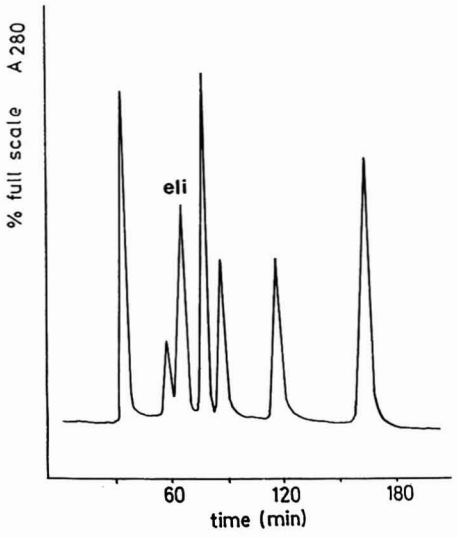

Fig. 4. Analytical size-exclusion FPLC of CEP on a Sepharose 12 HR HR10/30 column. For application, CEP was dissolved in 200 μl of 100 m*M* acetic acid. Elution was performed at a flow-rate of 0.3 ml min^{-1} with 100 m*M* acetic acid. Eli = pgt-elicitor.

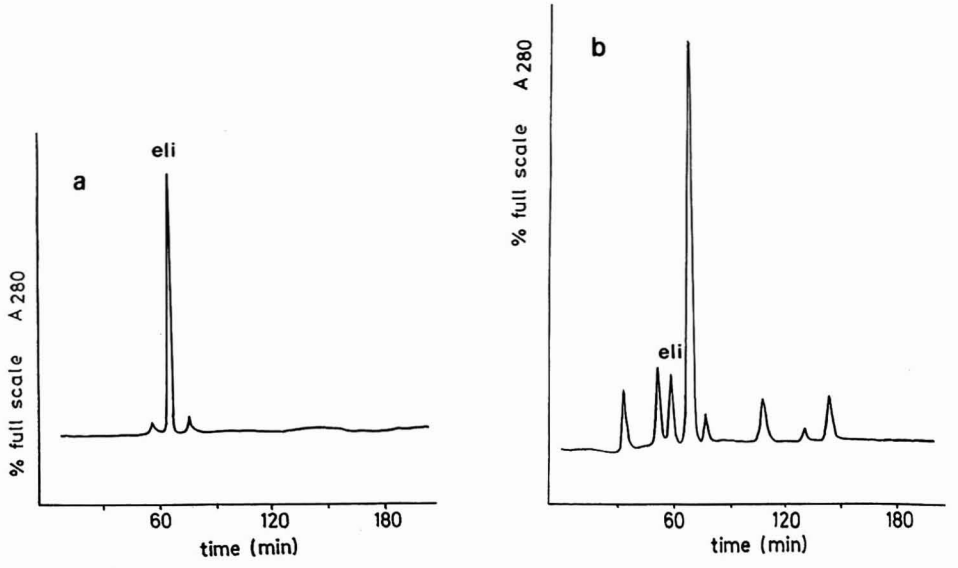

Fig. 5. (a) Analytical size-exclusion FPLC of fraction C exhibiting high elicitor activity, which was isolated by anion-exchange FPLC on a Mono Q HR 5/5 column (Fig. 2). 60 μg of the glycoprotein fraction A were dissolved in 200 μl of 100 m*M* acetic acid and applied to a Superose 12 HR 10/30 column. Elution was performed at a flow-rate of 0.3 ml min^{-1} with 100 m*M* acetic acid. (b) Analytical size-exclusion FPLC of fraction B exhibiting low biological activity, which was isolated by anion-exchange FPLC on a Mono Q HR 5/5 column (Fig. 1). A 60-μg amount of the glycoprotein fraction B was dissolved in 200 μl of 100 m*M* acetic acid and applied to a Superose HR 10/30 column. Elution was performed at a flow-rate of 0.3 ml min^{-1} with 100 m*M* acetic acid. Eli = pgt-elicitor.

shown to be highly reproducible but varying with each elicitor preparation. In order to prove whether the high-molecular-mass compounds were due to aggregation of glycoproteins, the material of CEP higher than 300 000 dalton was sampled over several runs and rechromatographed on a Superose 12 column eluted with 100 m*M* acetic acid. As expected, the complexes dissociated, yielding four new peaks including one peak corresponding to the pgt-elicitor (data not shown). Hence, the four glycoproteins with molecular weights from about 150 000–20 000 dalton obviously interact in a defined manner underlying a complex dissociation equilibrium.

Determination of purity

The results clearly demonstrate a correlation between increasing purity and specific elicitor activity during anion-exchange chromatography. The fractionation of CEP on Mono Q yielded a sharply separated peak C, indicating high purity of the pgt-elicitor (Fig. 1). This result did not correlate with gel electrophoretic analysis, indicating contamination by complex-forming glycoproteins as described above. Therefore, we additionally checked the purity of the pgt-elicitor isolated via anion-exchange FPLC by size-exclusion FPLC.

Under the conditions described above, the Mono Q anion-exchange FPLC fractions A, B, C and D were subjected to size-exclusion FPLC on a prepacked Superose 12 column. Interestingly, the elicitor activity of the fractions was strongly correlated with the peak height of a glycoprotein eluting at the same time as BSA (M_r 66 000). This glycoprotein was identical with the major component of fraction C and was therefore identified as pgt-elicitor (Fig. 5a). Fraction C additionally contained two minor (glyco)protein peaks with relative molecular masses of *ca.* 150 000 and 45 000 dalton. The latter might correspond to the broad band of low molecular weight glycoprotein(s) faintly stained below the pgt-elicitor after SDS-PAGE (Fig. 3, lane 5).

The FPLC Mono Q fractions A and B showed only small peaks corresponding to the pgt-elicitor, but additionally some larger peaks of other (glyco)proteins (Fig. 5b). These results confirm the conclusion that all active fractions share the pgt-elicitor as the only active compound. The elution profiles of all anion-exchange fractions during size-exclusion FPLC were highly reproducible. Therefore, we assume that specific complex formation occurs between the pgt-elicitor and other glycoproteins.

For evaluation of glycoprotein purity, size-exclusion FPLC at low pH was evidently superior to SDS-PAGE, as the relative amounts of different glycoproteins associated with the pgt-elicitor could be quantified. Interestingly, molecular weight determination of the pgt-elicitor by SDS-PAGE and size-exclusion FPLC led to nearly identical results. Often, the molecular size of glycoproteins is underestimated by SDS-PAGE and overestimated by size-exclusion chromatography [26].

Final purification of the pgt-elicitor

Pgt-elicitor was purified by size-exclusion FPLC on the analytical Superose 12 column. When subjected to PhastSystem SDS-PAGE, only a single protein (M_r 67 000) was stained by ultrasensitive silver staining (Fig. 6). In contrast to both kinds of anion-exchange FPLC, size-exclusion FPLC obviously resulted in maximum purification of the pgt-elicitor. However, as described above, pgt-elicitor formed large amounts of high-molecular-mass complexes which remained partly unresolved during size-exclusion FPLC, thus probably contributing to a considerable loss of active

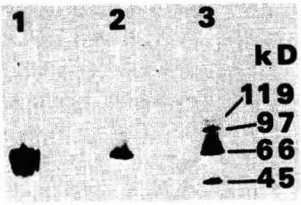

Fig. 6. SDS-PAGE of pgt-elicitor isolated via anion-exchange and size-exclusion MPLC. The (glyco)-protein fractions were separated by the PhastSystem in a gradient gel of 8–25% polyacrylamide and silver stained. Lanes: 1 = pgt-elicitor purified by Mono Q anion-exchange MPLC; 2 = pgt-elicitor purified by Superose 12 size-exclusion MPLC; 3 = molecular weight markers (kD = kilodalton).

material. Therefore, we do not recommend the scaling up of size-exclusion FPLC as a semi-preparative procedure for optimized elicitor purification.

Alternatively, elicitor purification by anion-exchange FPLC with Q-Sepharose Fast Flow medium presents an abundant source of highly active material. Further studies will concern the cleavage of the glycoprotein and the isolation of active molecular structures, thus leading to a final purification of elicitor-active components. As only the pgt-elicitor exhibits elicitor activity [11], slight contamination of inactive material will not affect studies on the physiological effects of the elicitor. Greater amounts of highly active material will also be helpful in continuing studies of the characterization of elicitor binding sites as putative receptor molecules in wheat plants [27].

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Application of high-performance liquid chromatography to the purification of the putative intestinal peptide transporter

WERNER KRAMER*, FRANK GIRBIG, ULRIKE GUTJAHR and IRINA LEIPE
Hoechst Aktiengesellschaft, Postfach 800320, 6230 Frankfurt am Main (F.R.G.)

ABSTRACT

A membrane protein of relative molecular mass (M_r) 127 000 was identified by photoaffinity labelling as (a component of) the uptake system for small peptides and β-lactam antibiotics in rabbit small intestine. This binding protein is a microheterogeneous glycosylated integral membrane protein which could be solubilized with non-ionic detergents and enriched by lectin affinity chromatography on wheat germ lectin agarose. For the final purification of this protein and separation from aminopeptidase N of M_r 127 000, fast protein liquid chromatography (FPLC) was used. Gel permeation, hydroxyapatite and hydrophobic interaction chromatography were not successful for the purification of the 127 000-dalton binding protein. By anion-exchange chromatography on a Mono Q column with either Triton X-100 or *n*-octylglucoside as detergent, a partial separation of the 127 000-dalton binding protein from aminopeptidase N was achieved. By cation-exchange chromatography on a Mono S HR 5/5 column at pH 4.5 using Triton X-100 as detergent also only a partial separation from aminopeptidase N could be achieved. If, however, Triton X-100 was replaced with *n*-octylglucoside, the binding protein for β-lactam antibiotics and small peptides of M_r 127 000 could be completely separated from aminopeptidase N. These results indicate that Triton X-100 should be avoided for the purification of integral membrane proteins because mixed protein-detergent micelles of high molecular weight prevent a separation into the individual membrane proteins. The putative peptide transport protein was finally purified by rechromatography on Mono S and was obtained more than 95% pure as determined densitometrically after sodium dodecyl sulphate gel electrophoresis. By application of FPLC even microheterogeneous membrane glycoproteins from the intestinal mucosa can be purified to such an extent that a sequence analysis and immunohistochemical localization with antibodies prepared from the purified protein is possible.

INTRODUCTION

The intestinal uptake of orally active α-amino-β-lactam antibiotics occurs by the transport system for small peptides in the brush border membrane of small intestinal enterocytes [1–4]. The uptake of di- and tripeptides and the uptake of α-amino-β-lactam antibiotics is stimulated by an inwardly directed H^+ gradient ($pH_{in} < pH_{out}$ [3–6]), which is generated by the combined action of an ($Na^+ + K^+$)ATPase in the basolateral membrane and an Na^+–H^+ exchanger in the brush border membrane of the enterocyte. By photoaffinity labelling of brush border membrane vesicles from the small intestine of rabbit, rat and pig with photoreactive derivatives of penicillins, cephalosporins and dipeptides, a membrane protein of apparent relative molecular mass (M_r) 127 000 was identified as (a component of) the intestinal peptide uptake system [4,7–9]. The photoaffinity labelling of this polypeptide and the uptake of orally

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active α-amino-β-lactam antibiotics was inhibited by small peptides and β-lactam antibiotics, whereas bile acids, amino acids and hexoses had no effect [4,7–9]. For a further characterization of the molecular structure, the amino acid sequence and the localization of this transporter by immunohistochemistry, highly purified transport protein must be accessible. In this paper we describe the application of fast protein liquid chromatography (FPLC) to the isolation of the putative transport protein responsible for the intestinal absorption of small peptides and β-lactam antibiotics.

EXPERIMENTAL

Materials

[^{3}H]Benzylpenicillin (specific radioactivity 18–31 Ci/mMol) was obtained from Amersham Buchler (Braunschweig, F.R.G.) and [^{14}C(U)]-D-glucose (specific radioactivity 252 mCi/mMol) from NEN (DuPont, Dreieich, F.R.G.). The tissue solubilizer Biolute S and scintillator Quickszint 501 were from Zinsser Analytic (Frankfurt, F.R.G.). Acrylamide, N,N′-methylenebisacrylamide, acrylamide–bisacrylamide premix (5% bisacrylamide), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium peroxodisulphate, Servalytes, Triton X-100, *n*-octyl-β-glucopyranoside and Serva Blue R 250 were obtained from Serva (Heidelberg, F.R.G.). Wheat germ lectin-agarose (WGA-agarose), DEAE-Sephacel and the columns Superose 6 HR 10/30, phenyl-Superose HR 5/5, alkyl-Superose HR 5/5, Mono Q HR 5/5 and Mono S HR 5/5 were obtained from Pharmacia–LKB (Freiburg, F.R.G.). Bio-Gel HT (hydroxyapatite) was from Bio-Rad Labs. (Munich, F.R.G.). N-Acetyl-D-glucosamine was bought from Sigma (Munich, F.R.G.). All other substances were of the highest purity available.

Animals

White rabbits (3–3.5 kg) (Tierzucht Kastengrund, Hoechst, Frankfurt, F.R.G.) were maintained on standard diets and tap water *ad libitum*.

Preparation of brush border membrane vesicles from rabbit small intestine

Brush border membrane vesicles from rabbit small intestine were prepared by the Mg^{2+} precipitation method [10] as described previously [4,6,9]. The final pellet of the vesicle preparation was suspended in the desired volume of 10 m*M* Tris–4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer (pH 7.4)–300 m*M* mannitol using a No. 27 gauge needle. The enrichments of the specific activities of the brush border marker enzymes leucine aminopeptidase N (E.C. 3.4.11.2) and γ-glutamyltransferase (E.C. 2.3.2.2) were 26 ± 5 and 23 ± 4 times, respectively. Protein was determined according to Bradford [11] using the Bio-Rad Labs. kit with bovine serum albumin as protein standard. The enzymatic activities of the marker enzymes were determined using Merckotest kits 3359 an 3394 (Merck, Darmstadt, F.R.G.). (One unit of aminopeptidase N is defined as the release of 1 μmol of *p*-nitroaniline from L-leucyl-*p*-nitroanilide and 1 unit of γ-glutamyltransferase as the release of 1 μmol of *p*-nitroaniline from L-γ-glutamyl-*p*-nitroanilide.) The quality of the vesicles was measured by the Na^+-dependent uptake of [^{14}C]D-glucose; the overshoot after 15 s of incubation was 25–40 compared with equilibrium. The vesicles were stored in liquid nitrogen for up to 4 weeks without loss of transport or enzymatic activity.

Purification of the intestinal peptide transport protein

All chromatographic steps were performed with a Pharmacia–LKB FPLC system (two P-500 high-precision pumps, LCC-500 PLUS liquid chromatography controller, P-1 peristaltic pump, Uvicord S UV monitor and SuperRac fraction collector). All buffers were filtered through RC 58 membrane filters (0.2 μm, 50 mm diameter; Schleicher & Schüll, Dassel, F.R.G.) and degased by bubbling with helium for 1 h. From all fractions of the chromatographic runs samples were removed for the determination of the enzymatic activity of aminopeptidase N and for the determination of the protein composition of the individual fractions by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Solubilization of brush border membrane vesicles

Brush border membrane vesicles (5–8 mg of protein, 150–300 μl) were solubilized for 30 min at 4°C either by addition of 1 ml of 1% (w/v) Triton X-100 solution or by addition of 1 ml of a 1% (w/v) *n*-octylglucoside solution. After 30 min of incubation an additional 1 ml of the corresponding detergent solution was added. The non-solubilizable material was removed by centrifugation at 48 000 *g* for 30 min.

WGA–agarose chromatography

The resulting supernatants containing solubilized brush border membrane proteins were loaded onto affinity columns (1 cm diameter containing 5 ml of WGA–agarose) equilibrated either with 10 m*M* Tris–HCl buffer (pH 7.4)–100 m*M* NaCl–0.1% (w/v) Triton X-100 or with 10 m*M* Tris–HCl buffer (pH 7.4)–100 m*M* NaCl–0.7% (w/v) *n*-octylglucoside. The flow-rate was 0.15 ml/min and the elution of proteins was monitored by ultraviolet absorption at 280 nm. After washing the columns with 19 ml of the corresponding buffers, the adsorbed proteins were eluted with 10 ml of a 100 m*M* solution of N-acetyl-D-glucosamine in the corresponding buffers. Fractions of 1.5 ml were collected. The protein fractions eluted with N-acetyl-D-glucosamine were stored at 4°C.

Hydrophobic interaction chromatography

For hydrophobic interaction chromatography, phenyl-Superose of alkyl-Superose columns were equilibrated with 50–100 m*M* sodium phosphate buffer (pH 7.4)–1 *M* ammonium sulphate containing either 0.03% (w/v) Triton X-100 or 0.7% *n*-octylglucoside. A 0.5-ml volume of the eluate from WGA–agarose chromatography adjusted to 1 *M* ammonium sulphate was applied at a flow-rate of 0.5 ml/min. Elution of adsorbed proteins was performed with a linear decreasing gradient from 1 to 0 *M* ammonium sulphate in 50 m*M* sodium phosphate buffer (pH 7.4) containing either 0.03% (w/v) Triton X-100 or 0.7% (w/v) *n*-octylglucoside in the experiments with Triton X-100 as detergent followed by a linear gradient from 0.03 to 1% Triton X-100.

Gel filtration

The Superose HR 10/30 column was equilibrated with 100 m*M* sodium phosphate buffer (pH 7.0) containing either 0.03% (w/v) Triton X-100 or 0.7% (w/v) *n*-octylglucoside. A 0.3-ml volume of the eluate from WGA–agarose chromatography were applied and elution of protein was performed at a flow-rate of 0.5 ml/min using

100 m*M* sodium phosphate buffer (pH 7.0) containing either 0.03% (w/v) Triton X-100 or 0.7% (w/v) *n*-octylglucoside.

Hydroxyapatite chromatography

Hydroxyapatite preswollen with six volumes of 10 m*M* sodium phosphate buffer (pH 6.8)–0.03% (w/v) Triton X-100 was filled into a column of 1 cm I.D. to a bed height of 30 cm. A 1.5-ml volume of the eluate from WGA–agarose chromatography equilibrated to 10 m*M* sodium phosphate buffer (pH 6.8)–0.03% (w/v) Triton X-100 with the aid of a PD-10 column (Pharmacia–LKB) was applied to the hydroxyapatite column. Elution was performed with a linear gradient from 10 to 400 m*M* sodium phosphate buffer at pH 6.8 with 0.03% (w/v) Triton X-100 as detergent at a flow-rate of 0.15 ml/min.

Anion-exchange chromatography on a Mono Q HR 5/5 column

The Mono Q HR 5/5 column was equilibrated with 10 m*M* Tris–HCl buffer (pH 7.4) containing either 0.03% (w/v) Triton X-100 or 0.7% (w/v) *n*-octylglucoside. A 1–50-ml volume of the eluate from WGA–agarose chromatography was applied with the aid of the 10- or 50-ml superloop sample applicators (Pharmacia LKB). The elution of adsorbed proteins was performed with linear NaCl gradients in the corresponding buffers at a flow-rate of 0.5 ml/min.

Cation-exchange chromatography on a Mono S HR 5/5 column

The Mono S column was equilibrated with 20 m*M* sodium acetate buffer (pH 4.5) containing either 0.03% (w/v) Triton X-100 or 0.7% (w/v) *n*-octylglucoside. The eluates from the WGA–agarose columns were diluted at least five-fold with the equilibration buffers. The pH of the resulting protein solution was adjusted to 4.5, if necessary. Up to 50 ml of protein solution (1.5–3 mg of protein) were applied to the column at a flow-rate of 0.5 ml/min. After loading of the protein solution, a linear gradient from 0 to 100 m*M* NaCl (in 10 ml of buffer) was applied. After elution with 20 ml of 20 m*M* sodium acetate buffer (pH 4.5)–100 m*M* NaCl–0.7% (w/v) *n*-octylglucoside or 0.3% (w/v) Triton X-100, a linear gradient from 100 to 600 m*M* NaCl was applied using 40 ml of the respective buffers. The fractions containing the 127 000-dalton binding protein (analysed by SDS-PAGE) were pooled, diluted five-fold with 20 m*M* sodium acetate buffer (pH 4.5)–0.7% (w/v) *n*-octylglucoside and rechromatographed on a Mono S HR 5/5 column. The adsorbed proteins were eluted with 20 ml of 20 m*M* sodium acetate buffer (pH 4.5)–0.7% (w/v) *n*-octylglucoside with a linear gradient from 0 to 400 m*M* NaCl.

Photoaffinity labelling

Photoaffinity labelling of brush border membrane vesicles was performed as described [4,9,12]. Brush border membrane vesicles were incubated for 2 min at 20°C in the dark with [^{3}H]benzylpenicillin and subsequently the suspension was irradiated at 254 nm for 2.5 min in a Rayonet RPR 100 photochemical reactor (Southern Ultraviolet, Hamden, CT, U.S.A.) equipped with sixteen RPR 2543-nm lamps. Subsequently, 1 ml of ice-cold 10 m*M* Tris–HEPES buffer (pH 7.4)–300 m*M* mannitol–4 m*M* phenylmethylsulphonyl fluoride–4 m*M* iodoacetamide–4 m*M* EDTA was added and the membranes were collected by centrifugation at 48 000 *g* for 30 min. After resuspension the pellets were solubilized with lysis buffer (see below) and submitted to two-dimensional electrophoresis.

SDS-PAGE

Prior to SDS-PAGE the proteins from brush border membrane vesicles and from the different fractions from chromatographic runs were precipitated by a modification of the procedure of Wessel and Flügge [13]. Up to 600 μl of probe were mixed with 600 μl of methanol and 200 μl of chloroform. After intense vortex mixing, the suspensions were centrifuged in 1.5-ml reaction tubes at 15 000 *g* for 5 min. The upper phase was removed and 500 μl of methanol were added. After centrifugation at 15 000 *g* for 5 min, the supernatant was discarded and the precipitated proteins were dried under vacuum and stored at −20°C until analysis by electrophoresis.

The dried protein precipitates were dissolved in 40–80 μl of 62.5 m*M* Tris–HCl buffer (pH 6.8)–2% (w/v) SDS–5% (w/v) 2-mercaptoethanol–10% (v/v) glycerol–0.001% (w/v) bromophenol blue by shaking in a vortex mixer for 1 h at about 30–35°C. After centrifugation at 15 000 *g* for 5 min, the clear supernatants were submitted to discontinuous SDS-PAGE on 0.7 × 200 × 150 mm or 1.5 × 200 × 150 mm slab gels using a Pharmacia–LKB LE 4/2 apparatus. Separation of proteins was performed at a constant voltage of 50 V at 10°C (current 20 mA for 1.5-mm gels). For the determination of molecular masses a mixture of standard proteins (Sigma) was used: myosin (205 000), β-galactosidase (116 000), phosphorylase B (97 400), bovine serum albumin (66 000), ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (24 000), trypsin inhibitor (20 100) and α-lactalbumin (14 200). The gels were fixed in 12.5% (w/v) trichloroacetic acid and subsequently stained with a solution of 0.08% (w/v) Serva Blue R 250–25% (v/v) methanol–8% (v/v) acetic acid. After destaining with several changes of a solution of 25% (v/v) methanol–8% (v/v) acetic acid the gels were stored in 5% (v/v) acetic acid. The gels were photographed and scanned with a CD 50 densitometer (Desaga, Heidelberg, F.R.G.). Gels containing radioactively labelled polypeptides were sliced into 2-mm pieces and submitted to liquid scintillation counting as described [4,9,12,14] or prepared for fluorography.

Two-dimensional gel electrophoresis

Brush border membrane vesicles or dried protein precipitates were dissolved in 50–75 μl of a solution of 2% (w/v) SDS–5% (w/v) 2-mercaptoethanol by vortex mixing at 30°C for 1 h. If necessary the samples were heated at 90°C for 5 min. After cooling (< 10°C), 100–150 μl of lysis solution [9 *M* urea–2% (w/v) Triton X-100–2% (v/v) Servalyte 2-11–5% (w/v) 2-mercaptoethanol] were added. After vortex mixing at 4°C for 30 min, the probes were centrifuged at 48 000 *g* for 30 min. The clear and particle-free supernatants were applied to isoelectric focusing performed in glass tubes (170 mm × 4 mm I.D.) with a gel height of 13 cm. The gels were prepared by polymerization of a freshly prepared solution of 5.5 g of urea, 2 ml of 10% (w/v) Triton X-100, 3.23 ml of water, 0.5 ml of 40% Servalyte 3-10 solution, 400 mg of acrylamide–bisacrylamide premix (5% bisacrylamide) and 25 μl of TEMED with 25 μl of a 10% (w/v) solution of ammonium peroxodisulphate. The polymerizing solution was overlayed with water-saturated isobutanol. After polymerization the tubes were mounted into a Bio-Rad Labs. Model 175 tube cell. A 100-μl volume of lysis buffer applied on the gel surface was carefully overlayed with 200 μl of a solution of 4 *M* urea–2% Servalyte 2-11 and subsequently prefocusing was performed for 500 V h with a maximum current of 1 mA per tube. Phosphoric acid (25 m*M*) and sodium

hydroxide (50 m*M*) solutions were used as cathode and anode buffers, respectively. After perfocusing, the lysis and overlay solutions were removed and the samples were applied. The sample solution was overlayed with 200 μl of 4 *M* urea–2% Servalyte 2-11 followed by 50 m*M* sodium hydroxide solution. Isoelectric focusing was performed for 10 000 V h at a maximum current of 1 mA per tube and a maximum voltage of 600 V. After 10 000 V h the voltage was increased to 800 V for 1 h and subsequently the gels were submitted to SDS-PAGE for the two-dimensional separation of proteins. The gel rods were applied to the separation gel of discontinuous SDS slab gels and overlayed with 5 ml of a warm (60°C) solution of 1% (w/v) agarose in 62.5 m*M* Tris–HEPES buffer (pH 5.8)–2% (w/v) SDS–5% (w/v) 2-mercaptoethanol–10% (v/v) glycerol–0.001% (w/v) bromophenol blue. After cooling of the agarose, elution of proteins from the focusing gel was performed at a voltage of 40 V and subsequently the separation of proteins was performed at 60 V. Fixing and staining of the gels were performed as described above.

Fluorography

The stained gels stored in 5% acetic acid were equilibrated in water for 2 h. Subsequently the gels were immersed for 20 min in a 1 *M* solution of sodium salicylate in 70% methanol [15]. After drying of the gels with a Bio-Rad Labs. gel dryer, the gels were exposed to Kodak-X-Omat AR film preflashed with red light [16,17] at −70°C.

RESULTS AND DISCUSSION

A strategy for the purification of the transport system responsible for the intestinal absorption of small peptides and β-lactam antibiotics from small intestinal brush border membrane vesicles must consider some specific characteristics of this transport system, as follows. Solubilization of brush border membrane vesicles with non-ionic detergents leads to a loss of binding affinity of the putative peptide transport protein for its substrates [18,19]; by photoaffinity labelling with [^{3}H]benzylpenicillin a nearly complete loss of binding affinity occurred after solubilization with Triton X-100, whereas a residual binding affinity of about 25% was found with *n*-octyl-glucoside as detergent. In addition, owing to the high proliferation rate of intestinal cells and their maturation and differentiation during their passage from crypt to villus and owing to the contact of the intestinal mucosa with digestive enzymes, the brush border membrane proteins are heterogeneous. The binding protein for β-lactam antibiotics and small peptides is a microheterogeneous glycoprotein.

Fig. 1A shows a two-dimensional gel of brush border membranes from rabbit small intestine. After photoaffinity labelling with photoreactive derivatives of penicillins, cephalosporins and dipeptides, the labelled 127 000-dalton binding protein is not found in a sharply focused spot on the two-dimensional gel; moreover, the radioactively labelled 127 000-dalton protein is distributed over a pH range of about 1 unit ranging from pH 5 to 6, as is evident by fluorography of two-dimensional gels (Fig. 1B).

Based on these findings some approaches to the purification of this putative transport protein can be ruled out. Affinity chromatography using immobilized ligands will presumably not be successful, as the ability for specific binding of the substrates is destroyed on solubilization of the membrane proteins. Owing to the

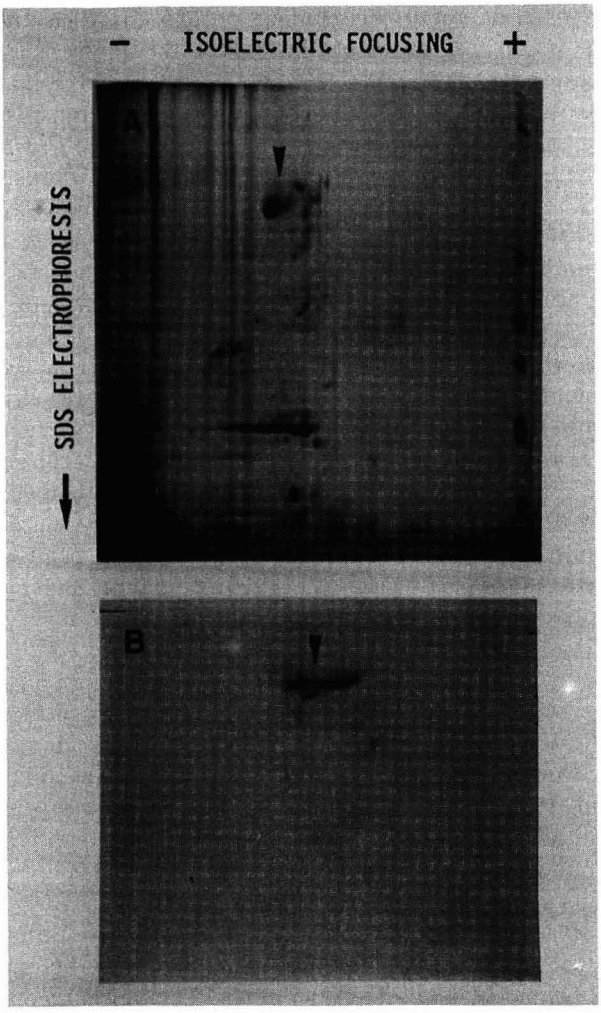

Fig. 1. Two-dimensional electrophoresis of rabbit small intestinal brush border membrane vesicles after photoaffinity labelling with [^{3}H]benzylpenicillin. A 1.5-mg amount of brush border membrane vesicle protein was photoaffinity labelled with 1.6 μM (15 μCi) [^{3}H]benzylpenicillin. After washing, the membrane proteins were submitted to two-dimensional electrophoresis with isoelectric focusing in the first and SDS-PAGE in the second dimension as described under Experimental. (A) Serva Blue R 250-stained polypeptides; (B) fluorogram.

heterogeneity of the 127 000-dalton binding protein a purification step by preparative isoelectric focusing, chromatofocusing or capillary electrophoresis would lead to a fractionation of the 127 000-dalton protein into many subfractions. The analysis of these subfractions would be too complicated, as the fractionation pattern depends on the individual preparations of membrane vesicles and further the amount of protein in the different fractions will be too low for a further detailed characterization. Therefore, we adopted a purification strategy using lectin affinity, ion-exchange, hydrophobic interaction, gel permeation and hydroxyapatite chromatography.

Owing to the glycoprotein structure of the binding protein for β-lactam antibiotics and small peptides of M_r 127 000, we tried affinity chromatography using immobilized lectins. On a wheat germ lectin agarose column the photolabelled 127 000-dalton protein was retained and could be eluted from the gel matrix with N-acetyl-D-glucosamine [18–20] together with the enzymatic activity of the brush border membrane-bound peptidase aminopeptidase N. As the involvement of brush border membrane-bound peptidases in peptide transport is a subject of controversy [21–25], a complete separation of the photolabelled 127 000-dalton protein from aminopeptidase N and other peptidases is essential for topological and immunohistological studies of the putative peptide transporter. By conventional ion-exchange chromatography using DEAE-Sephacel we could achieve a separation of both proteins with elution of aminopeptidase N prior to the elution of the 127 000-dalton binding protein [18–20]. The elution peaks of both proteins, however, partially overlapped; the second half of the elution peak of the 127 000-dalton binding protein contained about 5% of the initial aminopeptidase N activity. All attempts to achieve a complete separation of the 127 000-dalton protein from aminopeptidase N failed. Rechromatography of the eluted 127 000-dalton binding protein on DEAE-Sephacel did also not result in a further separation of aminopeptidase N and the peptide transporter. Therefore, in order to improve the yield of the 127 000-dalton binding protein and to obtain the peptide binding protein completely free from aminopeptidase activity, we developed an alternative purification procedure using FPLC.

Attempts to achieve a final purification by hydrophobic interaction chromatography were not successful; the eluted proteins from WGA–agarose chromatography were not adsorbed by alkyl-Superose whereas adsorption to phenyl-Superose occurred with Triton X-100 as detergent, However, no resolution into individual proteins could be achieved during the elution with either decreasing salt or increasing Triton concentrations. These findings suggest that the proteins are adsorbed to the column matrix predominantly by an interaction of the phenyl-Superose with Triton X-100–protein complexes [27]. The desorption of proteins occurs as mixed micelles of high molecular weight and therefore no separation into individual proteins occurred. From our experiments with the intestinal peptide transporter and other membrane-bound proteins, we conclude that hydrophobic interaction chromatography using detergents such as Triton X-100 which form micelles of high molecular weight is unsuitable for the purification of membrane proteins.

Gel permeation chromatography on Superose did not result in any fractionation into individual protein species and hydroxyapatite chromatography also failed as no adsorption to the hydroxyapatite matrix occurred.

As neither hydrophobic interaction, hydroxyapatite nor gel permeation chromatography was successful for the purification of the putative intestinal peptide transporter, ion-exchange chromatography with the strong ion exchangers Mono Q and Mono S was performed.

FPLC of the WGA–agarose eluates on Mono Q columns equilibrated with 10 m*M* Tris–HEPES buffer (pH 7.0)–0.03% (w/v) Triton X-100 and elution of the adsorbed proteins with a linear NaCl gradient did not result in a complete separation of the 127 000-dalton binding protein from aminopeptidase N. The best but still incomplete separation of both proteins was achieved with a flat NaCl gradient and an intermediate plateau at 100 m*M* NaCl. The replacement of Triton X-100 with

n-octylglucoside also had no significant influence on the resolution of the two proteins. As anion-exchange FPLC on Mono Q columns had no significant advantages for the purification of the intestinal peptide transporter compared with conventional chromatography on DEAE-Sephacel [18–20], we tried cation-exchange chromatography with the strong cation exchanger Mono S. The 127 000-dalton binding protein was adsorbed by the Mono S HR 5/5 column at pH <7. With 0.03% (w/v) Triton X-100 as detergent only a partial separation from aminopeptidase N could be achieved. If the non-ionic detergent Triton X-100 was completely omitted also in the affinity chromatographic step, a complete separation of the putative intestinal peptide transporter of M_r 127 000 from aminopeptidase N occurred (Fig. 2). An optimum and complete separation of both proteins was achieved in 20 m*M* sodium acetate buffer (pH 4.5)–0.07% (w/v) *n*-octylglucoside with a linear gradient from 0 to 100 m*M* NaCl followed by isocratic elution at 100 m*M* NaCl. Under these conditions the aminopeptidase N was completely desorbed from the column matrix whereas the 127 000-dalton binding protein still remained adsorbed (Fig. 2). The 127 000-dalton binding protein was subsequently eluted with a linear gradient from 100 to 600 m*M* NaCl. The linear gradient was essential for the separation of the 127 000-dalton binding protein from a polypeptide of M_r 112 000. With a steeper NaCl gradient only an incomplete separation of the 112 000-dalton protein from the putative peptide transporter was found and therefore the yield of pure 127 000-dalton binding protein decreased. SDS-PAGE of the different fractions from the Mono S chromatography showed that the amount of proteins eluting with aminopeptidase N activity is low compared with the amount of the 127 000-dalton binding protein eluted at higher

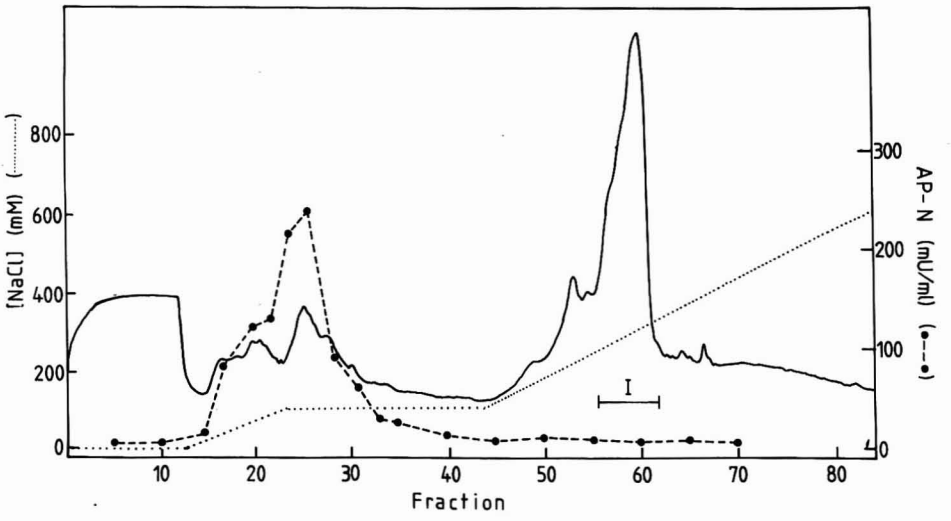

Fig. 2. Chromatography of the eluate from WGA–agarose chromatography on a Mono S HR 5/5 column with *n*-octylglucoside as detergent. The eluate from wheat germ lectin chromatography was fractionated on a Mono S HR 5/5 column using 20 m*M* sodium acetate buffer (pH 4.5)–0.7% *n*-octylglucoside as buffer. Proteins were eluted with an NaCl gradient as indicated. ———, UV absorption of proteins at 280 nm; ·····, shape of the NaCl gradient; ●– – –●, enzymatic activity of aminopeptidase N. The fractions containing the 127 000-dalton binding protein (indicated with I) were pooled and submitted to rechromatography.

NaCl concentrations. From 20 mg of brush border membrane protein about 200–300 μg of pure 127 000-dalton binding polypeptide could be obtained. The protein obtained from Mono S chromatography was finally purified and concentrated by rechromatography on a Mono S column. The final purity was greater than 95% (Fig. 3). Only by densitometry of SDS polyacrylamide gels was a small contamination of a protein of M_r 250 000 detectable. In Table I the results of the different chromatographic approaches for the purification of the putative intestinal peptide transporter are summarized.

Antibodies prepared against the 127 000-dalton binding protein purified according to the protocol described here completely precipitated the 127 000-dalton protein from solubilized brush border membrane vesicles whereas the aminopeptidase N activity remained in the soluble fraction. With these antibodies raised against the intestinal peptide transport protein purified by Mono S chromatography, a histo-

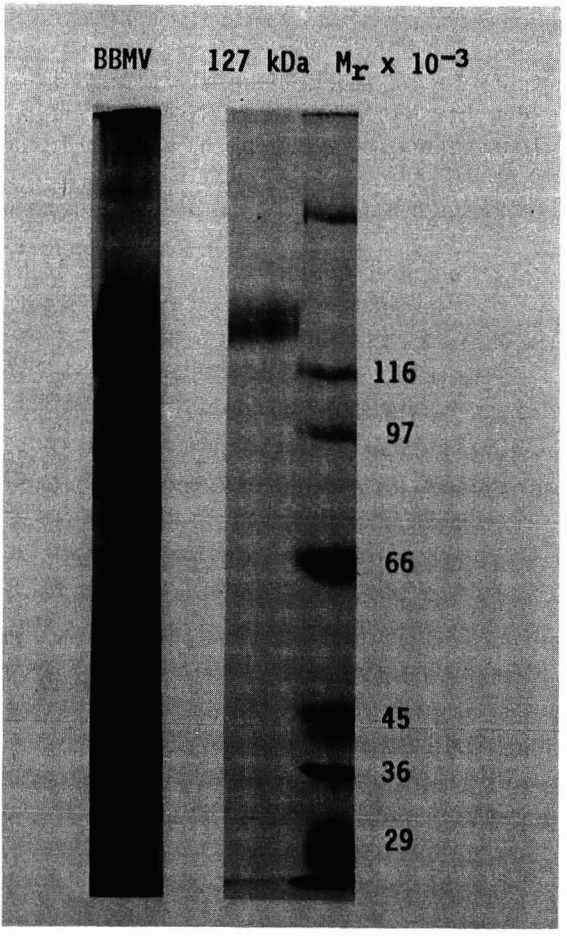

Fig. 3. SDS-PAGE of the putative intestinal peptide transporter purified by FPLC. Solubilized brush border membrane proteins from rabbit small intestine (BBMV) and the purified putative peptide transporter of M_r 127 000 were submitted to SDS-PAGE on a 7.5% gel. The numbers indicate the molecular masses of the standard proteins in kilodaltons (kDa).

TABLE I

CHROMATOGRAPHIC APPROACHES TO THE PURIFICATION OF THE PUTATIVE PEPTIDE TRANSPORTER FROM RABBIT SMALL INTESTINAL BRUSH BORDER MEMBRANES

Type of chromatography	Detergent[a]	Results[b]
WGA–agarose	Triton OG	Adsorption of the 127 000-dalton protein
DEAE-Sephacel	Triton OG	Separation of the 127 000-dalton protein and aminopeptidase N; contamination of 127 000-dalton protein with AP-N
Gel permeation, Superose 12	Triton OG	No separation into individual proteins
Hydroxyapatite	Triton OG	No adsorption to the matrix
Alkyl-Superose	Triton OG	No adsorption to the matrix
Phenyl-Superose	OG	No adsorption to the matrix
Phenyl-Superose	Triton	Adsorption, but no separation into individual proteins
Mono Q	Triton OG	Only partial separation of 127 000-dalton protein and AP-N (less than with DEAE-Sephacel)
Mono S	Triton	Only partial separation of 127 000-dalton protein and AP-N
Mono S	OG	Complete separation of 127 000-dalton protein and AP-N; final purity >95%

[a] Triton = Triton X-100; OG = *n*-octylglucoside.
[b] AP-N = Aminopeptidase N.

chemical localization of the intestinal peptide transport system in the small intestine and other organs and also sequencing of the putative peptide transporter should be possible.

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Efficient high-performance liquid chromatographic system for the purification of a halobacterial serine protease

WILMA SCHMITT*, URSULA RDEST and WERNER GOEBEL
Institut für Mikrobiologie und Genetik, Röntgenring 11, D-8700 Würzburg (F.R.G.)

ABSTRACT

Many of the extreme halophiles which belong to the archaebacteria produce extracellular proteases. The extracellular serine protease (designated ESP4) of *Halobacterium* sp. strain TuA4 was isolated in a pure state with a fast protein liquid chromatographic (FPLC) system. Because the enzyme is only stable at high ionic strength, it was necessary to develop a procedure that would allow a minimum Na^+ ion concentration of 0.3 *M* in each step. This is the first halophilic salt-dependent enzyme purified with FPLC. Two precipitation steps with PEG 6000 and acetone in combination with ion-exchange chromatography (CM-Sephadex, Mono Q HR 5/5) and hydrophobic interaction chromatography (phenyl-Superose HR 5/5) permitted the isolation of 216-fold purified ESP4 with a total recovery of 3%. The purified ESP4 was shown to possess a molecular weight of 60 000 dalton in sodium dodecyl sulphate–polyacrylamide gel electrophoresis, which correlates very well with the native molecular weight determined for this enzyme.

INTRODUCTION

Extremely halophilic bacteria which grow best in 20–30% NaCl belong to the third bacterial kingdom recently named archaebacteria. In certain respects these bacteria differ from other prokaryotes (eubacteria) and from eukaryotes [1].

Extracellular proteases are widely distributed among halophilic bacteria. Because they metabolize carbohydrates only slightly, these bacteria secrete proteolytic enzymes to degrade proteins from dead organisms in the natural salt environment [2]. Enzymes from extremely halophilic bacteria show a high degree of adaptation to extreme salt concentrations (4–5 *M* NaCl or KCl) and lose their activity at low ionic strength. This fact makes the development of a purification scheme very difficult, as one can only use procedures that are unaffected by high salt concentrations. The most highly purified and best characterized halobacterial proteins are structural proteins, such as the bacterioopsin [3], the gas vesicle protein [4], the cell envelope glycoprotein [5] and the ribosomal proteins [6]. These proteins are made in large amounts and can be isolated very easily. Only a few halobacterial enzymes have been purified because most of them are irreversibly denatured and inactivated after exposure to low salt concentrations. The purification methods that have generally been applied are salting-out mediated chromatography [7] and hydroxyapatite [8] and affinity chromatography [9], which allow high concentrations of salt. The first protease isolated from

halobacteria was the extracellular serine protease from *H. halobium* [9]. The method described here for the isolation of the extracellular serine protease (termed ESP4) from the mesophilic halobacterial isolate *Halobacterium* sp. strain TuA4 is new. In order to shorten the purification time we developed an efficient five-step procedure with two high-resolution steps using an automated fast protein liquid chromatographic (FPLC) system as last step of the purification scheme.

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade and were purchased from Merck (Darmstadt, F.R.G.), Roth (Karlsruhe, F.R.G.) and Sigma (Deisenhofen, F.R.G.). DEAE-Sephadex A-50, CM-Sephadex C-50, PD 10 columns (Sephadex G-25 M), arginine-Sepharose 4B, benzamidine-Sepharose 6B and the prepacked FPLC columns Superose 12 HR 10/30, Mono Q HR 5/5, phenyl-Superose HR 5/5 and Q-Sepharose HiLoad 16/10 were obtained from Pharmacia–LKB (Freiburg, F.R.G.). Sterile filters were from Sartorius (Göttingen, F.R.G.) and Gelman (Dreieich, F.R.G.).

Isolation and purification of ESP4

Halobacterium sp. strain TuA4 was isolated from Chott-el-Djerid, Tunisia, and purified in this laboratory. The cells were grown in rich medium containing 4.3 *M* NaCl, 0.12 *M* $MgSO_4$, 0.03 *M* KCl, 0.01 *M* trisodium citrate and 1% peptone (Oxoid) (pH 7.2) with shaking and illumination at 37°C to a cell density of 10^9 cells/ml. After 5 days the cells were harvested by centrifugation at 11 000 *g* for 20 min at 4°C. A 5-l volume of culture supernatant was filtered with a 0.45-μm Sartorius filter and then concentrated 100-fold by a Diaflow HP 10-20 hollow-fibre cartridge from Amicon (10 000-dalton cut-off). To 30 ml of the concentrated supernatant PEG 6000 was added from 60% stock solution in 3 *M* NaCl–10 m*M* $CaCl_2$–20 m*M* $MgCl_2$–50 m*M* Tris–HCl (pH 9.0) to a final concentration of 10%. After 30 min on ice, nucleic acids and membrane compounds were pelleted at 17 000 *g* for 15 min at 4°C. The PEG supernatant, which contained the protease activity, was then mixed with two volumes of acetone. The precipitation was complete after 20 min at −20°C and the precipitate was then centrifuged at 24 000 *g* for 20 min at 4°C. The pellet was suspended in 18 ml of 20% glycerol and desalted on PD 10 columns. The final buffer composition was 0.3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* 2-(N-morpholino)ethanesulphonic acid (MES) (pH 6.0)–20% glycerol. The eluate was then adsorbed in the batch on CM-Sephadex C-50 (50 ml in a beaker) equilibrated with the same buffer. After 30 min of adsorption the supernatant containing the protease activity was centrifuged at 39 000 *g* at 2°C for 20 min to remove remaining CM-Sephadex particles, desalted on PD 10 columns to 0.3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* Tris–HCl (pH 7.0)–20% glycerol, filtered with a 0.45-μm Acrodisc sterile filter and applied to a Mono Q HR 5/5 column (50 mm × 5 mm I.D.) equilibrated in 0.3 *M* NaCl–50 m*M* Tris–HCl (pH 7.0)–20% glycerol. All buffers used for FPLC were prepared with triply distilled water, filtered with a 0.22-μm Sartorius filter and then autoclaved. The protease activity was eluted with 28 ml of an increasing gradient of NaCl [0.3 to 1.5 *M* in 50 m*M* Tris–HCl (pH 7.0)–20% glycerol]. Protease-containing fractions were pooled, dialysed against 1.3 *M* Na_2SO_4–50 m*M* Tris–HCl (pH 7.0) and loaded onto a phenyl-Superose HR 5/5 column (50 mm ×

5 mm I.D.) that had been equilibrated in the same buffer. Protease activity was eluted with 18 ml of a decreasing linear gradient of Na_2SO_4 [1.3 *M* Na_2SO_4 to 10% ethylene glycol in 50 m*M* Tris–HCl (pH 7.0)]. After the final purification step phenylmethylsulphonyl fluoride (PMSF) (0.5 *M* in methanol) was added (final concentration 5 m*M*) to the purified enzyme to prevent autoproteolysis. The protease was precipitated by adding four volumes of saturated ammonium sulphate solution (69.7 g in 100 ml of triply distilled water). After 90 min on ice, the precipitate was centrifuged at 24 000 *g* at 4°C for 30 min. The samples were further analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Protease assay

Enzyme activity was determined by a modification of the Azocoll method described by Tsuboi *et al.* [10]. To 5 mg of Azocoll in 3 *M* NaCl–10 m*M* $CaCl_2$–20 m*M* $MgCl_2$–50 m*M* Tris–HCl (pH 7.0) were added 5–50 μl of enzyme solution. The samples were incubated at 56°C for 2 h and, after centrifugation at 12 000 *g* for 10 min, the released dye was measured at 520 nm with a Zeiss Model DM 4 spectrophotometer. One arbitrary unit (AU) of protease activity was defined as the amount of enzyme which causes an increase in absorbance of 0.1 under these conditions.

Batch adsorption

Adsorption of ESP4 to arginine-Sepharose 4B and benzamidine-Sepharose 6B was performed utilizing batch adsorption. A 100-μl volume of the chromatographic material was added to a series of Eppendorf tubes and equilibrated by washing three times with 1 ml of the following buffers: 3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* Tris–HCl (pH 6.0, 7.0, 8.0) and 0.3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* Tris–HCl (pH 6.0, 7.0, 8.0). After centrifugation, each tube was filled with the equilibration buffer to 200 μl and 50 μl of concentrated supernatant were then added. The gel was mixed for 10 min at room temperature and centrifuged at 12 000 *g* for 10 min. The protease activity in the supernatant was determined with Azocoll as previously described.

Chromatography on Q-Sepharose

The Q-Sepharose 16/10 HiLoad column (200 mm × 16 mm I.D.) was equilibrated with five bed volumes (100 ml) of 0.3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* Tris–HCl (pH 7.0)–20% glycerol (buffer A). Acetone precipitate was dissolved in the above buffer and 5 ml were applied to the column, after which the column was further washed with 20 ml of buffer A. The protease was eluted with 280 ml of an increasing gradient of NaCl from 0.3 to 1.5 *M* in 50 m*M* Tris–HCl (pH 7.0)–10 m*M* $CaCl_2$–20% glycerol at a flow-rate of 2.5 ml/min.

Test-tube method for selecting starting pH

The starting pH for anion-exchange chromatography was selected according to the method described by Pharmacia–LKB [11]. A 1-ml volume of enzyme solution in the appropriate buffer was added to each tube. After 30 min 100 μl of the supernatant were tested for enzyme activity. Desorption was carried out in 3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* Tris–HCl (pH 7.2). Volumes of 100 μl of the eluted samples were then tested for protease activity.

Protein assay

Protein concentration was determined by a modification of Bradford's method [12]. A 100-μl volume of 0.06% Coomassie Brilliant Blue G250 in 1.9% perchloric acid was mixed with 80 μl of distilled water and 20 μl of sample. After 2 min the absorbance was measured with an enzyme-linked immunoadsorbent assay (ELISA) reader (transmission wavelength 630 nm; reference wavelength 405 nm). Bovine serum albumin was used as a standard in the range 0–20 μg.

Gel electrophoresis

Proteins were analysed by discontinuous SDS-PAGE as described by Laemmli [13]. Silver staining of the gels was carried out according to the method of Blum *et al.* [14].

RESULTS AND DISCUSSION

As the extracellular protease of *Halobacterium* sp. strain TuA4 is a serine protease (see Table I), first attempts to purify the enzyme were made with affinity chromatography. Arginine-Sepharose 4B was shown to bind enzymes which contain serine at their active sites and which cleave proteins at the carboxyl end of arginine residues such as prekallikrein from bovine plasma [15]. Benzamidine-Sepharose 6B is another useful adsorbent specific for serine proteases such as trypsin and trypsin-related enzymes [16]. Both materials were tested with different ionic strength and pH conditions for binding of ESP4, but insufficient adsorption was obtained under the conditions tested, suggesting that the halophilic protease is not arginine/lysine-specific.

Fortunately, ESP4 is active and relatively stable in lower ionic strength buffers (0.3 *M* Na^+) if 20% gycerol is added as a stabilizing agent. As most of the halophilic proteins have a high content of acidic amino acids, anion-exchange chromatography seemed to be suitable for the purification of ESP4. The starting pH for anion-exchange chromatography was selected using the test-tube method as described by Pharmacia–LKB [11]. Adsorption on DEAE-Sephadex occurred within the pH range 5.0–7.5, suggesting that the isoelectric point of ESP4 is below 5.0 and the desorption was optimum at pH 7.0 (see Table II); thus anion-exchange chromatography was carried out in 50 m*M* Tris–HCl (pH 7.0) with the addition of 0.3 *M* NaCl and 20% glycerol. Binding of proteins to an anion-exchange material in the presence of 0.3 *M* NaCl has not been described previously, as most proteins elute under such conditions with the exception of highly acidic halobacterial proteins, which still bind to anion-exchangers in the presence of 0.3 *M* NaCl.

TABLE I

INHIBITION OF ESP4 WITH VARIOUS SERINE PROTEASE INHIBITORS

Inhibitor	Concentration (m*M*)	Inhibition (%)
PMSF	5	95
Diisopropyl fluorophosphate	5	100
Leupeptin	20	82

TABLE II

TEST-TUBE METHOD FOR SELECTING THE STARTING pH FOR ANION-EXCHANGE CHROMATOGRAPHY

pH	Enzyme activity after adsorption on DEAE-Sephadex (AU)	Enzyme activity without DEAE-Sephadex (AU)	Enzyme activity after desorption from DEAE-Sephadex (AU)
5.0	0.17	2.80	0.33
5.5	0.27	2.80	0.52
6.0	0.34	2.80	1.00
6.5	0.39	2.80	2.00
7.0	0.42	2.80	2.70
7.5	0.47	2.80	1.90

Furthermore, sodium chloride in the buffer could be replaced with sodium sulphate with little loss of activity, so that hydrophobic interaction chromatography could be applied for the purification of ESP4. The prepacked alkyl-Superose HR 5/5 FPLC column (50 mm × 5 mm I.D.) was tested first; ESP4 did not bind to the column in 1.3 *M* Na_2SO_4–50 m*M* Tris–HCl (pH 7.0), suggesting that the enzyme is not strongly hydrophobic. However, under the same conditions ESP4 was adsorbed on the more hydrophobic agent phenyl-Superose HR 5/5 (50 mm × 5 mm I.D.) column.

Based on these findings, a purification procedure could be developed that would maintain the protease in a minimum sodium ion concentration of 0.3 *M*. This is essential, because below 0.3 *M* Na^+ the protease activity is irreversibly lost. The crude concentrated supernatant is very complex and contains many proteins and peptones from the medium and nucleic acids and membrane compounds from "leaky" cells which lyse during the preparation. The nucleic acids and membrane compounds which can interfere with optimum separation on high-resolution FPLC columns were precipitated with 10% PEG 6000 in 3 *M* NaCl–10 m*M* $CaCl_2$–20 m*M* $MgCl_2$–50 m*M* Tris–HCl (pH 9.0). During this step the total enzyme activity increases about 1.5 fold, indicating that a more or less specific inhibitor is separated from the protease by PEG precipitation. About 48% of the protease activity could be precipitated by acetone precipitation (2:1, v/v) whereas other soluble proteins, especially smaller peptides, remained in the acetone supernatant. This step led to a 1.5-fold purification with a 48% total recovery. The loss of activity is high because the protease is exposed to low ionic strength in this step, but acetone precipitation is necessary at this point to remove the protease from PEG and peptone contamination.

The third step in the purification was the binding of contaminating proteins to CM-Sephadex C-50 in 0.3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* MES (pH 6.0)–20% glycerol. ESP4 has a net negative charge at pH 6.0 and did not adsorb on the column. Cation-exchange chromatography resulted in a 2.6-fold purification with a 28% total recovery.

The most effective purification steps were the anion-exchange chromatography on Mono Q HR 5/5 (Fig. 1) and hydrophobic interaction chromatography on phenyl-Superose HR 5/5 (Fig. 2) using an automated FPLC system from Pharmacia–

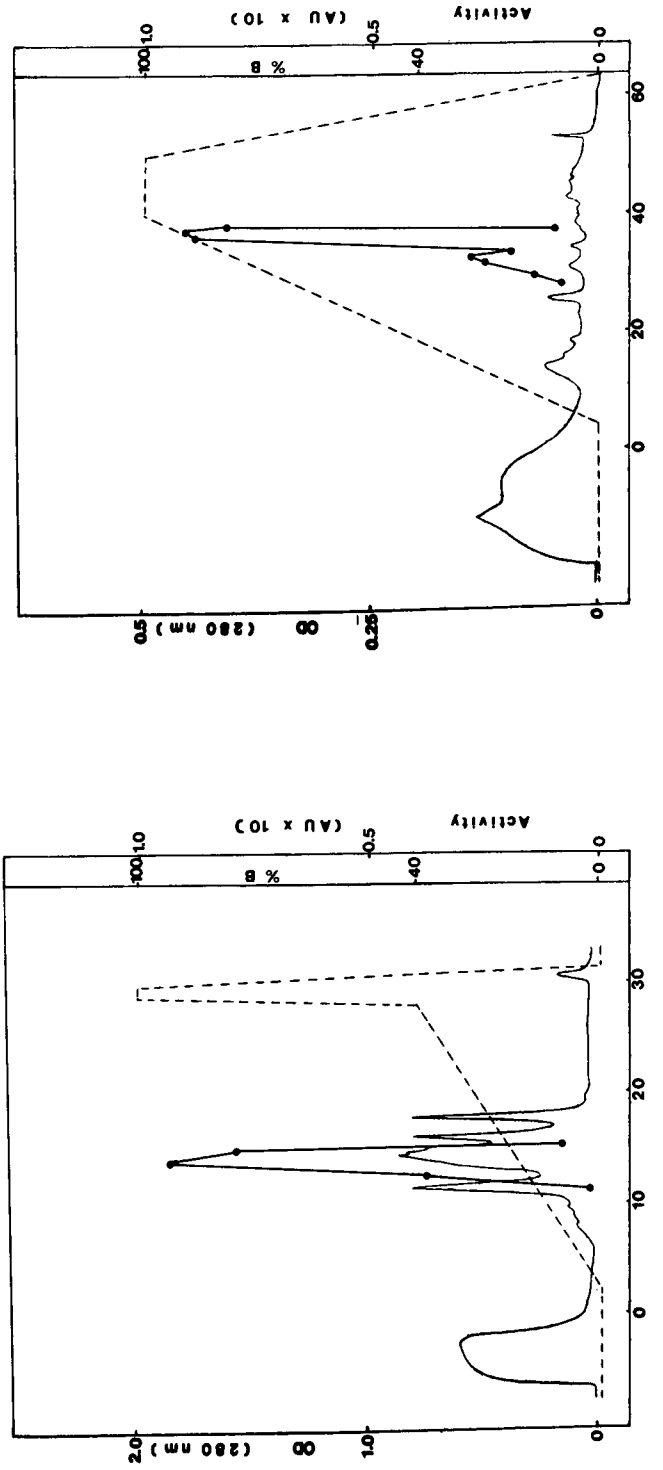

Fig. 1. Anion-exchange chromatography on Mono Q. 4 ml of enzyme solution were loaded onto the column and eluted in 1-ml fractions with an increasing sodium chloride gradient (– – –) at a flow-rate of 1 ml/min. A 50-μl volume of each fraction was assayed for protease activity (●—●). Protein absorbance was measured at 280 nm (———).

Fig. 2. Hydrophobic interaction chromatography on phenyl-Superose. 3.5 ml of the pooled activity from Mono Q was applied to the column. ESP4 was eluted in 0.4-ml fractions with an increasing ethylene glycol gradient (– – –; 0–10%) at a flow-rate of 0.4 ml/min. A 50-μl volume of each fraction was assayed for protease activity (●—●). Protein absorbance was measured at 280 nm (———).

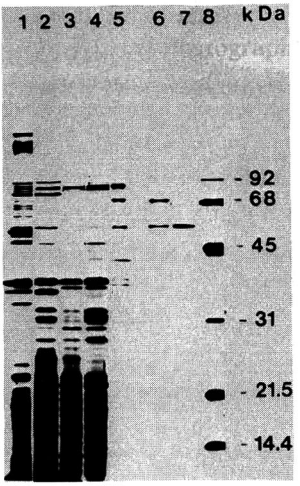

Fig. 3. Purification of ESP4 as monitored by SDS-PAGE. Proteins were removed at various steps of the purification procedure and loaded onto a 12% SDS polyacrylamide gel. Lanes: 1 = crude supernatant (2.5 μg); 2 = PEG supernatant (2.5 μg); 3 = acetone pellet (4.0 μg); 4 = CM-Sephadex eluate(3.0 μg); 5 = activity pool from Mono Q (1.0 μg); 6 = activity peak 1 from phenyl-Superose (1.0 μg); 7 = activity peak 2 from phenyl-Superose (0.5 μg); 8 = molecular weight markers (kDa = kilodalton).

LKB. These techniques resulted in a 177-fold purification with a 24% total recovery for Mono Q (only a 4% loss of activity in relation to the previous step) and a 216-fold purification with a 3% total recovery for phenyl-Superose. Such a degree of purification has never been achieved previously for halophilic enzymes purified using other procedures [9,17]. Especially the chromatography on Mono Q resulted in a 70-fold purification as this was the step where most of the contaminating non-halophilic proteins originating from the medium were separated from the protease. After chromatography on phenyl-Superose two protease activities were present, as shown in Fig. 2. The first peak is not pure, as shown in a 12% SDS polyacrylamide gel (Fig. 3), whereas from peak 2 only one protein band with a molecular weight of 60 000 dalton was detected in the gel. This value correlates very well with the native

TABLE III

PURIFICATION OF ESP4 FROM *HALOBACTERIUM* SP. STRAIN TuA4

Step	Volume (ml)	Total activity (AU)	Amount of protein (mg)	Specific activity (AU/mg)	Yield (%)	Purification factor
(1) Concentrated supernatant	30	4752	150	31.7	100	1
(2) PEG supernatant	36	7092	144	49.2	150	1.5
(3) Acetone pellet	18	2286	49.5	46.2	48	1.5
(4) CM-Sephadex	25	1320	16.25	81.2	28	2.6
(5) Mono Q	20	1120	0.2	5600	24	177
(6) Phenyl-Superose	6	144	0.02	6858	3	216

TABLE IV

COMPARISON OF ESP4 WITH THE EXTRACELLULAR SERINE PROTEASE OF *H. HALOBIUM* WITH REGARD TO THE SODIUM CHLORIDE CONCENTRATION

NaCl (*M*)	Enzyme activity (%)	
	H. halobium protease	ESP4
0	n.d.	0
0.3–20% glycerol	n.d.	70
0.5–20% glycerol	n.d.	86
0.5	n.d.	70
1	0	100
2	20	100
3	34	100
4.3	100	100

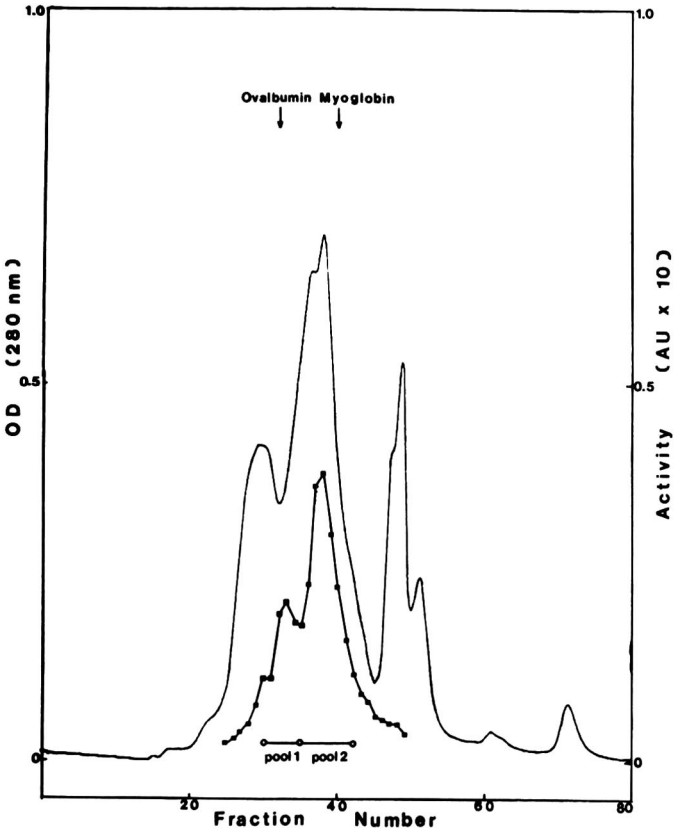

Fig. 4. Gel filtration of 100 μl of the resuspended acetone precipitate on a Superose 12 column. Fractions of 0.8 ml were collected and assayed for protease activity (■—■). The chromatography was carried out in 1 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* Tris–HCl (pH 7.2) at a flow-rate of 0.4 ml/min. Protein absorbance was measured at 280 nm (———).

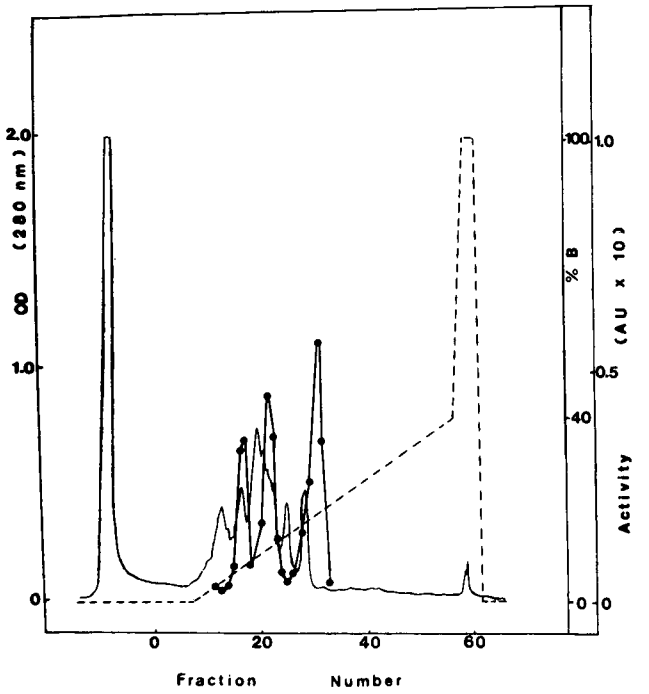

Fig. 5. Anion-exchange chromatography on Q-Sepharose. 5 ml of enzyme solution were applied to the column and eluted in 5-ml fractions with an increasing sodium chloride gradient (– – –) at a flow-rate of 2.5 ml/min. A 50-μl volume of each fraction was assayed for protease activity (●—●). Protein absorbance was measured at 280 nm (———).

molecular weight of ESP4 determined by glycerol gradient centrifugation and with the molecular weight determined by SDS-PAGE following active site labelling with [^{14}C]diisopropyl fluorophosphate. The results of the purification are summarized in Table III and Fig. 3.

ESP4 was shown to be completely different from the extracellular protease of *H. halobium* (see Table 1V), especially with regard to monovalent ionic concentration. Whereas the *H. halobium* protease is fully and irreversibly inactivated at concentrations below 2 *M* NaCl [9], ESP4 is active at concentrations up to 0.3 *M* NaCl. Also, the molecular weights of the two proteases are different: ESP4 exhibits a molecular weight of 60 000 dalton in SDS gel systems, whereas that of the protease isolated from *H. halobium* is 56 000 dalton, so ESP4 is obviously larger.

In some experiments, gel filtration on a Superose 12 HR 10/30 column (300 mm × 10 mm I.D.) in 1 *M* NaCl–10 m*M* $CaCl_2$–20 m*M* $MgCl_2$–50 m*M* Tris–HCl (pH 9.0) was used as a third step after acetone precipitation (Fig. 4). This step was not very effective because only small amounts of protein could be applied to the gel filtration column and the separation was not satisfactory. In order to purify large amounts of protein in a short time, the anion-exchange column Q-Sepharose HiLoad was tested (Fig. 5). We found that the protease activity separated into three different peaks which were very unstable (after 24 h at −20°C the activity was irreversibly lost)

so that further purification of the protease activities eluted from this column was not possible.

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Large-scale purification of prosomes from calf's liver

WOLFGANG TOMEK
Biologisches Institut, Universität Stuttgart, Pfaffenwaldring 57, D-7000 Stuttgart 80 (F.R.G.)
JACQUES BURI
Station de Virologie et d'Immunologie Moléculaires, INRA, Domaine de Vilvert, F-78350 Jouy-en-Josas (France)
and
RÜDIGER VALLON and HANS-PETER SCHMID*[a]
Biologisches Institut, Universität Stuttgart, Pfaffenwaldring 57, D-7000 Stuttgart 80 (F.R.G.)

ABSTRACT

Prosomes, cytoplasmatic ribonucleoprotein complexes containing small ribonucleic acid (19S small cytoplasmic RNPs), are ubiquitous in eukaryotic organisms. A new method for the preparation of prosomes in large amounts, starting with *ca.* 2 kg of calf's liver, is described. A combination of centrifugation and low- and high-pressure chromatography was used to purify intact particles. An alternative purification of prosomes with *Solanum tuberosum* agglutinin bound to divinyl sulphone-activated agarose is discussed. Calf's liver prosomes have a similar protein composition and RNA content to prosomes isolated from other tissues.

INTRODUCTION

Prosomes were first identified as cytoplasmic ribonucleoprotein complexes (19S scRNPs) in mouse and duck erythroblasts [1]. In the meantime they have been found in a great variety of eukaryotic cells ranging from plants to man [1–7]. The cellular function of prosomes is still unknown. Our group has recently demonstrated that prosomes are involved in the repression of translation of some viral mRNAs *in vitro* [8], and other workers found that they have specific proteinase or peptidase activity [9,10]. The fact that they exist in all eukaryotic organisms investigated so far suggests an important physiological function for the cell.

Prosomes consist of a specific set of proteins which band in Laemmli polyacrylamide gels in the range 19 000–35 000 dalton. Some of them, *e.g.*, the 27 000-dalton protein, were highly conserved during evolution, whereas others vary from species to species [2]. Some of the prosomal proteins are glycosylated [11]. Similarly, the content of small RNA of prosomes seems to be related but not identical among species. RNA is an intrinsic part of prosomes. We have recently shown that

[a] Present address: Université de Clermont II, Biochimie, B.P. 45, 24 Avenue des Landais, F-63170 Aubière, France.

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prosomal proteins protect RNA fragments of *ca.* 76–80 nucleotides against nuclease digestion [12].

Most interestingly, prosomes resist the strong detergent 1% sarcosyl and high ionic strengths. These properties were used for the purification of prosomes by a series of sucrose gradient centrifugations [1]. Here, we present a more effective method for isolating prosomes, using a combination of ultracentrifugation and low- and high-pressure chromatography, which leads to a high yield of prosomes. This enormously facilitates the study of the structure and function of these particles.

EXPERIMENTAL

Cell fractionation procedure

Calf's liver was removed from a freshly slaughtered calf, cut into small pieces and washed several times with cold buffer containing 20 m*M* Tris–HCl (pH 7.4)–5 m*M* $MgCl_2$–2 m*M* 2-mercaptoethanol–0.1 m*M* EDTA (pH 7.4)–20 m*M* KCl (wash buffer). All connective tissue was removed from the liver fragments, which were again washed twice with wash buffer and quickly dried on filter-paper.

Portions of 100–200 g were homogenized for 1 min in a Waring blender with 3 volumes of buffer H [20 m*M* Tris–HCl (pH 7.4)–5 m*M* $MgCl_2$–2 m*M* 2-mercaptoethanol–0.1 m*M* EDTA (pH 7.4)–20 m*M* KCl–200 m*M* sucrose]. Homogenates (5.8 l) were filtered through Miracloth and portions of 20 ml were then further homogenized by 20 strokes in a Dounce homogenizer and centrifuged for 15 min at 700 *g* at 4°C to sediment nuclei and unbroken cells. The postnuclear supernatant was centrifuged for 20 min at 3500 rpm at 4°C (GSA rotor, Sorvall RC 2-B) and the remaining supernatant was centrifuged again for 30 min at 10 000 rpm at 4°C (SS 34 rotor, Sorvall RC 2-B). The supernatant from this sedimentation (postmitochondrial supernatant) was transferred to Beckman Quickseal tubes (Ti 45 rotor) containing a cushion of 10 ml of 20% sucrose in wash buffer and centrifuged 2 h at 42 000 rpm at 4°C to sediment polysomes and other particles larger than 80S. The postribosomal supernatant (PRS) was centrifuged again in Beckman Quickseal tubes (Ti 45 rotor) containing a cushion of 10 ml of 30% sucrose in wash buffer (19 h, 42 000 rpm, 4°C).

The sediments containing postribosomal particles (PRPs) were resuspended in TBK 300 [20 m*M* Tris–HCl (pH 7.4)–5 m*M* $MgCl_2$–2 m*M* 2-mercaptoethanol–300 m*M* KCl], frozen at −80°C or immediately subjected to different chromatographic procedures.

One- and two-dimensional protein gel electrophoresis

One-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed according to ref. 13 and two-dimensional SDS-PAGE as described in ref. 14. Molecular weight markers were phosphorylase *b* (94 000 dalton), bovine serum albumin (68 000), ovalbumin (43 000), carboanhydrase (29 000), soybean trypsin inhibitor (20 000) and lactalbumin (14 000).

RNA extraction and RNA gel electrophoresis

Prosomes were treated with proteinase K (1 mg/ml) for 30 min at 37°C, then the RNA was extracted with chloroform–phenol according to ref. 15. RNA fragments were labelled at their 3′-ends with [^{32}P]pCp (Amersham, 3000 Ci/mmol) in a reaction

catalysed by T4-RNA-ligase [16]. RNA was analysed on 300-mm long 15% polyacrylamide gels containing 7 *M* urea–50 m*M* Tris–borate (pH 8.3)–2 m*M* EDTA. Electrophoresis was performed until the bromophenol blue reached the bottom of the gel.

Probing of protein blots with Solanum tuberosum agglutinin

Solanum tuberosum agglutinin (STA) was obtained from Kem-En-Tec. Streptavidin horseradish peroxidase complexes from Amersham were used as probes for the biotinylated lectin.

After separation on gels, prosomal proteins were transferred to nitrocellulose (Schleicher & Schüll BA 85) according to ref. 17. After the transfer, the remaining binding sites were blocked by incubation in 20 m*M* Tris–HCl (pH 7.4)–500 m*M* NaCl–2% Tween 20 for 2 min [11]. The nitrocellulose was then washed twice in Tris-buffered saline (TBS) [20 m*M* Tris–HCl (pH 7.4)–500 m*M* NaCl] for 5 min. After a short wash in lectin buffer [10 m*M* Tris–HCl (pH 7.4)–1 *M* NaCl–1 m*M* $CaCl_2$–1 m*M* $MgCl_2$–1 m*M* $MnCl_2$–2 m*M* NaN_3], the blots were incubated overnight with biotinylated STA (2 μg/ml) in the same buffer. The nitrocellulose sheets were again washed twice with TTBS (TBS containing 0.05% of Tween 20) for 5 min and incubated with streptavidin-horseradish peroxidase complexes diluted with TTBS for 1 h. After washing three times in TBS, the blots were assayed for peroxidase activity by incubation with peroxidase substrate buffer [50 m*M* sodium citrate (pH 5.0)–0.25 mg/ml carbazole–0.5% hydrogen peroxide). The reaction was stopped by rinsing with tap water and the nitrocellulose was dried between Whatman filter-papers.

Affinity chromatography with immobilized Solanum tuberosum agglutinin

STA was covalently bound to divinyl sulphone-activated agarose according to ref. 18. The activated agarose was washed three times with 1.4 *M* potassium phosphate (pH 8.6). A 1-ml volume of the gel was shaken gently with 1 mg of STA for 16 h at room temperature. The gel mixture was packed into an HR 5/5 column (Pharmacia–LKB) and washed thoroughly with lectin buffer.

Prosomes were applied to the column in the same buffer. Bound material was eluted with lectin buffer containing 1 *M* N-acetylglucosamine.

RESULTS AND DISCUSSION

To accelerate the investigations on the structure and function of the prosomes, we developed a rapid and effective method for the purification of these RNP particles. We chose calf's liver, because it is easy to dissect and contains well defined cells, mostly hepatocytes. We started the purification with 2030 g of calf's liver, using about 6 l of wash buffer during dissection and preparation of the liver fragments and 5.8 l of buffer H for the homogenization of the fragments. The 100 000 *g* sediments containing ribosomal subunits, free mRNPs, small cytoplasmic RNPs and proteins larger than 10S (PRPs) were further purified by anion-exchange chromatography and gel filtration.

Approximately 50 ml of PRP suspension in TBK 300 (1500 A_{280} units) were applied to 100 ml of Q-Sepharose Fast Flow anion exchanger in a C 26/40 column (Pharmacia–LKB) equilibrated with TBK 300. Particles and unbound proteins were washed off with the same buffer until the absorbance at 280 nm reached a constant

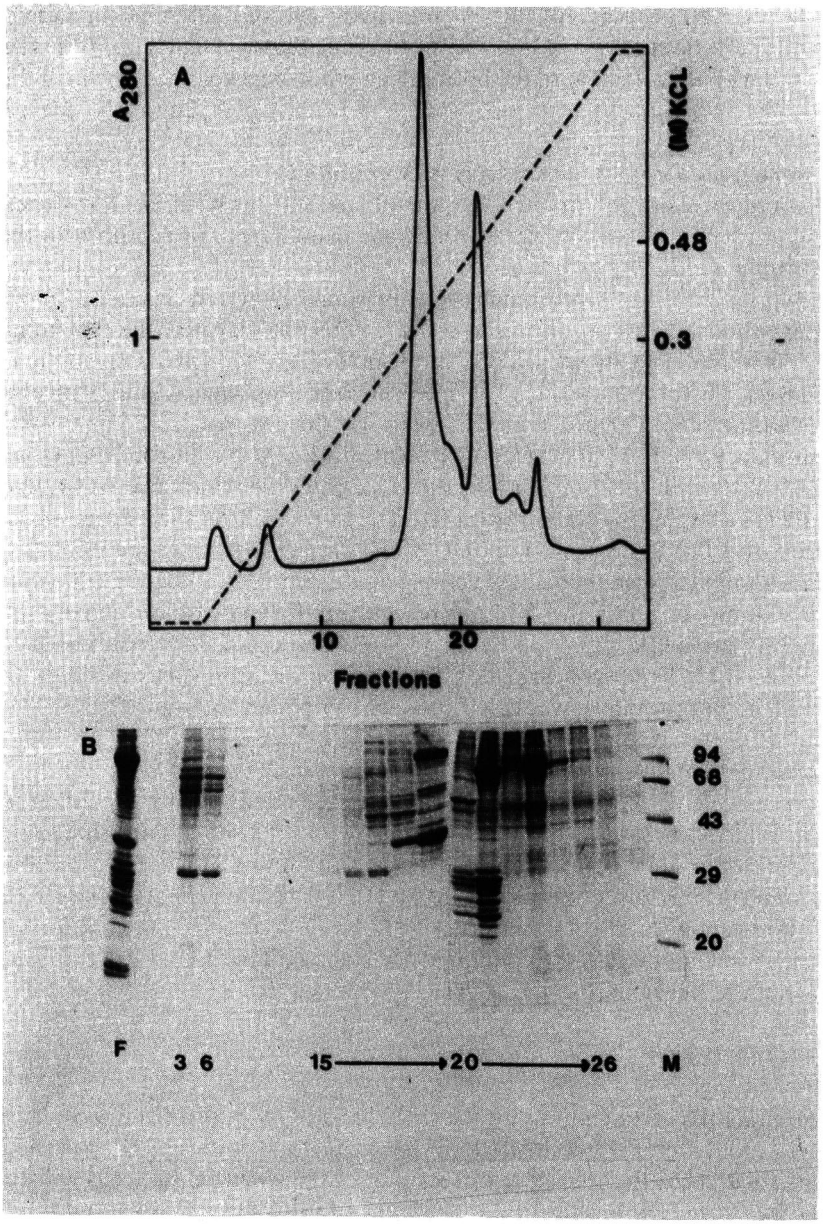

Fig. 1. Chromatogram of particles eluted from the Q-Sepharose Fast Flow applied to an FPLC Mono Q HR 5/5 column. (A) Bound particles were eluted with a linear salt gradient ranging from 0 to 600 m*M* KCl in Tris–HCl buffer (pH 7.4). Dashed line, KCl concentration; solid line, absorbance at 280 nm (A_{280}). (B) Protein composition of the fractions obtained by Mono Q chromatography. Fractions 20 and 21 contain prosomes. The proteins were analysed by one-dimensional SDS-PAGE in 12.5% polyacrylamide gels and stained with Coomassie brilliant blue. F = Protein composition of the suspension applied to the column; M = molecular weight marker proteins in kilodaltons.

value near the baseline. Fractions containing prosomes were eluted by a step gradient to 600 m*M* KCl and detected by SDS–PAGE (data not shown). To concentrate the eluate, containing prosomes, the particles were sedimented by ultracentrifugation (19 h, 235 000 *g*). The sediments were resuspended in FPLC buffer A [20 m*M* Tris–HCl (pH 7.4)–5 m*M* $MgCl_2$–7 m*M* 2-mercaptoethanol] and 20–30 A_{280} units were applied to a fast protein liquid chromatography (FPLC) Mono Q column (HR 5/5, Pharmacia–LKB). A linear salt gradient up to 600 m*M* KCl was formed. Prosomes were eluted at 480 m*M* KCl with a pronounced peak of absorbance in fractions 20 and 21, while other particles and proteins were eluted in fractions 16–19 (Fig. 1A and B).

To obtain highly concentrated amounts of prosomes, fractions 20 and 21 from five Mono Q runs were collected, diluted to a final concentration of 300 m*M* KCl and again loaded on a Mono Q column. After a short wash with TBK 300, a step gradient to 500 m*M* KCl was applied. Under these conditions, all particles bound on the exchanger were recovered in 1 ml, the void volume of the column. In the final step, prosomes were purified to homogeneity by gel filtration, using an FPLC Superose 6 column (HR 10/30, Pharmacia–LKB) equilibrated with Tris–HCl (pH 7.4) buffer containing 480 m*M* KCl. As demonstrated in Fig. 2, almost 90% of the injected

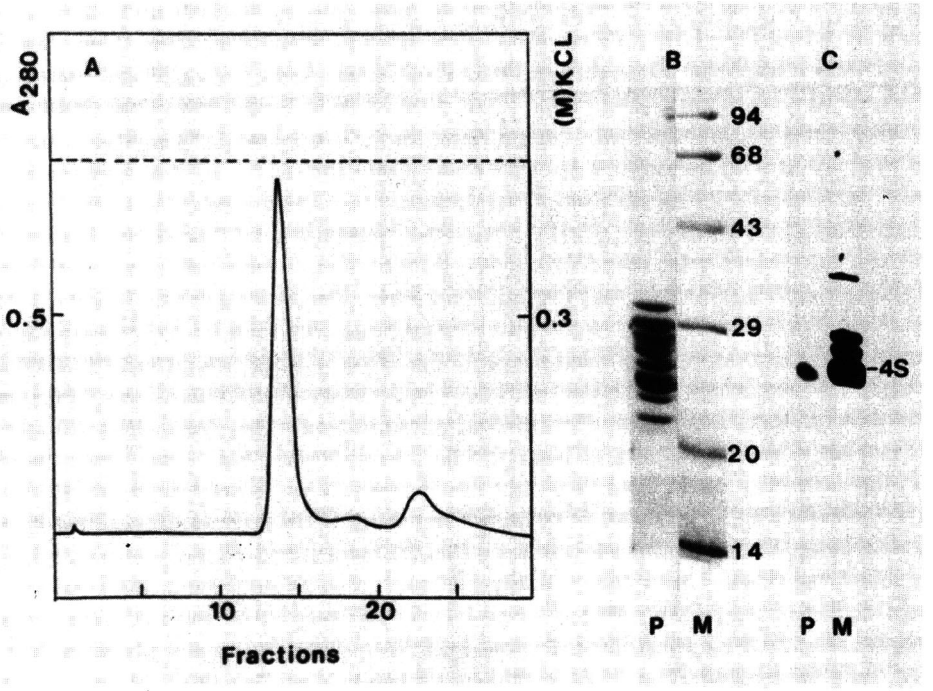

Fig. 2. Purification of prosomes by gel filtration with FPLC Superose 6. (A) Prosomal fractions (2 A_{280} units) isolated and concentrated by Mono Q chromatography were passed through a Superose 6 column equilibrated with Tris–HCl buffer (pH 7.4) containing 480 m*M* KCl. Dashed line, KCl concentration; solid line, absorbance at 280 nm (A_{280}). (B) Eluted particles of fractions 13 and 14 (prosomes) were analysed in 12.5% polyacrylamide gels [13] and proteins were stained with Coomassie brilliant blue (P). M = Molecular weight markers in kilodaltons. (C) RNA was extracted from prosomes as described under Experimental and analysed by urea–SDS-PAGE. RNA bands were rendered visible by autoradiography. M = t-RNA from brewers yeast; P = prosomal RNA.

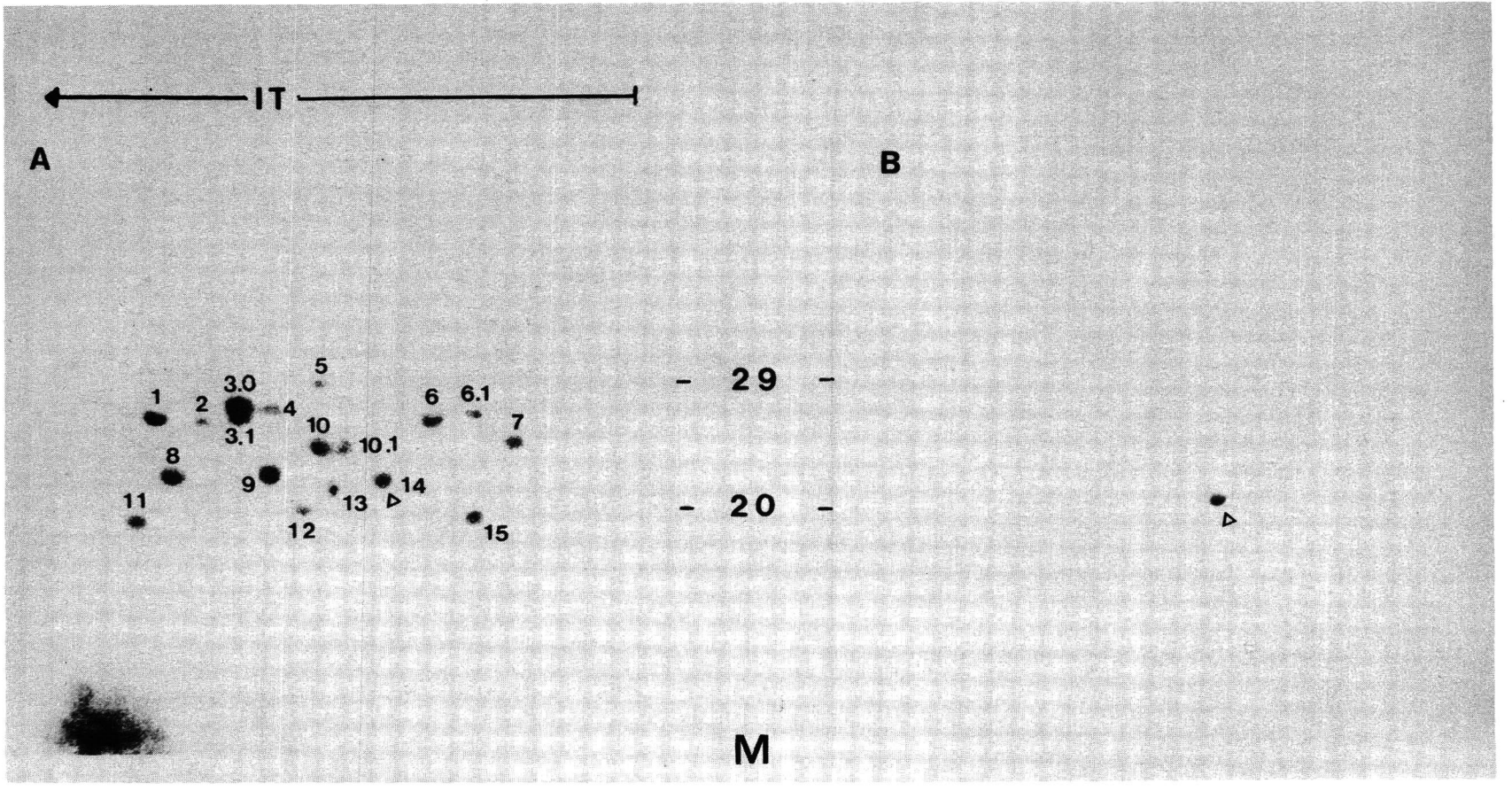

Fig. 3. Two-dimensional protein blot of prosomes probed with STA. Prosomes, purified by gel filtration (Fig. 2) were analysed by two-dimensional gel electrophoresis [14]; then proteins were transferred to nitrocellulose [17]. Blotted proteins were probed with biotinylated STA in lectin buffer. Bound STA was detected by streptavidin-horseradish peroxidase assayed for peroxydase activity. (A) Prosomal proteins stained with Coomassie blue; (B) two-dimensional protein blot probed with STA; (▽) prosomal protein reacting with STA.

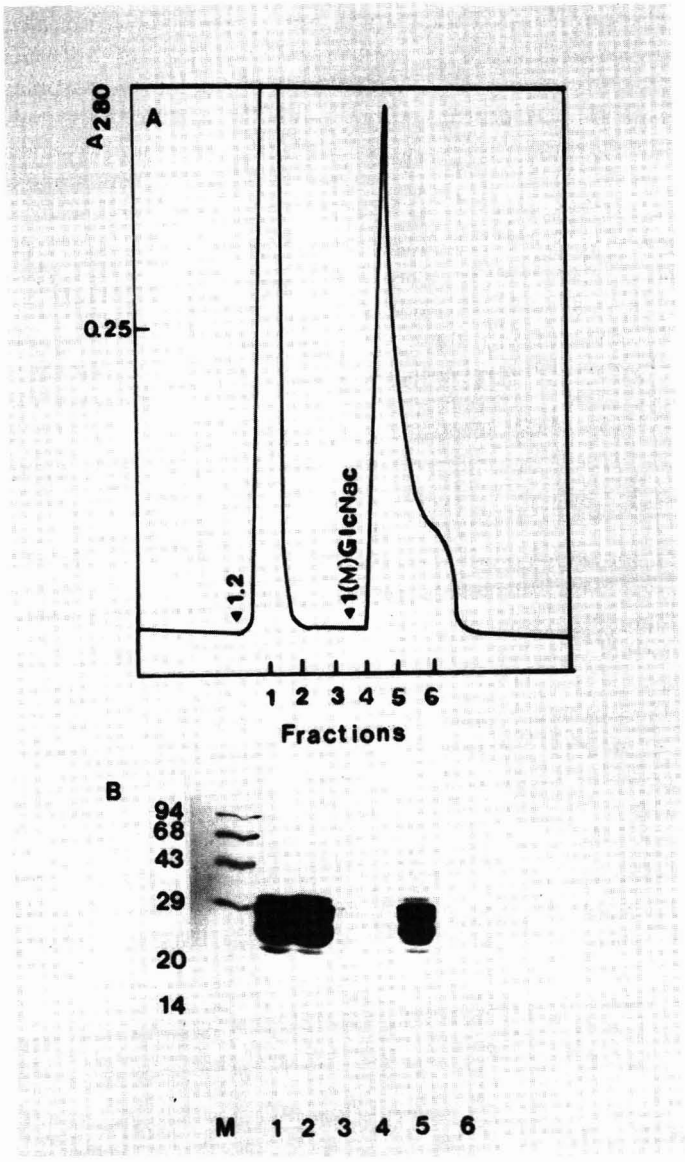

Fig. 4. Affinity chromatography of prosomes on STA agarose. (A) Prosomes (1.5 A_{280} units) purified by gel filtration on a Superose 6 column were applied to divinyl sulphone-activated agarose with immobilized STA. The column was washed with lectin buffer. Bound particles were eluted with 1 *M* N-acetylglucosamine in lectin buffer. 1.2 = Valve position: sample load. (B) Protein composition of bound and unbound prosomes from STA agarose. The proteins were analysed by SDS-PAGE in 12.5% polyacrylamide gel and stained with Coomassie brilliant blue. M = Molecular weight marker proteins in kilodaltons.

particles were eluted as a homogeneous fraction with a retention of 13.5 ml, which corresponds well with a molecular weight of 630 000 dalton and a sedimentation constant of 19S, estimated by ultracentrifugation.

Calf's liver prosomes, purified according to the FPLC procedure, revealed a typical protein pattern when subjected to one- and two-dimensional SDS-PAGE (Figs. 2B and 3A). Differences between the protein patterns of calf's liver prosomes purified via different sucrose gradient centrifugations and FPLC-purified prosomes were not observed (data not shown). In addition, we tested FPLC-purified prosomes for the presence of RNA. As shown in Fig. 2C, they contain RNA in the region of 4S. RNA with the same sedimentation value can be isolated from prosomes purified by sucrose gradient centrifugation [1,2]. This indicates that both methods lead to identical particles. However, the purification by FPLC is faster than the isolation of prosomes by various steps of sucrose gradient centrifugation. With the technique described above we obtained at least 79.8 mg of prosomes from 2030 g calf's liver in 3 weeks.

We propose an alternative method for the purification of prosomes, which is based on the glycosyl residues of prosomal proteins. We recently described the detection of glycosylated proteins of prosomes isolated from erythropoietic mouse cells by the following lectins [11]: Con A, specific for mannosyl- and glycosyl-containing residues; LPA, specific for neuraminic acid; and STA, specific for N-acetylglucosamine.

Interestingly, only one prosomal protein was detected by STA. This corresponds well with the results obtained from calf's liver prosomes, presented in Fig. 3 (protein No. 14). A column with immobilized STA was used for affinity chromatography (Fig. 4). It was found that prosomes were able to bind to immobilized STA via glycosyl residues of one individual prosomal protein. Prosomes were eluted from the matrix with lectin buffer containing 1 *M* N-acetylglucosamine as a competitor. This method could be useful for the isolation of prosomal subcomplexes containing protein No. 14.

ACKNOWLEDGEMENTS

We thank Dr. G. Adam for divinyl sulphone-activated agarose and Professor Dr. K. Köhler for helpful discussions. This work was supported by grants from the Deutsche Forschungsgesellschaft (Schm 620/1-2) and by the Hasselblad foundation.

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Purification of human placental prostaglandin 15-hydroxydehydrogenase

B. TOMBACH*, R. KUSSELER and W. SCHLEGEL
Universitätsfrauenklinik, Albert-Schweitzerstrasse 33, 4400 Münster (F.R.G.)

ABSTRACT

The NAD+-linked prostaglandin 15-hydroxydehydrogenase, which is responsible for the physiological inactivation of prostaglandins by catalysing the first step in the catabolism, was isolated and purified 995-fold from human placenta. The introduction of two new chromatographic steps in the purification procedure is responsible for an achieved specific activity of 1791 mU/mg. The molecular mass of the enzyme, as estimated by fast protein liquid chromatography, was 24 500 dalton. Sodium dodecyl sulphate discontinuous gel electrophoresis of the denatured enzyme revealed a molecular mass of 24 000 dalton. These data suggest that the enzyme consists of a single polypeptide chain.

INTRODUCTION

The enzyme prostaglandin 15-hydroxydehydrogenase (E.C. 1.1.1.141) was discovered in 1964 by Ånggard and Samuelsson [1] when they demonstrated that homogenates from swine lung converted prostaglandins to 15-dehydroprostaglandins. Subsequently an enzyme named 15-hydroxyprostaglandin dehydrogenase was isolated in 1966 and purified 11-fold from the lung cytosol fraction by Ånggard and Samuelsson. Since that time, the enzyme has been identified in several tissues [2–7], followed by a number of successful purifications of the enzyme from bovine lung [8–10], swine lung [11–13], swine kidney [14] and placenta [15–20]. Considerable differences concerning the molecular mass and the enrichment of the enzyme were noted in those publications.

This paper reports the purification of prostaglandin 15-hydroxydehydrogenase from human placenta in highly purified state, using an improved rapid purification technique with fast protein liquid chromatography (FPLC). Sodium dodecyl sulphate (SDS) discontinuous gel electrophoresis of the enzyme was performed to establish the degree of purification.

Using this highly purified protein, studies of the amino acid sequence of the prostaglandin 15-hydroxydehydrogenase might now be possible, giving more information concerning the active site of the enzyme and its regulation.

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EXPERIMENTAL

Materials

DEAE-Trisacryl-M, Sephadex G-75, Blue Sepharose CL-6B and Superose 12 were purchased from Pharmacia-LKB (Uppsala, Sweden), prostaglandin E_2 (PGE_2) from Sigma (Munich, F.R.G.) and NAD^+ and all other reagents from Merck (Darmstadt, F.R.G.).

Spectrophotometric assay

The activity of prostaglandin 15-hydroxydehydrogenase was measured spectrophotometrically following the formation of NADH at 340 nm.

All assays were conducted with blank controls (denatured enzyme solutions which had been boiled for 30 min). The reaction mixture contained 100 m*M* Tris–HCl buffer (pH 7.4), 20 m*M* NAD^+, 4.7 m*M* PGE_2 and 10–400 μl of enzyme in a total volume of 1 ml. The incubation was carried out in the absence of prostaglandin at 37°C for 5 min and the reaction was initiated by the addition of prostaglandin. One unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of 1 μmol of NADH per minute under standard assay conditions.

Protein determination

The protein concentration was determined by the measurement of the absorbance at 280 and 260 nm followed by calculation according to Kalckar [21]. Low concentrations of protein were determined by the method of Bradford [22] using bovine serum albumin (BSA) as a standard.

Electrophoresis

SDS discontinuous gel electrophoresis was performed in 13% gels by the method of Laemmli [23]. The protein bands were stained with silver nitrate.

Fast protein liquid chromatography

The molecular mass of prostaglandin 15-hydroxydehydrogenase was determined using FPLC (Pharmacia). The gel filtration column (30 × 1 cm I.D., Superose 12) was calibrated with dextran blue, BSA, ovalbumin, chymotrypsinogen A and ribonuclease A. Prostaglandin 15-hydroxydehydrogenase was identified by measuring the activity in each fraction.

Enzyme isolation and purification

The method used for the isolation and purification of prostaglandin 15-hydroxydehydrogenase is a modification of the method described by Schlegel and Greep [16].

Step 1. Preparation of placenta up to the first chromatography step. All purification steps were carried out at 0–5°C unless specified otherwise. Two fresh human placentas were placed on crushed ice, dissected free from membranes, washed several times with ice-cold buffer A [20% (v/v) glycerol–10 m*M* potassium phosphate–250 m*M* saccharose–1 m*M* EDTA–1 m*M* dithiothreitol (DTT) at pH 7.4] and cut into small pieces.

After homogenization with an MSE mixer and in an Ultra-Turrax (IKA-Werk,

Stauffen, F.R.G.) for 60 s, the homogenate was centrifuged at 17 000 *g* for 30 min. Cold 1 *M* acetic acid was added to the supernatant solution until the pH had decreased to 5.1 and the solution was allowed to stand overnight in a refrigerator for quantitative precipitation. The solution was centrifuged at 17 000 *g* for 30 min.

The precipitate was suspended in buffer B[20% (v/v) glycerol–10 m*M* potassium phosphate–1 m*M* EDTA–1 m*M* DTT at pH 7.4] and homogenized in a glass vessel with a PTFE pestle and the pH was adjusted to 7.4 with 1 *M* NaOH. After stirring for 1 h the solution was centrifuged at 28 000 *g* for 30 min and the supernatant solution was used for the next step.

Step 2. DEAE-Trisacryl-M chromatography. The supernatant solution was applied to a column of DEAE-Trisacryl-M (21 × 5 cm I.D.) that had previously been equilibrated against buffer B. The column had been washed with 400 ml of buffer B and the elution was carried out with a linear gradient of 1 *M* potassium chloride in buffer B at a flow-rate of 120 ml/h (chamber 1 contained 250 ml of buffer B, chamber 2 contained 250 ml of buffer B with 1 *M* KCl). Fractions of 10 ml were collected and those having enzyme activity were pooled to form the DEAE pool in a total volume of 170 ml.

Step 3. Ammonium sulphate precipitation I. Solid ammonium sulphate was added with stirring to the DEAE pool to achieve 65% saturation. After 16 h at 4°C the suspension was centrifuged at 23 000 *g* for 30 min and the precipitate was dissolved in 50 ml of buffer D [20% (v/v) glycerol–100 m*M* potassium phosphate–1 m*M* EDTA–1 m*M* DTT at pH 7.4]. This solution is termed AS-precipitation I.

Step 4. Sephadex G-75 chromatography. AS-precipitation I was applied to a column of Sephadex G-75 (100 × 5 cm I.D.) equilibrated with buffer D and chromatographed with the same medium. Fractions having enzyme activity were pooled to form the Sephadex G-75 pool in a total volume of 240 ml.

Step 5. Ammonium sulphate precipitation II. This step was done in the same way as the ammonium sulphate precipitation I. After centrifugation the precipitate was dissolved in 50 ml of buffer B. That solution is termed AS-precipitation II.

Step 6. Blue Sepharose CL-6B chromatography. AS-precipitation II was applied to a column of Blue Sepharose CL-6B (25 × 1.5 cm I.D.) that had previously been equilibrated against buffer B. The column was then washed with 50 ml of the same buffer and the enzyme was eluted with a linear gradient of 2 m*M* NADH in buffer B at a flow-rate of 8.0 ml/h (chamber 1 contained 50 ml of buffer B, chamber 2 contained 50 ml of buffer B with 2 m*M* NADH). Fractions having enzyme activity were pooled to form the Blue Sepharose pool in a total volume of 10 ml. Before using that pool in the next step, it was dialysed against buffer E [5% (v/v) glycerol–100 m*M* potassium phosphate–10 m*M* EDTA–5 m*M* DTT at pH 7.4].

Step 7. Fast protein liquid chromatography on Superose 12. The last step of the purification was carried out using the FPLC technique. A gel filtration column (30 × 1 cm I.D) of Superose 12 was used, which was equilibrated with buffer E. The enzyme was eluted at a flow-rate of 0.3 ml/min and a pressure of 0.9 MPa. The fraction size was 0.5 ml. The active fraction is termed the Superose fraction.

RESULTS

Enzyme purification

The purification steps of the placental prostaglandin 15-hydroxydehydrogenase,

TABLE I

PURIFICATION OF HUMAN PLACENTAL PROSTAGLANDIN 15-HYDROXYDEHYDROGENASE

Fraction	Volume (ml)	Protein (mg)	Activity (mU)	Specific activity (mU/mg)	Enrichment	Yield (%)
(1) 28 000 *g* supernatant	200	6060	10 880	1.8	–	100
(2) DEAE pool	170	399.6	8704	21.8	12.1	80
(3) $(NH_4)_2SO_4$ precipitation I	50	248	8000	32.3	17.9	73.5
(4) Sephadex G-75 pool	240	101.4	5352	52.8	29.3	49.2
(5) $(NH_4)_2SO_4$ precipitation II	46	51.3	3901	76	42.2	35.9
(6) Blue sepharose pool	10	1.5	1312	874.7	486	12.1
(7) Superose fraction	0.5	0.068	121.8	1791	995	1.1

resulting in a 995-fold enrichment and a yield of 1.1%, are listed in Table I. The specific activity of the purified enzyme was 1791 mU/mg.

The chromatography on Blue Sephrose CL-6B and the FPLC were the most effective steps, leading to a high purity of the protein.

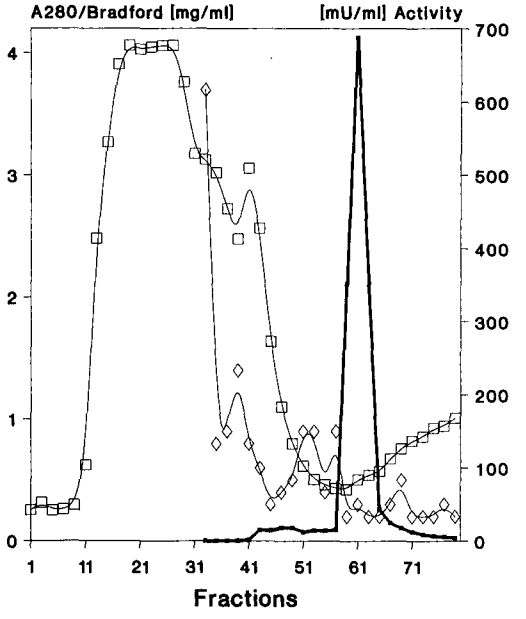

Fig. 1. Elution profile of Blue Sepharose CL-6B. □ = Protein determination by measurement of the absorbance at 280 nm; ◇ = protein determination by the method of Bradford using BSA as standard; ■ = prostaglandin 15-hydroxydehydrogenase activity.

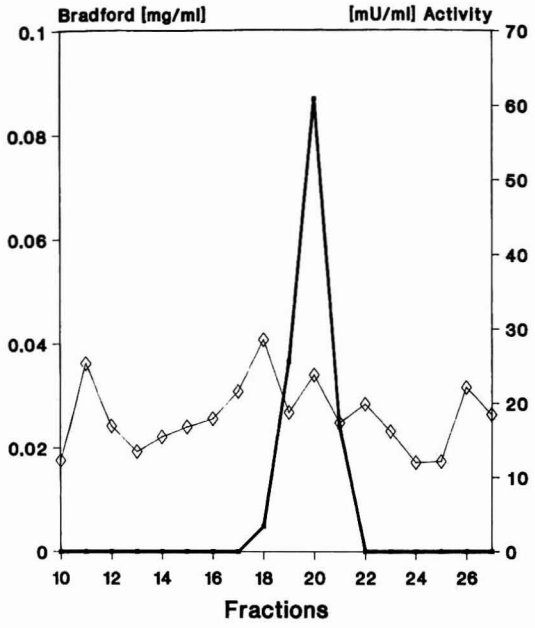

Fig. 2. Elution profile of Superose 12 using the FPLC technique. ◇ = Protein determination by the method of Bradford using BSA as standard; ■ = prostaglandin 15-hydroxydehydrogenase activity.

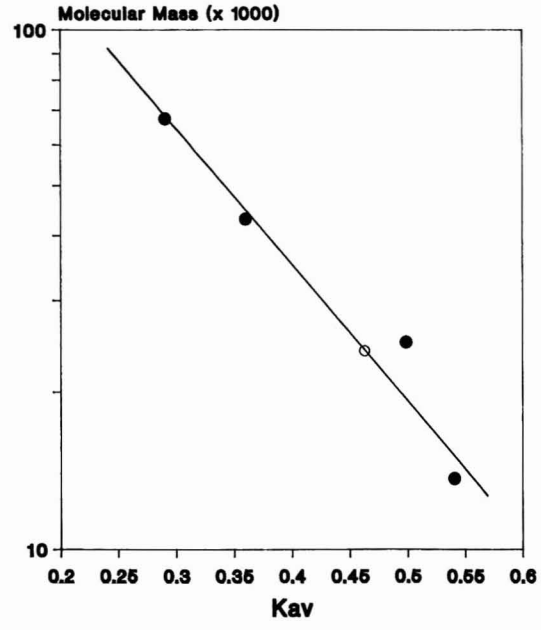

Fig. 3. Molecular mass determination of prostaglandin 15-hydroxydehydrogenase by FPLC. 500 μl of purified enzyme were applied to a Superose 12 gel filtration column (30 × 1 cm I.D.). (●) Protein standards: 1 = BSA (67 000 dalton); 2 = ovalbumin (43 000 dalton); 3 = chymotrypsinogen A (25 000 dalton); 4 = ribonuclease A (13 700 dalton). (○) Prostaglandin 15-hydroxydehydrogenase (24 500 dalton). The partition coefficient K_{av} is defined by $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume, V_0 the void volume and V_t the total volume.

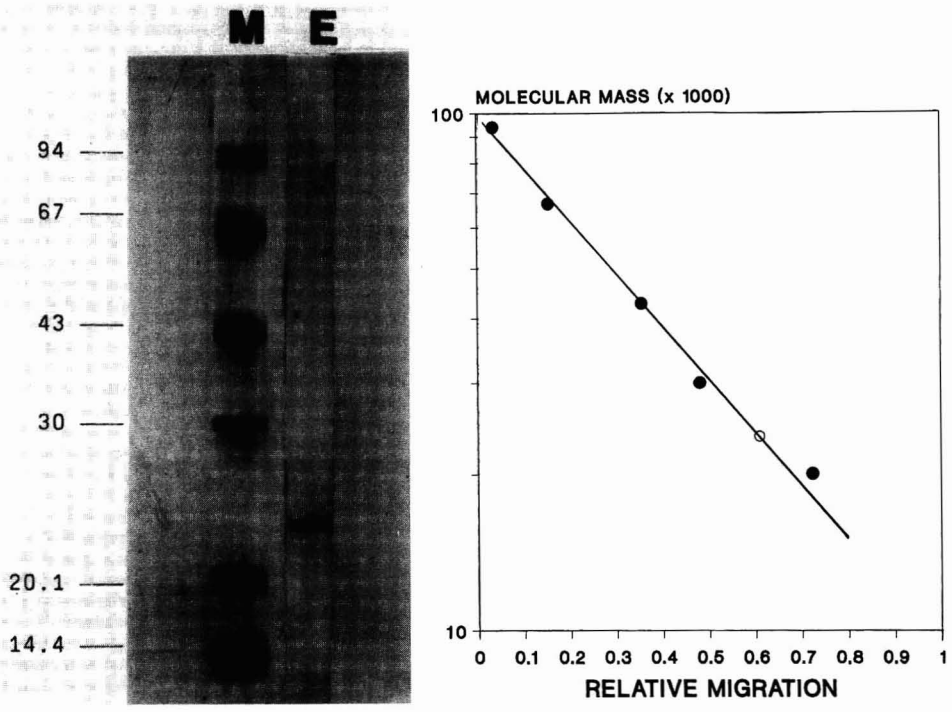

Fig. 4. SDS-gel electrophoresis of prostaglandin 15-hydroxydehydrogenase. The lines denote the positions of the marker proteins (molecular masses, indicated in kilodalton). (M) Phosphorylase *b* (94 000 dalton), BSA (67 000 dalton), ovalbumin (43 000 dalton), carboanhydrase (30 000 dalton), trypsin inhibitor (20 100 dalton), α-lactalbumin (14 400 dalton). (E) Purified enzyme.

Fig. 5. Molecular mass determination of prostaglandin 15-hydroxydehydrogenase by SDS gel electrophoresis. (●) Protein standards: 1 = phosphorylase *b* (94 000 dalton); 2 = BSA (67 000 dalton); 3 = ovalbumin (43 000 dalton); 4 = carboanhydrase (30 000 dalton); 5 = trypsin inhibitor (20 000 dalton). (○) Prostaglandin 15-hydroxydehydrogenase (24 000 dalton).

An NADH gradient was introduced into the Blue Sepharose CL-6B procedure as it released the enzyme in a sharp peak from the column, leading to a 486-fold enrichment. A typical elution profile of Blue Sepharose CL-6B is shown in Fig. 1.

The elution profile of the progressive purification step on Superose 12 using the FPLC technique is shown in Fig. 2.

Determination of molecular mass

Using the FPLC technique, a molecular mass of 24 500 dalton was established for prostaglandin 15-hydroxydehydrogenase (Fig. 3).

SDS discontinuous gel electrophoresis of the denatured enzyme revealed a single band with a molecular mass of 24 000 dalton (Fig. 4). The calculation is based on the retention factor of the enzyme in relation to protein standards (Fig. 5).

DISCUSSION

Prostaglandins play an important physiological role in the female reproduction system. However, still not much is known about the regulation of the prostaglandin metabolism. As placentae tissues are the richest sources of the prostaglandin 15-hydroxydehydrogenase, it was feasible to use placentae for purification of the enzyme. The modified purification procedure resulted in an enzyme fraction with a specific activity of about 1800 mU/mg protein, which is about three times higher than reported previously [16]. Recently, Jarabak and Watkins [20] described a purified enzyme fraction with a specific activity of 8300 mU/mg. Their results are not comparable with ours, however, as they used PGE_1 as the substrate in their test system. There is also a difference in calculating the enrichment of enzyme, as we used the 28 000 *g* supernatant as the basic for calculation, because it is difficult and not reliable to measure NADH production in a mass of tissue samples.

The purification steps 6 and 7 resulted in an extremely high enzyme enrichment, which should be promising for advances in studies on amino acid sequence analysis.

The linear gradient of NADH on Blue Sepharose CL-6B led to an 11.5-fold enrichment in one step, which could not be achieved without NADH.

The concentration of glycerol could be reduced from 20 to 5% (v/v) in the elution buffer of the Superose 12 without any loss of enzyme activity. In order to conserve enzyme activity we had to increase the concentration of EDTA from 1 to 10 m*M* and that of DTT from 1 to 5 m*M* in the buffer. Frozen enzyme solutions in that buffer at −20°C were stable for more than 8 months. The fact that glycerol was not important for the protection of the highly purified enzyme may also indicate that prostaglandin 15-hydroxydehydrogenase is not organized in subunits.

Jarabak and Watkins [20] suggested that the enzyme is split into two inactive identical or nearly identical subunits with a molecular mass of 25 500–26 000 dalton. These findings could not be confirmed in our studies. A molecular mass of 24 500 dalton for the purified enzyme could be established determined by FPLC. The molecular mass of the denatured enzyme, obtained by SDS gel electrophoresis, was almost identical with that obtained by FPLC. These results provide strong evidence that the enzyme consists of a single polypeptide chain with a size of *ca.* 24 000 dalton.

Preliminary studies on the amino acid content of the enzyme demonstrated Amadori products, which indicate that there are carbohydrate molecules associated with the protein structure (unpublished results).

Further studies on the organization of the prostaglandin 15-hydroxydehydrogenase, especially the analysis of the amino acid sequence, are necessary in order to understand the physiological control of the prostaglandin metabolism.

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Purification of the membrane-form variant surface glycoprotein of *Trypanosoma brucei*

DIETMAR SCHELL and PETER OVERATH*
Max-Planck-Institut für Biologie, Correnssstrasse 38, D-7400 Tübingen (F.R.G.)

ABSTRACT

The membrane-form variant surface glycoprotein (mfVSG) is anchored in the plasma membrane of African trypanosomes by a diacylglycerol residue. On cell rupture the anchor is rapidly cleaved by an endogenous phospholipase C. A purification procedure is described which results in native mfVSG devoid of lipase activity. A total membrane fraction is prepared in the presence of the SH-inhibitor *p*-chloromercuribenzenesulphonic acid (pCMBS). Membrane proteins are solubilized in the presence of pCMBS and the detergent Zwittergent 3-12, conditions which inhibit the activity of the phospholipase. mfVSG is then purified by successive chromatography on rabbit anti-VSG affinity and cation-exchange columns (25% yield). The isolated protein is electrophoretically pure and partitions into the detergent phase on Triton X-114 phase separation, proving that it retains the diacylglycerol anchor.

INTRODUCTION

African trypanosomes, exemplified by *Trypanosoma brucei*, are unicellular flagellates which cause human sleeping sickness and Nagana in cattle. The surface of one cell is covered by 10^7 copies of a single protein, the membrane-form variant surface glycoprotein (mfVSG) which is anchored in the membrane by a glycosylphosphatidylinositol residue (GPI [1,2]). On cell rupture, an endogeous GPI-specific phospholipase C rapidly converts mfVSG to its soluble form (sVSG) and diacylglycerol [3–6]. In a recent investigation [7], sVSG was used as a model for analysing the denaturation–renaturation properties of a glycoprotein. In order to extend such biophysical studies to the membrane-form VSG, a purification procedure yielding native protein devoid of phospholipase C was required. Previous protocols for purifying mfVSG have used denaturing conditions [8–12], whereas the preparation described here was obtained under non-denaturing conditions and was free from both sVSG and phospholipase C.

EXPERIMENTAL

Materials

N^{α}-*p*-Tosyl-L-lysine chloromethyl ketone (TLCK) (cat. no. 17013), *p*-chloromercuribenzenesulphonic acid (pCMBS) (cat. No. 16900), N-(2-hydroxymethyl)-piperazine-N′-(2-ethanesulphonic acid) (HEPES) (cat. No. 25245), 2-(N-morpho-

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lino)ethanesulphonic acid (MES) (cat. No. 29834), Triton X-100 (cat. no. 37238) and Triton X-114 (cat. No. 37243) were purchased from Serva (Heidelberg, F.R.G.), octyl glucoside (cat. No. O-8001) from Sigma (Deisenhofen, F.R.G.) and Zwittergent 3-12 (cat. No. 693015) from Calbiochem (Frankfurt, F.R.G.).

Cells

Trypanosoma brucei (variant clone MITat 1.2 [1]) was raised in rats and purified by DEAE-cellulose chromatography [13].

Antibodies

sVSG purified by DEAE-cellulose chromatography and isoelectric focusing [14] was used for the immunization of a New Zealand white rabbit [15]. The immunoglobulin G (IgG) was isolated from serum by ammonium sulphate precipitation and DEAE-cellulose chromatography. The purified antibody was coupled to activated CH-Sepharose 4B (Pharmacia–LKB, Freiburg, F.R.G.) as described by the manufacturer.

Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [16] using 4.5% acrylamide in the stacking gel and 10% in the separation gel. Proteins were rendered visible by staining with Coomassie Brilliant Blue R-250.

Detergent extract of membrane proteins

Cells ($4 \cdot 10^{10}$) were lysed in 40 ml of ice-cold 5 m*M* HEPES–0.1 m*M* TLCK–10 m*M* pCMBS (pH 7.0) using a Branson sonifier at 60 W for 2 min. The lysate was centrifuged at 4°C and 105 000 *g* for 1 h. The pellet was taken up in 40 ml of 5 m*M* HEPES–25 μM TLCK–2.5 m*M* pCMBS (pH 7.0), centrifuged and extracted with 40 ml of 5 m*M* HEPES–0.1 m*M* TLCK–5 m*M* pCMBS–20 mg/ml Zwittergent 3-12 (pH 7.0) by sonication. Centrifugation yielded a clear detergent extract.

Affinity chromatography

The detergent extract was adjusted to 150 m*M* NaCl using a 1.5 *M* stock solution. A 10-ml volume of rabbit anti-VSG-Sepharose 4B with 10 mg of antibody per ml of gel was packed into an HR 10/10 column. The purification was performed in a fast protein liquid chromatographic (FPLC) system (Pharmacia–LKB). The column was equilibrated with phosphate-buffered saline (PBS)–0.2% Triton X-100 at a flow-rate of 0.5 ml/min. A 15-ml volume of the detergent extract was loaded from a 50-ml Superloop at a rate of 0.3 ml/min. The column was washed with 100 ml of PBS–0.2% Triton X-100 at 0.5 ml/min and the VSG was eluted in 2-ml fractions with 200 m*M* glycine–0.2% Triton X-100 (pH 2.5) at 0.5 ml/min in tubes containing 100 μl of 1 *M* Tris–Cl (pH 8.0). The column was used again after re-equilibration in PBS–Triton X-100 or stored in the presence of 0.1% sodium azide.

Cation-exchange chromatography

The pooled mfVSG-containing fractions were dialysed against buffer A [20 m*M* MES–0.2% Triton X-100 (pH 6.0)]. The sample was applied to a MonoS HR 5/5

cation-exchange column (50 mm × 5 mm I.D., bed volume 1 ml) equilibrated with 10 ml of buffer A. The whole cation-exchange purification was run at 1 ml/min. The column was washed with 10 ml of buffer A and then 10 ml of buffer B [20 m*M* MES–1% octylglucoside (pH 6.0)]. The bound proteins were eluted with 20 ml of a linear salt gradient of 0–400 m*M* NaCl using buffer B and buffer C [20 m*M* MES–1% octylglucoside–1 *M* NaCl (pH 6.0)]. At the end of the gradient the column was washed with buffer C and re-equilibrated with buffer A. mfVSG-containing fractions were pooled after assessing the purity of mfVSG by SDS-PAGE.

Protein determination

Protein was measured by the method of Peterson [17] using bovine serum albumin as a protein standard.

Triton X-114 phase separation

Phase-separation experiments [18] were carried out using 50 μl of precondensed Triton X-114 (2% in PBS) and 5 μl of the protein sample. A detergent concentration of 0.2% Triton X-100 in the protein solution did not interfere with the phase separation.

RESULTS AND DISCUSSION

The objective of the purification procedure was the isolation of native, phospholipase C-free mfVSG which could be used for subsequent physico-chemical studies. As mfVSG and the phospholipase show a similar behaviour on ion-exchange columns, an affinity column of immobilized anti-VSG antibody was chosen as a first

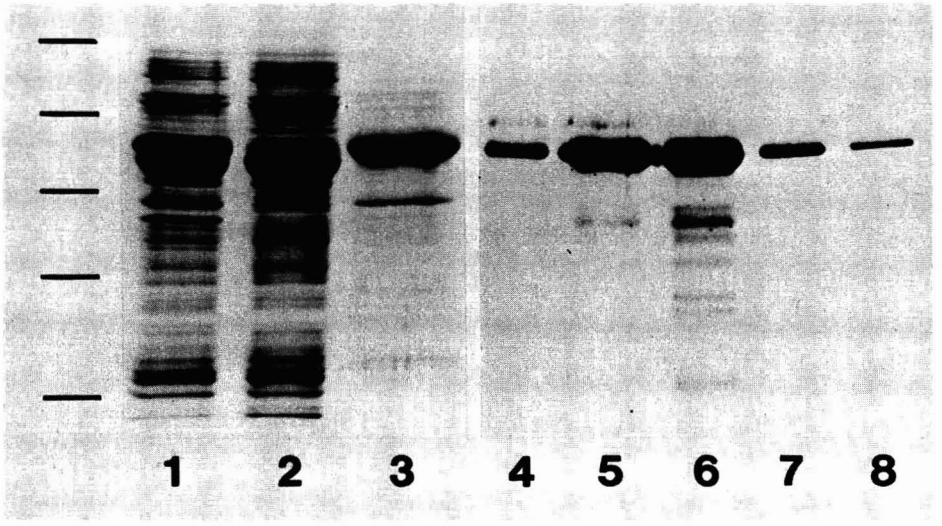

Fig. 1. SDS-PAGE of mfVSG. Lanes: 1 = homogenate of whole cells; 2 = supernatant of the homogenate after centrifugation (soluble proteins); 3 = detergent extract of membrane proteins after centrifugation; 4–8 = protein pattern produced by successive fractions eluted from the rabbit anti-VSG affinity column with glycine (pH 2.5). Molecular mass standards from top to bottom 116 000, 66 000, 45 000, 29 000 and 14 300 dalton.

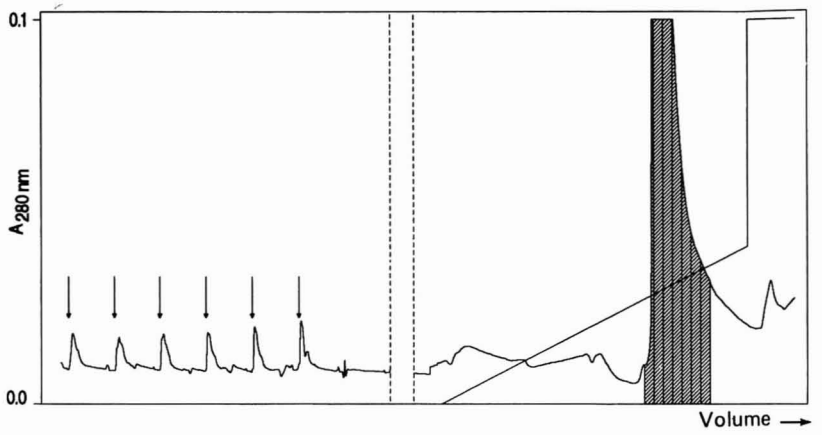

Fig. 2. Cation-exchange chromatography on a Mono S column: sequential sample application (6 times 500 μl), washing with buffer A. The detergent exchange to buffer B is not shown (space between vertical dotted lines); elution of mfVSG (shaded fractions) with a linear salt gradient. The graph is corrected to a common baseline for buffer A (before the dotted lines) and buffer B (after the dotted lines).

step for separating the glycoprotein from the enzyme. The cells were lysed in the presence of the detergent Zwittergent 3-12 and high concentrations of the sulphydryl reagent pCMBS in order to inhibit the phospholipase [4,12]. The detergent extract was

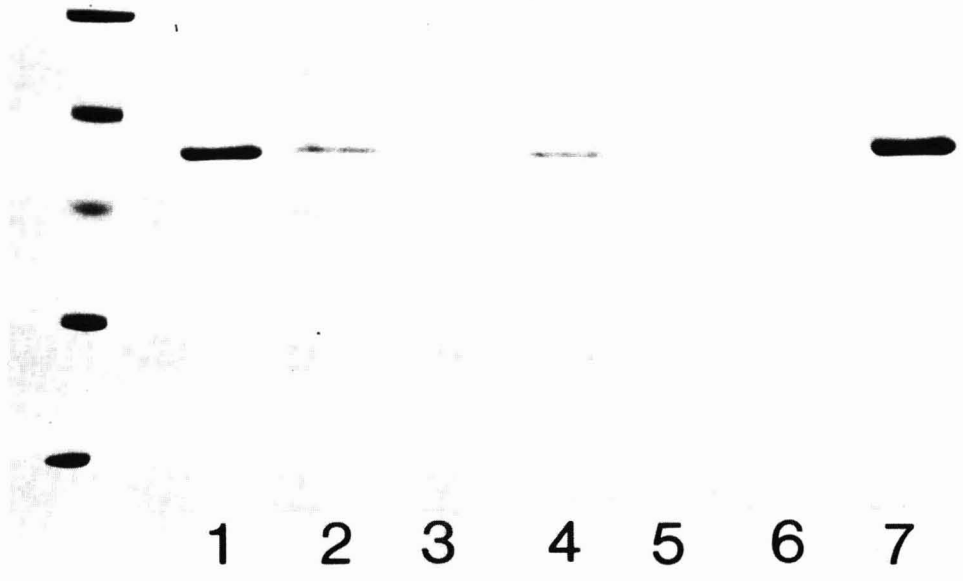

Fig. 3. Triton X-114 phase separation of mfVSG and sVSG. mfVSG eluted from the Mono S column was either directly applied to an SDS polyacrylamide gel (lane 1) or first subjected to a Triton X-114 phase separation (lane 2, detergent phase; lane 3, aqueous phase). Alternatively, mfVSG was first incubated for 1 h at 39°C and then subjected to a phase separation (lane 4, detergent phase; lane 5, aqueous phase). Lanes 6 and 7 refer to the detergent and aqueous phases, respectively, of a sample of sVSG. Molecular mass standards as in Fig. 1.

passed over the affinity column; mfVSG was eluted with glycine buffer (pH 2.5) and immediately neutralized. Fig. 1 shows the SDS-PAGE of the starting material and of the fractions eluted from the affinity column. The prominent band at an apparent M_r of 55000 is mfVSG. A multitude of minor impurities could be removed by chromatography on a cation-exchange column to which the protein binds at pH 6.0 and can be eluted at about 290 m*M* NaCl (Fig. 2). If the protein was eluted with a salt gradient using the detergent Triton X-100 instead of octylglucoside, the mfVSG eluted at 150 m*M* NaCl. On either column about half of the mfVSG could not be recovered. The final product (6 mg of protein, yield *ca.* 25%) was pure as judged by SDS-PAGE (Fig. 3, lane 1), even on highly overloaded gels (not shown).

In order to investigate whether the isolated product was mfVSG, *i.e.*, that it retained the diacylglycerol anchor, a phase separation using Triton X-114 [18] was performed. As shown in Fig. 3 (lanes 2 and 3), the protein was entirely recovered in the detergent phase as expected for an amphiphilic molecule. This pattern was not changed if the protein was first incubated for 1 h at 39°C (lanes 4 and 5). According to this criterion, mfVSG is free from phospholipase C. As a control, sVSG was entirely recovered in the aqueous phase (lanes 6 and 7). The fact that mfVSG was entirely recovered in the detergent phase is a strong argument for its native state because the denatured protein is recovered in the aqueous [19].

In conclusion, the procedure yields mfVSG devoid of contaminating GPI-specific phospholipase C in adequate yield.

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Purification of the glycoprotein glucose oxidase from *Penicillium amagasakiense* by high-performance liquid chromatography

HENRYK M. KALISZ*, JÖRG HENDLE and ROLF D. SCHMID
Department of Enzyme Technology, GBF–Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, D-3300 Braunschweig (F.R.G.)

ABSTRACT

Fast protein liquid chromatography (FPLC) in combination with ion-exchange chromatography on a Mono Q column was used to purify glucose oxidase from *Penicillium amagasakiense* to homogeneity. Purification was performed with a mixed pH and salt gradient, with 20 m*M* phosphate buffer (pH 8.5) as starting buffer (A) and 50 m*M* acetate buffer (pH 3.6) with 0.1 *M* NaCl as elution buffer (B). Elution conditions were optimized to permit the simultaneous purification and separation of the glucose oxidase isoforms. Three peaks, each consisting of 1–2 isoforms and exhibiting a homogeneous titration curve profile, were resolved with a very flat linear gradient of 5.0–5.1% B in 40 ml. Three more peaks, each consisting of several isoforms, were eluted at 10%, 30% and 100% B. Optimization of the elution conditions and separation of the glucose oxidase isoforms was only possible because of the rapidity of each purification step and the high resolution provided by FPLC and Mono Q.

INTRODUCTION

The glycoprotein glucose oxidase (GO) (E.C. 1.1.3.4) is of considerable commercial importance [1–3]. GO catalyses the oxidation of glucose to D-glucono-1,4-lactone. The enzyme is produced by several filamentous fungi, those from *Aspergillus niger* and *Penicillium* species being commercially the most important. Despite its commercial importance, structural information about the enzyme is not available, owing to the lack of growth of crystals suitable for X-ray analysis. The large carbohydrate moiety, accounting for 10–16% of the molecular mass of GO, is believed to inhibit the crystallization process. Cleavage of the carbohydrate moiety from *A. niger* GO was shown to be an important prerequisite step for the growth of crystals suitable for X-ray diffraction analysis [4].

However, other factors, such as sample purity and homogeneity, are important criteria for crystallization, as contaminants can hinder the crystallization proces [5]. Development of purification techniques, such as fast protein liquid chromatography (FPLC), together with high-resolution liquid chromatographic resins, such as MonoBeads, has greatly enhanced protein purification procedures [6,7]. Consequently, the time required for each purification step has been greatly reduced and peak

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resolution significantly increased. Further, optimum purification conditions can be quickly established.

This paper decribes the application of FPLC to the purification of GO from *Penicillium amagasakiense*. The enzyme was purified to homogeneity on a Mono Q column, using a mixed pH and salt gradient. In addition, the procedure served to separate the multiple GO isoforms.

EXPERIMENTAL

Materials

GO from *P. amagasakiense* was purchased from Nagase Biochemicals (Osaka, Japan). All chemicals were obtained from Merck (Darmstadt, F.R.G.) and PhastGel polyacrylamide gels from Pharmacia–LKB (Uppsala, Sweden).

Methods

GO activity was assayed at 420 nm by the method of Sahm *et al.* [8] using 2,2′-azino-di(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as dye and 0.1 *M* glucose as substrate. Assays were performed in 0.1 *M* acetate buffer (pH 6) at 25°C under oxygen saturation.

Protein was determined by the method of Bradford [9] using Coomasie Brilliant Blue G reagent (Bio-Rad Labs.) with bovine serum albumin as a standard.

Polyacrylamine gel electrophoresis (PAGE) was performed either in the presence (dissociating conditions) or in the absence (non-dissociating conditions) of sodium dodecyl sulphate (SDS) on 10–15% or 8–25% gradient gels, respectively, using the Phast System (Pharmacia–LKB) according to the manufacturer's instructions [10]. Isoelectric focusing was performed in the pH range 4.0–6.5 according to Olsson *et al.* [11]. Electrophoretic titration curves were generated by performing electrophoresis perpendicular to a stable pH gradient (in the pH range 3.0–9.0) according to Haff *et al.* [12], as described by Jacobson and Skoog [13]. Gels were silver stained by the method of Butcher and Tomkins [14].

Purification

Purification was performed with a Pharmacia FPLC unit equipped with two P-500 pumps, an LCC-500 controller and an LKB 2238 Univord SII ultraviolet monitor, fitted with a 280-nm filter. Chromatograms were recorded with an LKB 2210 two-channel recorder. Samples were collected with a FRAC-100 fraction collector. GO was dissolved in and dialysed against the starting buffer and applied to a Mono Q HR 5/5 column pre-equilibrated with the appropriate buffer. All samples and buffers were filtered through 0.22-μm Millex-GV_{13} (Millipore) or cellulose acetate (Sartorius) filters before use. GO-containing fractions were pooled, desalted and concentrated as described previously [4]. Sample purity and homogeneity were assessed electrophoretically. Unless stated otherwise, a flow-rate of 2 ml/min was used.

RESULTS AND DISCUSSION

Following the successful crystallization of the purified and deglycosylated *A. niger* GO [4], similar success was envisaged with the *P. amagasakiense* GO. However,

despite its purification to homogeneity by native and SDS-PAGE [15], the deglycosylated enzyme could not be crystallized, probably owing to its isoelectric heterogeneity [16]. Therefore, a reduction in the number of its isoforms before deglycosylation was considered important.

Ion-exchange chromatography on a Mono Q column was used for the purification of *P. amagasakiense* GO. The influence of the steepness of the salt gradient on GO elution at different pH values was initially tested. The resolution of the GO isozymes was poor under all conditions tested.

However, the resolution was significantly improved by using a mixed pH and salt gradient, with 20 m*M* phosphate buffer (pH 8.5) as starting buffer and 100 m*M* acetate buffer (pH 3.6) with 200 m*M* NaCl as elution buffer (Fig. 1A). The resolution of the three peaks was further improved by a flattening of the linear gradient from 0–25% B in 15 ml to 5–10% B in 15 ml (Fig. 1B).

A major improvement in the resolution of the GO isoforms was achieved with 50 m*M* acetate buffer (pH 3.6) and 100 m*M* NaCl as elution buffer (Fig. 2a). Minor changes in the slope of the gradient dramatically affected the elution profile of GO. Thus, a flattening of the linear gradient from 5–10% B in 15 ml to 5.0–5.1% B in 12 ml permitted the resolution of three sharp peaks (Fig. 2b). Optimum resolution was achieved with a linear gradient of 5.0–5.1% B in 40 ml at a flow-rate of 1 ml/min (Fig.

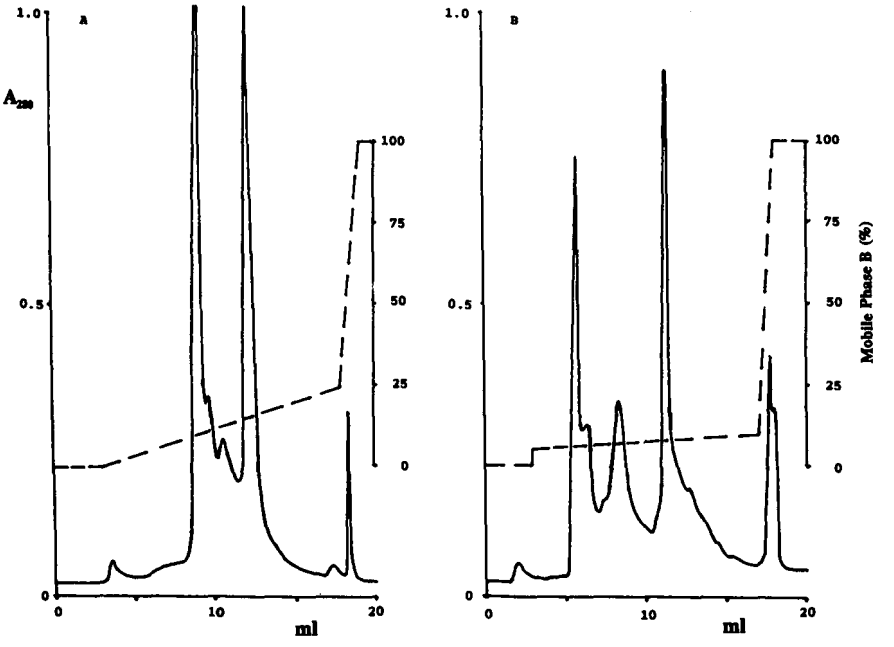

Fig. 1. Influence of a mixed pH and steepness of the salt gradient on the separation of *Penicillium amagasakiense* GO isoforms. GO was applied to a Mono Q column in 20 m*M* potassium phosphate buffer (pH 8.5) (buffer A) and eluted with 100 m*M* sodium acetate buffer (pH 3.6)–0.2 *M* NaCl (buffer B) with a gradient of (A) 0–25% B in 15 ml and (B) 5–10% B in 15 ml.

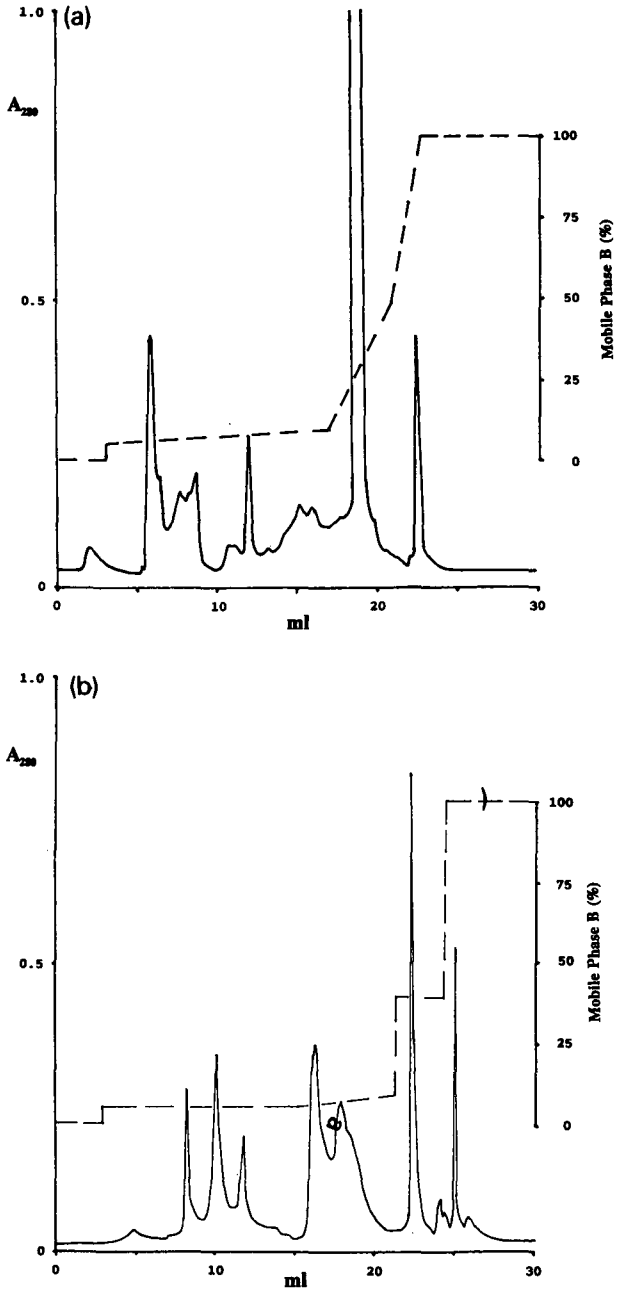

Fig. 2. Influence of a mixed pH and steepness of the salt gradient on the separation of *Penicillium amagasakiense* GO isoforms. GO was applied to a Mono Q column in 20 m*M* potassium phosphate buffer (pH 8.5) (buffer A) and eluted with 50 m*M* sodium acetate buffer (pH 3.6)–0.1 *M* NaCl (buffer B) with a gradient of (a) 5–10% B in 15 ml, 10–50% B in 4 ml and 50–100% B in 2 ml and (b) 5.0–5.1% B in 12 ml, 5.1–10% B in 6 ml then in stepwise increments at 30% B and 100% B.

3A). The three GO peaks each consisted of a maximum of two isoforms (Fig. 3B). Further, these samples exhibited a homogeneous profile in the pH range 3–9 on an electrophoretic titration curve (Fig. 4). The homogeneous *P. amagasakiense* GO isoform which eluted as peak 1 at 5% B has been successfully crystallized, with the crystals diffracting to at least 2.2 Å resolution [17]. Detailed analysis of the properties of each isoform, especially protein and carbohydrate composition and stability, is currently in progress.

Peaks eluted at or above 10% B were heterogeneous with respect to both their isoelectric forms and titration curve profiles. This heterogeneity, in contrast to that of *A. niger* GO [4], could not be eliminated or reduced by deglycosylation. This implies that, in contrast to the *A. niger* GO [18], variations in the carbohydrate content

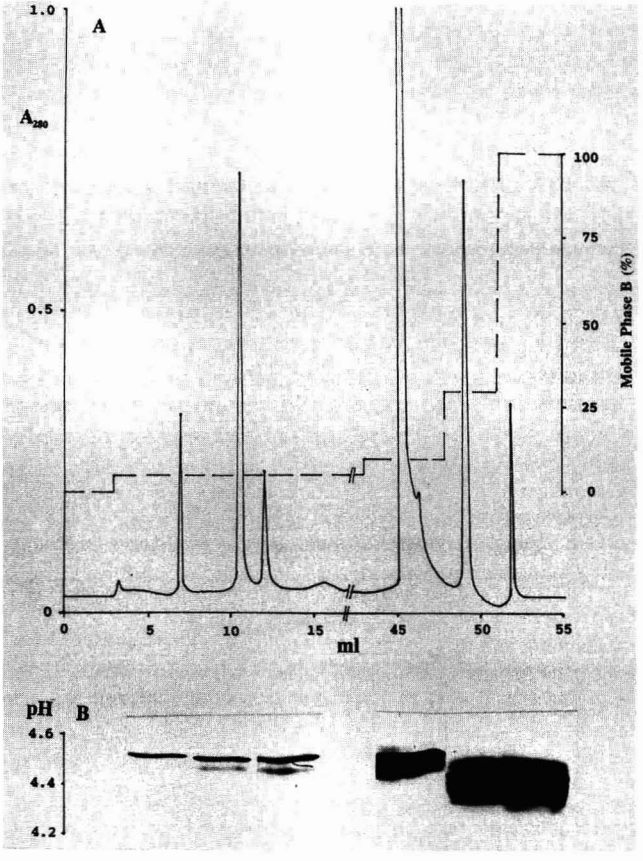

Fig. 3. Separation of *Penicillium amagasakiense* GO isoforms (A) on a Mono Q column and (B) by isoelectric focusing. GO was applied to a Mono Q column in 20 m*M* potassium phosphate buffer (pH 8.5) (buffer A) and eluted with 50 m*M* sodium acetate buffer (pH 3.6)–0.1 *M* NaCl (buffer B) with a linear gradient of 5.0–5.1% B in 40 ml, then in stepwise increments at 10% B, 30% B and 100% B. Isoelectric focusing was performed in the pH range 4.0–6.5, as described under Experimental.

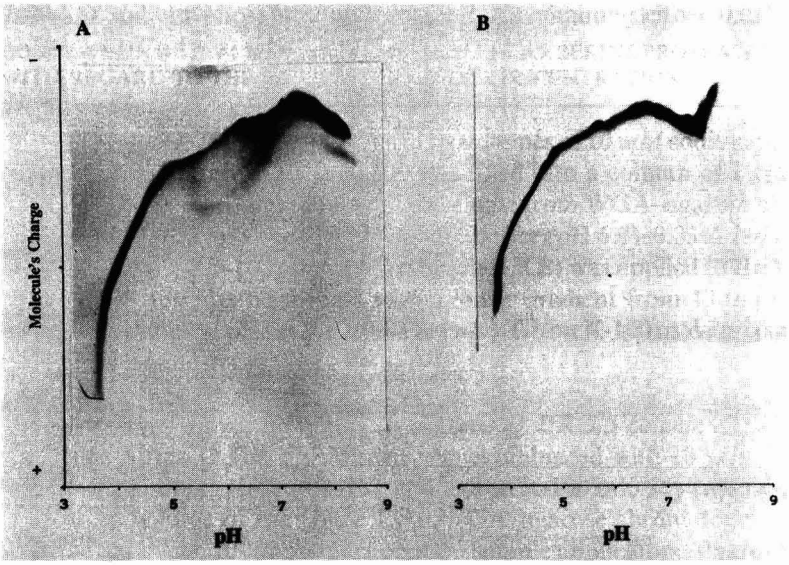

Fig. 4. Electrophoretic titration curve of *Penicillium amagasakiense* GO (A) before and (B) after purification (peaks 1–3) under the conditions as in Fig. 3. The electrophoretic titration curve was generated by prefocusing the ampholytes across a Phast IEF gel [10], then by running electrophoresis perpendicular to the pH gradient [12,13]. The sample components migrate towards the anode or cathode according to their net charge at any particular pH [6].

probably do not contribute to the heterogeneity of the *P. amagasakiense* GO. A reduction in heterogeneity is being attempted by further modifications of the buffer composition and elution conditions.

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Analytical fractionation of microsomal cytochrome P-450 isoenzymes from rat liver by high-performance ion-exchange chromatography

PETER H. ROOS
Institut für Physiologische Chemie I, Abteilung Bioenergetik, Geb. MA 2/136, Ruhr-Universität Bochum, 4630 Bochum (F.R.G.)

ABSTRACT

Ion-exchange Fast Protein Liquid Chromatography (FPLC) on Mono Q and Mono S was optimized for the analytical separation of microsomal cytochrome P-450 species from rat liver. The effects of detergent, pH, gradient profile and column load on resolution are demonstrated. Successive application of anion- and cation-exchange chromatography leads to eleven separated P-450 fractions. The altered microsomal P450 pattern after treatment of rats with various inducers is reflected by distinct elution profiles. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and enzymatic analysis imply that several FPLC fractions contain more than one P-450 species. Preliminary results are presented showing the suitability of immobilized metal affinity chromatography (IMAC) for general P-450 fractionation and thus for the further resolution of Mono Q and Mono S fractions. Scale-up for preparative P-450 fractionation is easily done by adapting the optimized analytical FPLC procedures to Q- and S-Sepharose Fast Flow.

INTRODUCTION

The cytochrome P-450 enzyme family includes a large number of different protein species [1] which share as a common feature the ability to form a $P\text{-}450_{reduced}$–CO complex with a specific absorption at about 450 nm [2]. The number of known P-450 species in rat liver microsomes is more than 20 and is still increasing, as illustrated by the recent isolation of two new forms [3]. Because of structural and functional similarities of the P-450 isozymes, purification is often difficult and results in low yields. For example, up to six chromatographic steps are needed to obtain purified P-450IIE1 (= P-450j) [4].

The apparent molecular weight [sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)] of the P-450 species is in the range 46 000–57 000 dalton with a clustering at about 51 kilodalton. Therefore, SDS-PAGE is often insufficient as a criterion for P-450 homogeneity and has only a limited value for P-450 identification. In addition to similar molecular weights, P-450 species can exhibit similar or overlapping spectroscopic [5], immunochemical [6,7] and enzymatic properties [8,9]. These circumstances make it difficult to establish a simple method for analysing the pattern of a complex P-450 mixture such as rat liver microsomes. Because individual

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P-450s cannot be identified by a single criterion, any analytical procedure ends up being a combination of different methods.

High-resolution chromatographic techniques such as high-performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) have not often been used for P-450 purification. Their analytical application is restricted to only a few studies [10,11] and FPLC has been chosen for this purpose only by Sakaki *et al.* [12] to demonstrate the distinctness of purified P-450 species and by Kastner and Schulz [13] to differentiate inductor effects on the P-450 pattern of liver microsomes of marmoset monkeys.

Because the P-450 pattern in a given species or individual varies with age [14], sex [15], physiological status [16] and/or the action of drugs and xenobiotics [17], a simple analytical system would be useful in follow-up studies of developmental or pathological events or in diagnostics to evaluate xenobiotica exposure. In this paper we present results concerning primarily the first step in the analytical procedure, *viz.*, the chromatographic resolution of a complex P-450 mixture by FPLC.

EXPERIMENTAL

Animals and animal treatment

Sprague–Dawley rats (200–250 g) were purchased from Lippische Versuchstierzucht (Extertal, F.R.G.). They were induced by phenobarbital or β-naphthoflavone as described by Guengerich and Martin [18]. Induction by hexachlorobenzene (HCB) was done by three intraperitoneal injections (20 mg of HCB per 0.5 ml of corn oil) on three subsequent days. Liver dissection followed on the next day.

Preparation and solubilization of liver microsomes

Rat liver microsomes were prepared by the method of Guengerich [19] with additional 0.4 m*M* phenylmethylsulphonyl fluoride (PMSF) in all the solutions used. For microsome solubilization the suspension was adjusted to 2 mg/ml of protein with 100 m*M* Tris–HCl (pH 7.7), 20% glycerol, 1 m*M* EDTA, 1 m*M* dithioerythritol (DTE), 0.4 m*M* PMSF and 0.8% Lubrol PX and stirred for 30 min at 4°C. Insoluble material was removed by centrifugation at 105 000 *g* for 1 h at 4°C. The clear supernatant was passed through a 0.2-μm filter (Minisart NML, SM 16534, Sartorius) prior to FPLC fractionation. To test the solubilization efficiency of detergents we used the same procedure and buffer system including the detergents as specified in Table I. After centrifugation the supernatant was analysed for protein and P-450 content (see below).

Chromatography

Analytical and preparative separations were performed with an automatic FPLC system from Pharmacia (Uppsala, Sweden) equipped with two pumps, a gradient controller, various valves and a fraction collector. Protein and haemoprotein were detected by continuous simultaneous monitoring at 280 and 417 nm. Data processing and storage were performed with a personal computer (Atari PC3) equipped with a PC Integration Pack (Softron, Gräfeling, F.R.G.). For analytical separations 1-ml columns (HR 5/5) of the strong ion exchangers Mono Q (anion) and Mono S (cation) were used. The composition of the equilibration and elution buffers

was as follows: Mono Q-A, 20 m*M* Tris–HCl (pH 7.7), 20% glycerol, 1 m*M* EDTA, 1 m*M* DTE, 0.2 m*M* PMSF, 0.2% Lubrol PX; Mono Q-B, same as Mono Q-A + 1 *M* NaCl; Mono S-A, same as Mono Q-A but with 20 m*M* 3-(N-morpholino)propanesulphonic acid (pH 7.0) instead of Tris–HCl; Mono S-B, same as Mono S-A + 1 *M* NaCl.

Preparative fractionations were carried out in FPLC columns (HR 16/10 and HR 10/10; Pharmacia) filled with 20 ml of Q- or 7.5 ml of S-Sepharose Fast Flow, respectively, using the same buffer systems as for Mono Q or Mono S separations.

Immobilized metal affinity chromatography (IMAC) was performed in 1-ml FPLC columns (HR 5/5) filled with Chelating Sepharose Fast Flow. Charging the gel with metal ions was done according to the manufacturer's user's manual [20]. Buffers for elution by pH gradient were IMAC-A1 for equilibration, consisting of 50 m*M* sodium phosphate (pH 7.0), 500 m*M* NaCl, 20% glycerol and 0.2% Lubrol PX, and IMAC-B1 for elution, with the same composition as IMAC-A1 but adjusted to pH 3.8. Buffers for elution with a competitive ligand were IMAC-A2 for equilibration, consisting of 50 m*M* sodium phosphate (pH 7.2), 500 m*M* NaCl, 20% glycerol and 0.2% Lubrol PX, and IMAC-B2 for elution with the same composition as IMAC-A2 but with an additional 2 *M* NH_4Cl.

Column treatment. The monobead ion exchangers were washed and re-equilibrated immediately after each run by subsequent application of 5 ml of buffer A, 10 ml of buffer B and 5 ml of buffer A (buffers as specified above). Because of increased back-pressure the columns were extensively washed after about 20 runs by the following procedure: (1) alternate application of 0.5 ml of 1 *M* NaOH and 2 ml of water in the reverse flow direction until the back-pressure remained constant; (2) washing with 5 ml of 0.5% trifluoroacetic acid in acetonitrile and subsequently 5 ml of water; and (3) re-equilibration as described above. The Chelating Sepharose was washed with 5 ml of 100 m*M* EDTA (pH 7.2) and 5 ml of water after each run and equilibrated with Zn^{2+} by passing 2 ml of 200 m*M* $ZnSO_4$ solution through the column.

Analytical methods

Column fractions were analysed by SDS-PAGE (8% acrylamide) on slab gels according to Laemmli [21]. Protein bands were rendered visible by silver staining [22]. The P-450 and protein content of the fractions were determined by the methods of Omura and Sato [2] and Lowry *et al.* [23].

Testosterone metabolizing activity

For partial removal of detergent, FPLC fractions were incubated with Biobeads (Bio-Rad Labs., Munich, F.R.G.) (20 mg of beads per mg of protein) for 1 h at 4°C. To evaluate the testosterone-metabolizing activity the sample was supplemented with 10 μg/ml of dilauroylphosphatidylcholine, 125 μ*M* of testosterone and 50 μ*M* of cumene hydroperoxide and incubated for 10 min at 37°C [24]. The steroids were extracted with diethyl ether and the organic phase was evaporated under nitrogen. The residue was dissolved in 0.1 ml of ethanol and analysed by reversed-phase HPLC (Supelcosil RP C_{18}, 5 μm, 150 × 4.6 mm I.D.) with a linear gradient from methanol–acetonitrile–water (43:1.1:55.9) to methanol–acetonitrile–water (75:1.9:23.1). Detection was at 254 nm.

Chemicals and gels

Lubrol PX, benzalkonium chloride, dodecylammoniopropanesulphonate, hexadecyltriethylammonium bromide and other analytical-reagent grade chemicals were purchased from Sigma (Deisenhofen, F.R.G.), except for 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) (Calbiochem, Frankfurt, F.R.G.), Brij 35, sodium cholate, sodium phenobarbital (Serva, Heidelberg, F.R.G.), hexachlorobenzene (Fluka, Neu-Ulm, F.R.G.), cumene hydroperoxide (Aldrich, Steinheim, F.R.G.) and HPLC standards of the hydroxylated testosterones 6β-, 7α- and 16β-hydroxytestosterone (Steraloids, Wilton, NH, U.S.A.).

Gel materials (Mono Q, Mono S, Q-Sepharose Fast Flow, S-Sepharose Fast Flow and Chelating Sepharose Fast Flow) were purchased from Pharmacia.

RESULTS AND DISCUSSION

Selection of suitable detergent

The first step in designing the analytical procedure is screening for a suitable detergent (for membrane solubilization and chromatography), which has to fulfil the following criteria: (1) high efficiency to solubilize microsomal P-450, (2) compatibility with the chromatographic procedure and (3) negligible effects on the enzymatic activity of P-450. Of the different types of detergents, *i.e.*, cationic, anionic, zwitterionic and non-ionic, only members of the last three categories are suitable for P-450 studies because cationic detergents lead to a complete conversion of P-450 to P-420 or irreversible damage of P-450 even at low concentrations ($\leqslant$0.02%). Therefore, we excluded the following detergents from further investigations: benzalkonium chloride, dodecylammoniopropanesulphonate and hexadecyltriethylammonium bromide.

As shown in Table I, acceptable P-450 solubilization is obtained with sodium

TABLE I

EFFICIENCY OF MICROSOME SOLUBILIZATION BY DETERGENTS

Microsomes were solubilized in detergent-containing buffer as described under Experimental. After centrifugation (100 000 *g*), the protein and P-450 content of the supernatant were determined. The figures give the percentage of recovered material.

Detergent	Solubilization (%)	
	Protein	P-450
0.5% Cholate	62	97
0.3% Cholate	72	71
0.1% Cholate	51	35
0.8% Lubrol	69	96
0.4% Lubrol	59	93
0.2% Lubrol	47	79
0.5% CHAPS	43	83
0.3% CHAPS	20	63
0.1% CHAPS	21	40
0.8% Brij 35	74	88

cholate, CHAPS, Lubrol PX and Brij 35. Sodium cholate is widely used in P-450 studies but it is not suitable for analytical separations on the strong anion exchanger Mono Q. Its firm binding to the gel material causes unpredictable shifting of retention times and thus leads to irreproducible chromatographic patterns. This observation is in accordance with the results of Kastner and Schulz [13].

The effect of the three remaining detergents on the chromatographic resolution of P-450 species was further examined using a standard gradient (Fig. 1). Three reasons led us to use Lubrol PX for further investigations: (1) the resolution is superior to that in the presence of CHAPS (Fig. 1); (2) monitoring of protein elution at 280 nm is possible; and (3) enzymatic activities are maintained, as shown by Guengerich and Martin [18] and our own results (see below).

In contrast to our results, Kastner and Schulz [13] found that the chromatographic resolution is higher in the presence of CHAPS than of Lubrol PX. Further, we observed complete binding of P-450 to Mono Q in the presence of CHAPS, whereas the pass-through fraction of Kastner and Schulz [13] contained about 25% of applied P-450. An explanation may be species-specific differences in the chromatographic properties of P-450s from rats and marmoset monkeys.

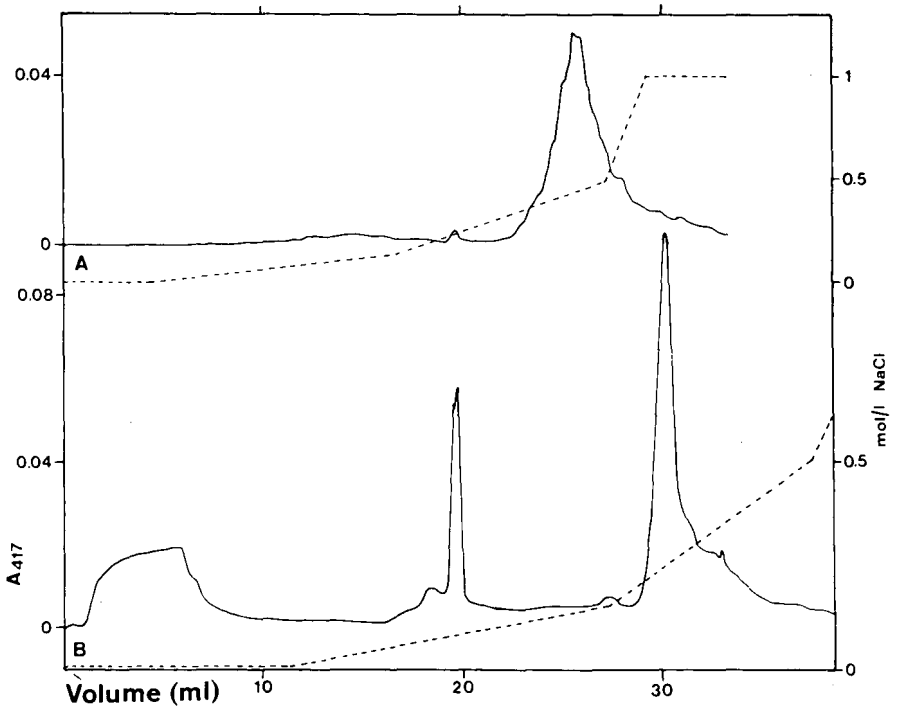

Fig. 1. Separation of P-450 species from detergent-solubilized liver microsomes of phenobarbital-treated rats by anion-exchange FPLC (Mono Q HR 5/5). (A) Sample, 3.5 nmol of P-450 (1.9 mg of protein) in 0.15% CHAPS, 20 m*M* Tris–HCl (pH 7.7), 20% glycerol, 1 m*M* EDTA, 1 m*M* DTE, 0.4 m*M* PMSF. (B) Sample, 4.6 nmol of P-450 (1.7 mg of protein) in the buffer as in (A) but with 0.2% Lubrol PX instead of CHAPS. The equilibration buffer is identical with the corresponding sample buffer. The elution buffers contain 1 *M* NaCl in addition. Flow-rate, 1 ml/min. Solid line, absorbance at 417 nm; broken line, NaCl gradient.

Optimization of the chromatographic procedure

Analytical separations of complex P-450 mixtures by ion-exchange chromatography have been attempted to only a limited extent [10–12]. Preliminary results of our own work were published recently [25]. Prerequisites for a convenient method are reproducibility and high resolution in combination with short separation times. In addition, the sensitivity of detection should be high enough to allow the analysis of small amounts of applied samples.

Anion-exchange chromatography (Mono Q)

Figs. 1B and 2A show that the A_{417} elution profile does not reflect the complexity of the underlying P-450 pattern. Similar gradient systems were also used by

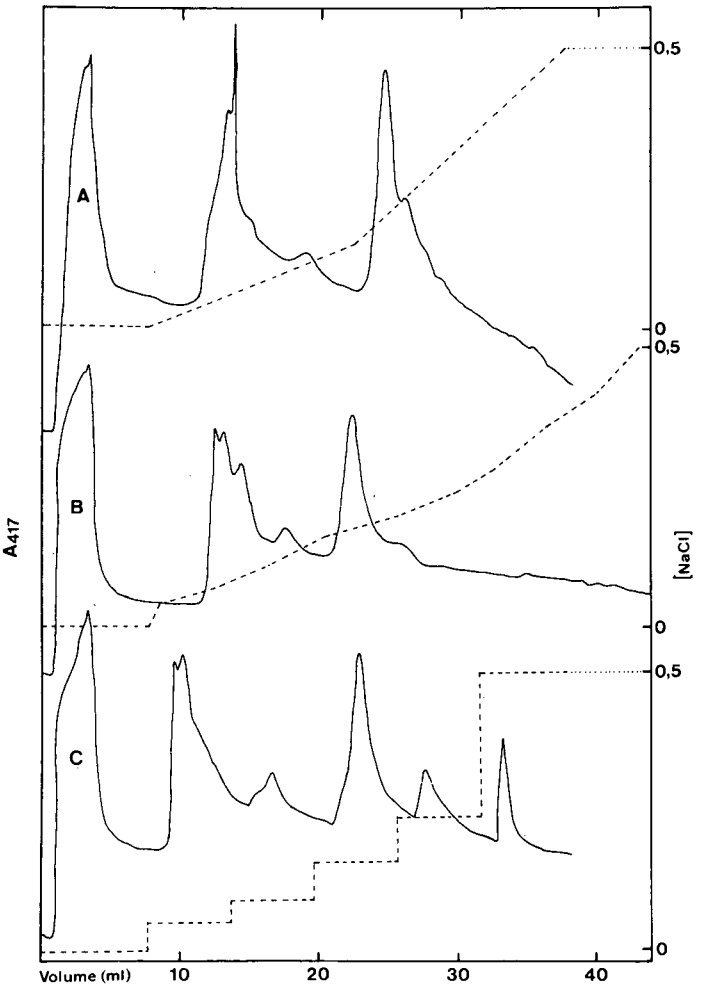

Fig. 2. Effect of the gradient form on the resolution of P-450 species by anion-exchange FPLC on Mono Q HR 5/5. Sample, 1.25 nmol P-450 in Lubrol-containing buffer as specified in Fig. 1A. Rats were treated with phenobarbital and β-naphthoflavone. Flow-rate, 1 ml/min. Solid line, absorbance at 417 nm; broken line, NaCl gradient.

Kastner and Schulz [13] for preparative P-450 separation and by Sakaki *et al.* [12] for the separation of purified P-450 species. The resolution of complex P-450 mixtures, however, requires more complex gradients.

Optimization of resolution is achieved by varying the gradient form as shown in Fig. 2. A pronounced improvement of resolution is obtained by switching from continuous to stepwise gradients. Connecting the discrete concentration steps by linear gradients maintains the analytical character of the elution mode and leads to a further increase in resolution (Fig. 3). Using the optimized method we obtained eight separated P-450 fractions including the material passing through the Mono Q column. P-420 (only sometimes present in our preparations) almost exclusively appears in the Mono Q pass-through fraction. This is in accordance with the observations of Kastner and Schulz [13].

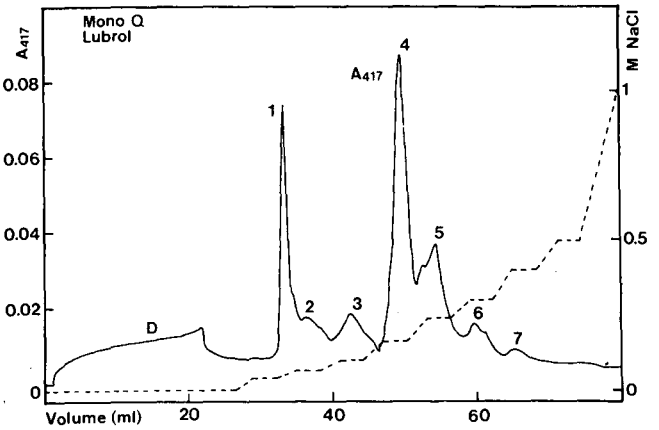

Fig. 3. Chromatographic resolution of P-450 species by optimized gradient elution on Mono Q. Column, Mono Q HR 5/5. Sample, Lubrol-solubilized liver microsomes of rats pretreated with Phenobarbital. P-450 content, 3.8 nmol. Buffers as described under Experimental. D = Unbound material in the pass-through fraction.

The chromatographic resolution especially of peaks 4 and 5 is influenced by the amount of P-450 applied (Fig. 4). Acceptable separation of both peaks is obtained with 1 nmol of P-450, an amount which allows the quantitative spectroscopic determination of P-450 and qualitative analysis by SDS-PAGE of individual Mono Q fractions. Increasing the amount of applied P-450 finally leads to fusion of peaks 4 and 5 (Fig. 4C). Therefore, we routinely use 1–2 nmol of P-450 for analytical separations.

Resolution and reproducibility of chromatographic patterns is maintained even after 20 or more successive runs with simple intermediate reequilibration steps (see Experimental). Retention volumes of individual P-450 peaks are highly reproducible with an averaged variation of 0.6% (n = 8) based on the total gradient volume [25]. As an example, retention times of the Mono Q peaks 4 and 5 obtained with liver microsomes of rats pretreated with various inducers are listed in Table II.

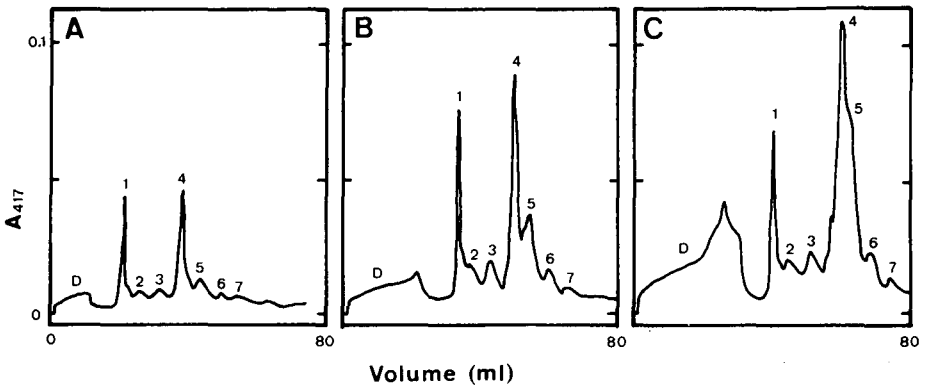

Fig. 4. Influence of the amount of applied P-450 on the elution profile. Column, Mono Q HR 5/5. Sample, Lubrol-solubilized microsomes of phenobarbital-treated rats. (A) 1 nmol of P-450; (B) 3.8 nmol of P-450; (C) 6.5 nmol of P-450. Gradient profile as in Fig. 3. Buffers as specified under Experimental.

Cation-exchange chromatography (Mono S)

The relative amount of P-450 in the Mono Q pass-through fraction depends on the type of induction, *i.e.*, the microsomal P-450 pattern (Table III) and is in the range of 5 to 35% of applied P-450. This fraction can be further separated on the FPLC cation exchanger Mono S. Using MOPS buffer pH 7.0 as described in Experimental the Mono Q pass-through fraction is completely bound to Mono S. This is in agreement with the results of Imaoka *et al.* [26] who used combined anion and cation exchange HPLC for preparative P-450 separation. Chromatographic P-450 resolution of Mono S is more sensitive to pH compared to Mono Q with an optimum at pH 7.0 (not shown). Improvement of resolution is achieved by using a stepwise instead of a linear NaCl gradient (Fig. 5).

Mono S as the first chromatographic step for P-450 separation of Lubrol-solubilized liver microsomes leads to a pass-through fraction containing about 70% of

TABLE II

FRACTIONATION OF MICROSOMAL P-450 (1 nmol) FROM RATS TREATED WITH VARIOUS INDUCERS: COMPARISON OF THE MONO Q PEAKS 4 AND 5

Lubrol-solubilized microsomes (1 nmol P-450) were fractionated on Mono Q HR 5/5 using the optimized chromatographic procedure (Fig. 3). Elution profiles (retention times, peak areas) were analysed by the PC Integration Pack (Softron). P-450 content was determined spectroscopically.

Inducer[a]	Retention time (min)		Area (mV ml)		P-450 (pmol)		Ratio 4:5	
	Peak 4	Peak 5	Peak 4	Peak 5	Peak 4	Peak 5	Area	P-450
PB	47.04	53.54	78.19	25.67	90	80	3.03	1.13
β-NF	47.47	53.92	88.31	40.50	80	250	2.18	0.32
HCB	47.68	53.77	88.29	53.44	74	190	1.65	0.39
m-un	47.48	53.60	72.17	36.95	99	180	1.95	0.63

[a] See Table III.

TABLE III

COMPARATIVE P-450 DETERMINATION IN MONO Q FRACTIONS OBTAINED WITH LUBROL-SOLUBILIZED MICROSOMES OF RATS TREATED WITH VARIOUS INDUCERS

Solubilized microsomes were fractionated on Mono Q HR 5/5 using the optimized chromatographic procedure (see Fig. 3). Peak fractions were pooled and their P-450 contents determined spectroscopically. The figures give the percentage of applied P-450.

Inducer[a]	P-450 (nmol)	P-450 in Mono Q fractions (%)							
		D[b]	1+2	3	4	5	6	7	Total
PB	1.05	24.8	12.4	8.6	8.6	7.6	—[c]	—[c]	63.8
β-NF	0.98	23.5	8.2	10.0	8.2	25.5	3.1	3.1	81.6
HCB	0.96	7.8	10.2	5.1	7.7	19.8	—[c]	—[c]	50.6
INH	0.96	33.6	10.3	6.8	6.8	20.6	1.6	—[c]	80.2
m-un	0.96	15.6	5.1	10.3	10.3	18.8	3.1	—[c]	63.5

[a] PB = Phenobarbital; β-NF = β-naphthoflavone; HCB = hexachlorobenzene; INH = isonicotinic acid hydrazide; m-un = untreated male rats.

[b] D = Pass-through fraction.

[c] P-450 content too low for spectroscopic determination.

applied P-450. Similarly, Kastner and Schulz [13] obtained ≥80% of unbound P-450 by fractionation of liver microsomes of phenobarbital-induced marmoset monkeys. This amount of unbound P-450 corresponds to the amount bound by Mono Q, provided that the pH of the sample is adjusted to 7.7. Thus, successive combination of Mono Q and Mono S provides a powerful tool for P-450 separation, resulting in eleven P-450 fractions.

Unfortunately, weak FPLC ion exchangers (such as DEAE or CM) based on monobeads are not yet available. Therefore, we had to restrict our studies to the strong ion exchangers Mono Q and Mono S. For protein elution we used NaCl throughout our studies. Kastner and Schulz [13] found that lithium perchlorate gives a better resolution than sodium chloride but results in unstable P-450 fractions.

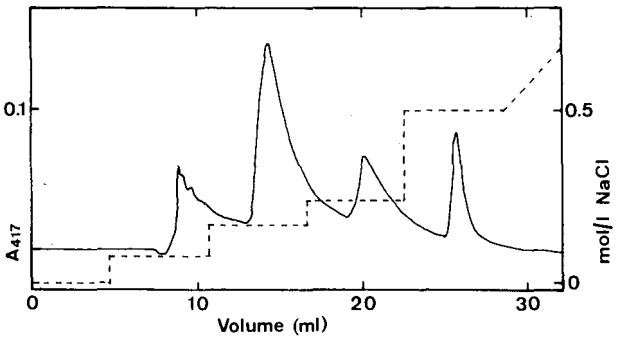

Fig. 5. Subsequent separation of Mono Q pass-through fraction on Mono S HR 5/5. Sample, 0.83 nmol of P-450 of a Mono Q pass-through fraction obtained with liver microsomes of phenobarbital-treated rats. Buffers as indicated under Experimental. Solid line, absorbance at 417 nm; broken line, NaCl gradient. Note that there is no 417-nm absorbing material in the pass-through fraction.

Analysis of FPLC fractions and induction effects

The use of Lubrol PX instead of Emulgen or Triton allows protein elution to be monitored at 280 nm. By simultaneous and continuous detection at 417 nm, haemoprotein-containing fractions are easily recognized. Peak fractions are combined and further analysed by spectroscopy, SDS-PAGE and determination of testosterone metabolizing activity.

Routinely, the P-450 content of individual Mono Q fractions is determined spectroscopically via the $P\text{-}450_{reduced}$–CO complex [2]. Typical results are shown in Table III with the corresponding chromatograms in Fig. 6. Variation of the chromatographic patterns is dependent on the type of P-450 inducer. Three points should be stressed: (1) the amount of unbound P-450 in the pass-through fraction varies with different inducers; (2) chromatograms normalized on a protein basis differ in absolute peak heights, especially for peaks 1, 4 and 5; and (3) the area ratio of peaks 4 and 5 is a good indicator of the induction type (Table II). Detailed data on the latter point will be published in a forthcoming paper [27]. In addition to cytochrome P-450, microsomes contain another haemoprotein, cytochrome *b*5, which elutes in peak 4 at about 160 m*M* NaCl, causing the discrepancy between the peak area at 417 nm and the spectroscopically determined P-450 content relative to other fractions (Table II).

The chromatographic separation of proteins by Mono Q is demonstrated by SDS-PAGE in the P-450 relevant molecular weight range of 46 000–58 000 dalton (Fig. 7). Obviously, some fractions contain more than one P-450 species. Their

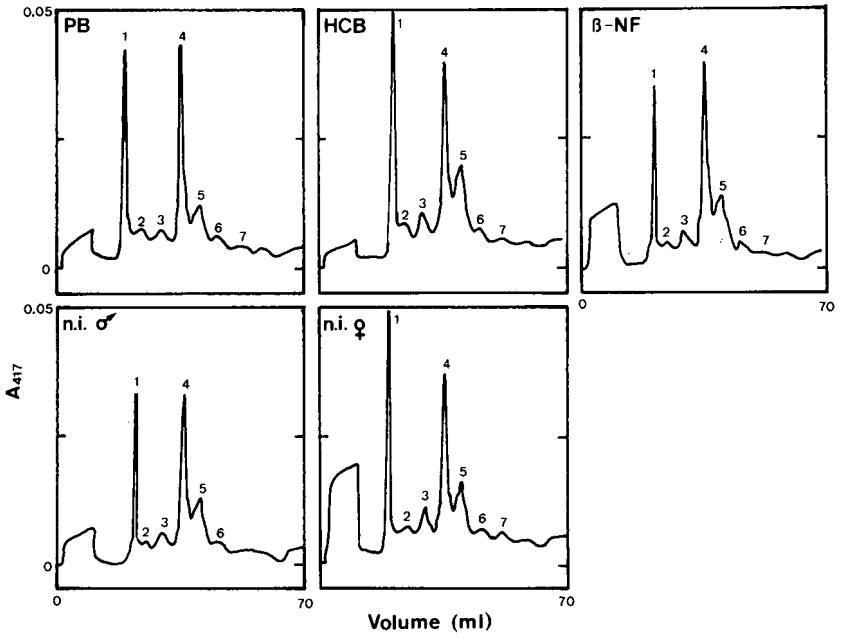

Fig. 6. Analytical fractionation of microsomal P-450 from male rats pretreated with various inducers or untreated rats. Column, Mono Q HR 5/5. Samples, Lubrol-solubilized liver microsomes containing 1 nmol of P-450 each. Gradient profile as in Fig. 3. PB = Phenobarbital; HCB = hexachlorobenzene; β-NF = β-naphthoflavone; n.i. = not induced.

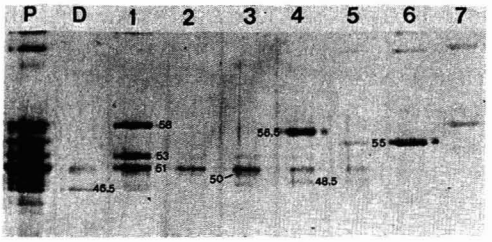

Fig. 7. SDS-PAGE of Mono Q fractions. Samples, fractionated liver microsomes of phenobarbital-treated rats (1 nmol of P-450). Numbers correspond to peaks as in Fig. 3. P = Unfractioned sample. The P-450 relevant molecular weight range between 46 and 58 kilodalton is magnified. Apparent molecular weights are assigned to individual protein bands.

resolution requires a supplementary fractionation step based on a different chromatographic principle (see below). On the other hand, several fractions exhibit similar enzymatic activities and SDS-PAGE patterns (Table IV, Fig. 7). This may be due to the existence of closely related isozyme or allozyme forms. Relevant results by other workers support this possibility. A P-450 preparation homogeneous by SDS-PAGE could be resolved into three fractions by anion-exchange HPLC [28]. Sakai *et al.* [29] detected three immuno-identical P-450 species in rat liver microsomes after phenobarbital induction. These forms probably coincide with multiple P-450b species described by Vlasuk *et al.* [30] and Oertle *et al.* [31].

Data from testosterone metabolism point in the same direction because several fractions catalyse the formation of identical products, especially 6β- and 7α-hydroxytestosterone. This is in partial agreement with data published by Funae and Imaoka [32], who found 6β-hydroxylase activity in five fractions obtained by anion-exchange

TABLE IV

TESTOSTERONE METABOLITES PRODUCED BY MONO Q FRACTIONS OBTAINED WITH LUBROL-SOLUBILIZED MICROSOMES OF RATS TREATED WITH PHENOBARBITAL

Enzymatic activity of Mono Q fractions was determined as described under Experimental. Fraction numbers correspond to those in Fig. 5. Metabolites: testosterone derivatives hydroxylated at the indicated positions.

Fraction	Testosterone metabolite									
	unidentified metabolite	6α-	6β-	7α-	2?β-	15β-	16α-	16β-	18	A[a]
Sample	−	+	+	+	+	+	+	+	+	+
D[b]	−	−	+	+	−	−	−	−	−	−
1	+	−	+	+	−	−	−	−	−	+
2	+	−	−	+	+	−	+	+	−	+
3	+	−	+	+	−	−	−	−	−	+
4	+	+	+	+	−	+	−	−	−	+
5	−	−	+	+	−	−	−	−	−	−
6	−	−	+	+	−	−	−	−	−	−
7	−	−	+	+	−	−	−	−	−	−

[a] A = Androstenedione.
[b] D = Pass-through fraction.

HPLC. Formation of 7α-hydroxytestosterone, however, was restricted to one fraction only. Our finding is especially interesting because 7α-hydroxylation was ascribed to P-450IIA1 (P-450a) only [33]. A recently described new P-450 species (P-450m) [3], however, catalyses the same reaction. The results point to isozymic or allozymic heterogeneity of these P-450 species. Detailed data on testosterone metabolism by individual FPLC fractions will be published in a forthcoming paper [34].

Immobilized metal affinity chromatography (IMAC)

For further resolution of Mono Q fractions containing more than one P-450 species, we chose immobilized metal affinity chromatography. Here we present some preliminary results to demonstrate the applicability of this method to P-450 fractionation.

The chelating gel (Chelating Sepharose Fast Flow) can be charged with different transition metal ions [20]. Of the set tested by us (Ni^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}), only Zn^{2+} proved to be useful for our purposes. The recovery of spectroscopically intact P-450 eluted from the column was very low or zero using any of the other cations. In addition to the nature of the immobilized metal ion, the kind of eluting

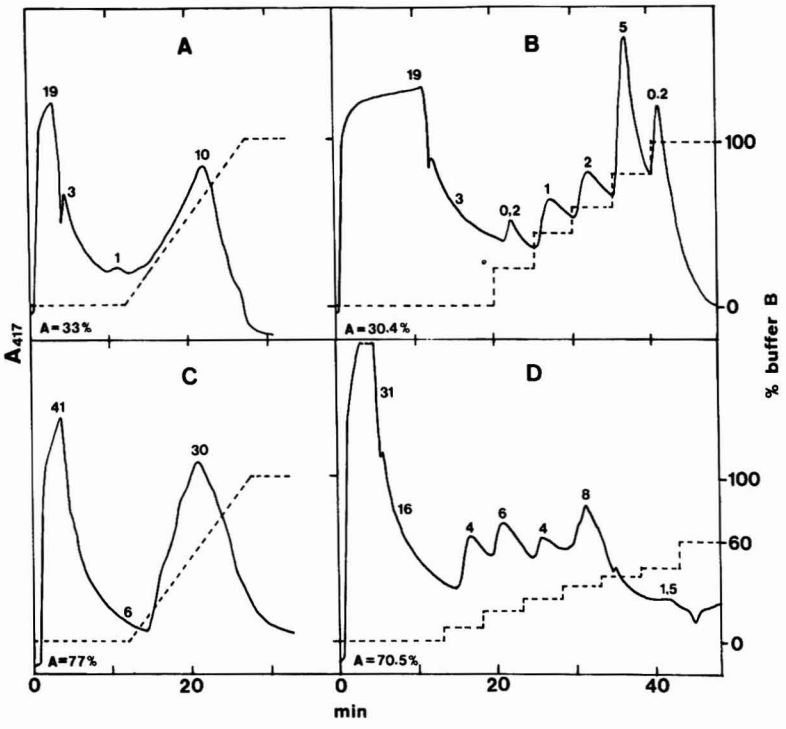

Fig. 8. Fractionation of P-450 species by immobilized metal affinity chromatography (IMAC). Column and gel material, FPLC HR 5/5 filled with 1 ml of Chelating Sepharose Fast Flow. Immobilized cation, Zn^{2+}. Sample, liver microsomes of phenobarbital-treated rats prefractionated on 8-aminooctyl-Sepharose. (A) 0.77 nmol of P-450, linear pH gradient; (B) 3.5 nmol of P-450, stepwise pH gradient; (C) 0.96 nmol of P-450, linear NH_4Cl gradient; (D) 3.1 nmol of P-450, stepwise NH_4Cl gradient. Buffers as specified under Experimental. Gradient composition is given by the broken line as a percentage of buffer B in the eluting buffer. Solid line, absorbance at 417 nm. Numbers, percentage of applied P-450.

agent has a strong effect on recoveries. If elution is performed with decreasing pH (7.0 to 3.8) the P-450 recovery is about 30% compared with 70–100% with an ammonium chloride gradient (0–2 mol/l) (Fig. 8). In analogy with ion-exchange chromatography, improvement of resolution is attained by stepwise gradient elution (Fig. 8B and D).

Scale-up

The results of the optimized analytical ion-exchange procedures suggest their application for preparative purposes. Considering the minute amounts of many P-450

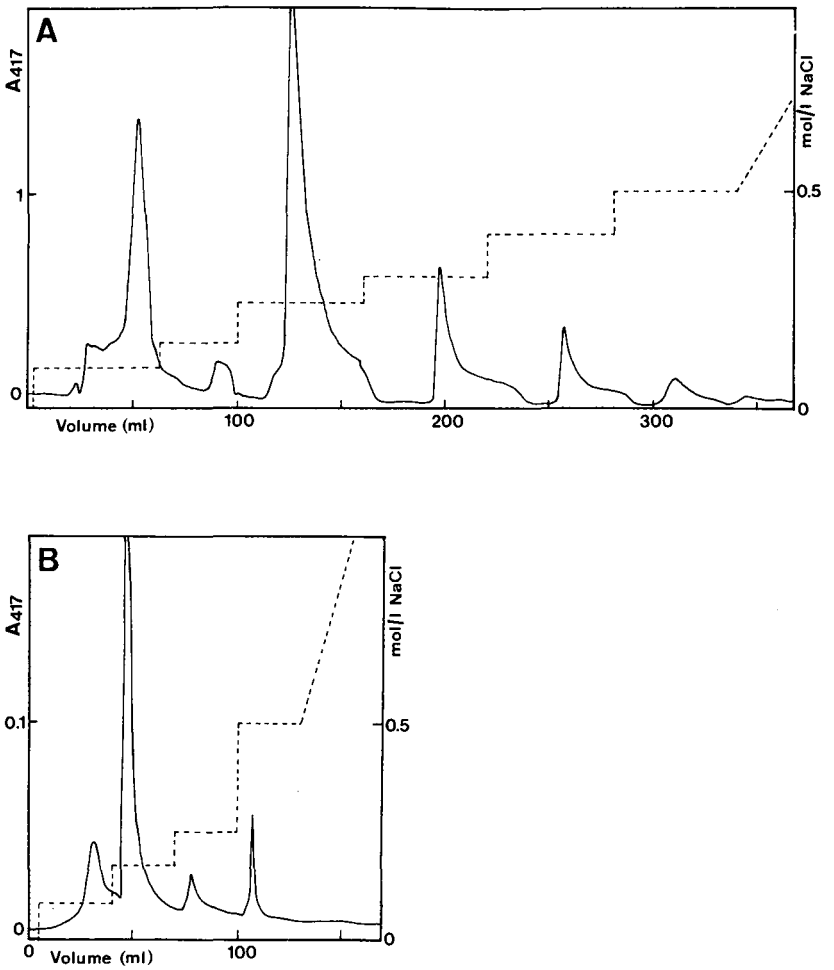

Fig. 9. Preparative fractionation of microsomal P-450 species of phenobarbital-treated rats on Q-Sepharose Fast Flow and S-Sepharose Fast Flow. (A) Column and gel material, FPLC HR 16/10 filled with 20 ml of Q-Sepharose Fast Flow. Sample, 190 nmol of P-450. Buffers as specified under Experimental for Mono Q. Flow-rate, 1 ml/min. Pass-through fraction not shown. (B) Column and gel material, FPLC HR 10/10 filled with 7.5 ml of S-Sepharose Fast Flow. Sample, pass-through fraction of the Q-Sepharose run shown in (A). Buffers as specified under Experimental for Mono S. Flow-rate, 1 ml/min. Solid lines, absorbance at 417 nm; broken lines, NaCl gradient.

species in crude microsomal extracts, columns with high protein binding capacity are required for the initial fractionation steps. The optimized chromatographic procedures for Mono Q and Mono S can be easily adapted to the equivalent Sepharose Fast Flow gels using conventional chromatography columns still taking advantage of the automatic FPLC system. As shown in Fig. 9, gradient steps slightly modified compared with those of the analytical runs can be applied to Q- and S-Sepharose, leading to a comparable resolution. Differences in the elution patterns of Mono Q and Q-Sepharose may be due to the physically and chemically distinct gel matrices, different column geometries and different gradient steps.

CONCLUSION

Fractionation of microsomal proteins by ion-exchange FPLC results in a high resolution of P-450 isoenzymes. This method can be used for both preparative and analytical fractionations. The purpose of these studies is the evaluation of xenobiotically caused effects on the organism by the analysis of microsomal P-450 patterns. We believe that high-resolution FPLC in combination with other more specific analytical methods currently being established will be an appropriate tool for this purpose.

ACKNOWLEDGEMENTS

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Purification of a recombinantly produced transmembrane protein (gp41) of HIV I

ERWIN SOUTSCHEK*, BRIGITTE HÖFLACHER and MANFRED MOTZ
Mikrogen GmbH, Westendstrasse 125, 8000 München 2 (F.R.G.)

ABSTRACT

The transmembrane protein gp41, a component of the viral envelope of HIV I, and its analogue gp36 of HIV II are important antigens for the sensitive and specific detection of anti-HIV antibodies. The immunodominant region of the protein gp41, which reacts with 100% of sera of infected persons, was produced by gene technological means in *Escherichia coli*. The protein accumulates in the form of insoluble inclusion bodies in the bacterial cell. Purification strategies for this aggregated material depend mainly on the isolation of these "inclusion bodies" and subsequent washing procedures. Growth conditions of the recombinant *E. coli* cells and the method of the cell disruption are important for the efficiency of purification and the recovery of the antigen. Owing to the insolubility of the expressed antigen, a significant concentration of recombinant gp41 was possible by extracting the soluble cell components. For this purpose, mild detergent solutions and low-molarity chaotropic buffer solutions were used. After final solubilization in 8 *M* urea buffer at pH 12.5, further chromatographic purification steps followed. The reduction of disulphide bridges with β-mercaptoethanol or dithiothreitol was important. Gel filtration on a Sephacryl S-200 or Superose 12 column and/or ion-exchange chromatography on a DEAE-Sepharose Fast Flow or Mono Q HR (5/5) column finally resulted in the desired purity of the antigen.

INTRODUCTION

A group of closely related retrovirus isolates designated human T-cell lymphotrophic virus type III (HTLV-III), AIDS-related virus (ARV) and lymphadenopathy-associated virus (LAV), collectively referred to as human immunodeficiency virus (HIV), has been identified as the primary etiologic agent of the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC) [1,2]. The infectious virus particle contains RNA wherein the genetic information is encoded. On invasion into susceptible cells the genetic information is transcribed into DNA. Integration into the chromosome occurs along with the production of virus-specific proteins. Known viral proteins are the group-specific antigens (gag), the pol encoded antigens (protease, reverse transcriptase, integrase), the envelope (env) antigens (gp120, gp41) and the regulatory proteins TAT, REV, VIF and NEF [3].

The transmission of the HI virus occurs in most instances by blood-to-blood contact. After a period of a few weeks or months seroconversion can be observed by detection of antibodies against viral proteins, namely p24 and env (gp120, gp41) derived antigens.

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Anti-gp41 antibodies are currently the most important marker for HIV-I, as this protein reacts with virtually all sera positive for HIV-I. To date, no cross-reactivity has been found and relatively few variances in the amino acid sequence of differing isolates are known [4–8]. A recombinant antigen was produced in *Escherichia coli* by expressing almost the complete nucleotide sequence encoding the outwardly directed region of the transmembrane protein gp41 of HIV-I [9,10]. The purified recombinant protein is intended to be used as diagnostic reagent in a new confirmatory assay based on the Western blot principle.

EXPERIMENTAL

Extraction of the recombinant protein

An overnight culture of *E. coli* (strain JM 83 or JM 109) in L-broth [10 g of tryptone (Difco), 5 g of yeast extract (Difco), 5 g of NaCl, pH 7.4] harbouring the expression plasmid (pUC18RSp, pLIN41 or pDS2BPs) was diluted (1:50) and grown further for 3 h at 37°C to an absorbance at 578 nm of 0.7–0.8. After this the lac promoter was induced with 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) (Biomol) for an additional 3–4 h to express the recombinant antigen. The cells were harvested by centrifugation for 10 min at 8000 *g* (4°C). After washing in lysis buffer [50 m*M* Tris–HCl (Boehringer) (pH 8.0 at 25°C)–2 m*M* EDTA–1 m*M* dithiothreitol (DTT) (Biomol)–5% glycerol] the cells were resuspended (4 ml/g wet weight) in the same buffer [with the addition of 0.4 mg/ml lysozyme and 1 m*M* phenylmethylsulphonyl fluoride (PMSF) (Sigma)]. The suspension was incubated for 30 min and centrifuged for 45 min at 12 000 *g*. The pellet was resuspended in lysis buffer containing 0.1% Triton X-100 (Serva) and 0.1 m*M* PMSF (25 ml/g wet weight). The suspension was stirred for 30 min and Benzonase (DNase) (Merck) (1 μl per 25 ml) was added to reduce the viscosity due to the DNA release. NaCl was added to a final concentration of 1 *M* and the suspension was stirred for 2 h prior to centrifugation for 30 min at 12 000 *g*. The pellet fraction containing the "inclusion bodies" was suspended (10 ml/g wet weight) in detergent buffer [50 m*M* Tris–HCl (pH 8.0)–2 m*M* EDTA–2 m*M* DTT–0.1 m*M* PMSF] containing 1.5% of octyl β-D-glucopyranoside (OGP) (Biomol) and stirred for 2 h. The suspension was centrifuged for 45 min at 12 000 *g* and the pellet fraction was washed either with 2, 4 or 6 *M* urea solution [in 50 m*M* Tris–HCl (pH 8.0)–2 m*M* EDTA–2 m*M* DTT–0.1 m*M* PMSF]. Finally, the pellet was solubilized in 8 *M* urea (Merck) in 20 m*M* Tris–HCl (pH 8.0)–2 m*M* DTT. To achieve complete solubilization the pH was increased to 12.5 by adding 1 *M* NaOH.

Purification of the solubilized antigen

For gel filtration, an S-200 Sephacryl HR column (100 × 1.6 cm I.D.) or a Superose 12 HR 10/30 column (300 × 10 mm I.D.) (Pharmacia) in 8 *M* urea buffer [50 m*M* Tris–HCl (pH 8.0)–2 m*M* DTT] were used. Fractions containing the recombinant protein were then purified to apparent homogeneity by reversed-phase high-performance liquid chromatography (HPLC) on a ProRPC 15-μm HR 16/10 or Mono Q HR 5/5 column (Pharmacia). The ProRPC column was developed with an organic solvent gradient [0 to 100% acetonitrile–isopropanol (2:1); flow-rate 1 ml/min]. The protein-containing solution was acidified prior to injection with 0.1% trifluoroacetic acid (TFA). The Mono Q column was developed with a 20 m*M*

ethanolamine buffer containing 8 *M* urea (pH 9.8) with a gradient from 0 to 1 *M* NaCl (flow-rate 1 ml/min). The fast protein liquid chromatographic (FPLC) columns (Pharmacia–LKB) were attached to an inert HPLC system consisting of a Model 2249 gradient pump and a Model 2150 UV monitor from Pharmacia–LKB.

Alternatively, the solubilized antigen was loaded directly on a DEAE-Sepharose Fast Flow column (25 × 2.3 cm I.D.). The column was developed with a gradient from 0 to 1 *M* NaCl in 8 *M* urea [20 m*M* ethanolamine (pH 9.8)–2 m*M* DTT–0.1 m*M* PMSF; flow-rate 0.5 ml/min].

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Discontinuous SDS-PAGE was performed according to Laemmli [11] using a 3% stacking gel and a 17% running gel. Separated proteins were transferred by electroblotting between two graphite plates in blotting buffer [0.2 *M* glycine–25 m*M* Tris base–20% (v/v) methanol (pH 8.2)] for 1 h onto nitrocellulose. The transferred proteins can be renderd visible by staining the nitrocellulose with Ponceau S (Serva). The nitrocellulose sheets were blocked with dry milk powder solution [5% dry milk powder–0.05% Tween 20–10 m*M* Tris–HCl (pH 7.5)–150 m*M* NaCl–0.1% NaN_3]. For detection of specific bands an HIV serum pool was used (inactivated for 45 min at 58°C). Non-specific bands were rendered visible by incubation with a high-titered anti-*E. coli* rabbit serum. Bound antibodies were detected by incubation with peroxidase conjugated anti-human immunoglobulin G (IgG) (Dakopatts) or anti-rabbit IgG (Dakopatts) followed by peroxidase-catalysed conversion of 3,3′-diamino-benzidine tetrahydrochloride (Sigma) and H_2O_2 (0.05%) to an insoluble brown pigment [10].

RESULTS AND DISCUSSION

The plasmid vector pUC18st was used to express an Rsa–Ssp-restriction fragment from the envelope gene of an HIV-1 isolate (WF1.13) closely resembling the BH10 [12] isolate. The expressed fragment encodes the exterior part of the trans-membrane protein gp41 omitting the amino-terminal fusion peptide, including amino acid residues 531–674 [12]. It was ligated into pUC or pLIN vectors and *E. coli* (strain JM83 or JM109) cells were transformed with this plasmid construct. The expressed antigen should have an approximate molecular mass of about 17 000 dalton and, depending on the plasmid construct, from 4 to 15 foreign amino acids attached to the amino terminus.

For efficient expression of the protein and good recovery, different growth conditions were examined. Double concentrated L-broth supplemented with 0.2–0.5% glycerine was finally used for cell growth. The lac promoter was induced by IPTG as indicated under Experimental. The cells were washed in lysis buffer and then lysed by addition of lysozyme and Triton X-100 with constant stirring. Alternatively, sodium deoxychlolate was used for destabilizing the cell membrane [13,14]. The cell pellet of 1–2 l fermentation broth could be easily disrupted with the Triton X-100 method. Cell pellets from up to 10 l are more conveniently handled by mechanical (French press) or ultrasonification methods with additional lysozyme treatment. The cell disruption should be nearly complete (> 99%) otherwise the following purification

steps are hindered. To this viscous suspension DNase (Benzonase) was added to reduce the viscosity due to the release of chromosomal DNA. The addition of a protease inhibitor such as PMSF throughout the purification procedure was important to reduce degradation of the expressed protein. After adding NaCl to a final concentration of 1 *M*, the suspension was centrifuged for 30 min at 12 000 *g* (4°C) and the expressed protein was found in the pellet fraction. With this step most of the soluble impurities can be separated (Fig. 1).

The pellet fraction was then extracted with octyl β-D-glucopyranoside solution to remove *E. coli* membrane proteins from the inclusion bodies (Fig. 1). Neither the extractions with solutions containing increasing Triton X-100 concentrations (0.1–2%) nor octyl β-D-thioglucopyranoside (1.5%) showed the same good efficiency (data not shown). The suspension was centrifuged again at 12 000 *g* and the enriched "inclusion bodies" were suspended in chaotropic buffer solutions with increasing molarity [up to 8 *M* urea or 7 *M* guanidinium hydrochloride (GHCl) in 50 m*M* Tris–HCl (pH 8.5)–5 m*M* DTT].

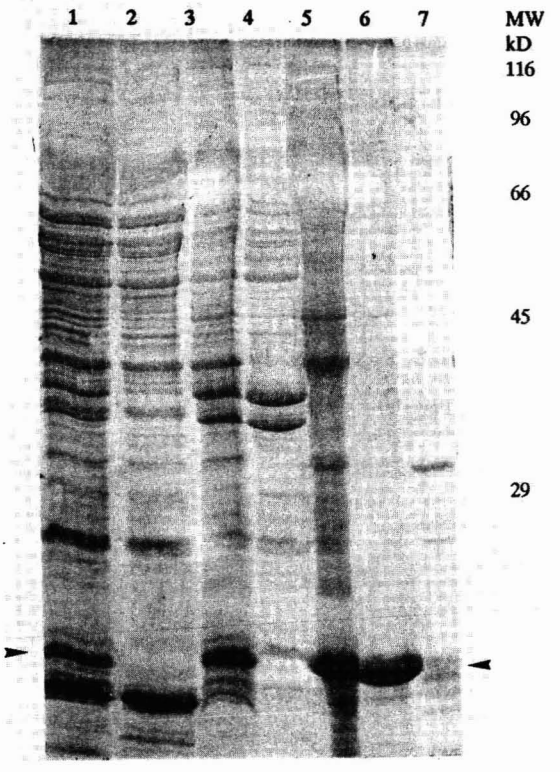

Fig. 1. Coomassie brilliant blue-stained 17% SDS-polyacrylamide gel of total *E. coli* proteins and isolated inclusion bodies. Molecular masses (MW) are indicated on the right, in kilodalton (kD). Lanes: 1 = total cell proteins from JM109/pDS2BPs resuspended in lysis buffer with addition of lysozyme; the position of the recombinant gp41 is marked with an arrow; 2 = lysis supernatant after 1 *M* NaCl extraction; 3 = lysis pellet resuspended in octyl β-D-glucopyranoside (OGP) buffer; 4 = supernatant of the OGP extraction; 5 = pellet of OGP extraction resuspended in 6 *M* urea buffer; 6 = pellet of 6 *M* urea extraction resuspended in 8 *M* urea buffer (pH 8.5); 7 = supernatant of 6 *M* urea extraction.

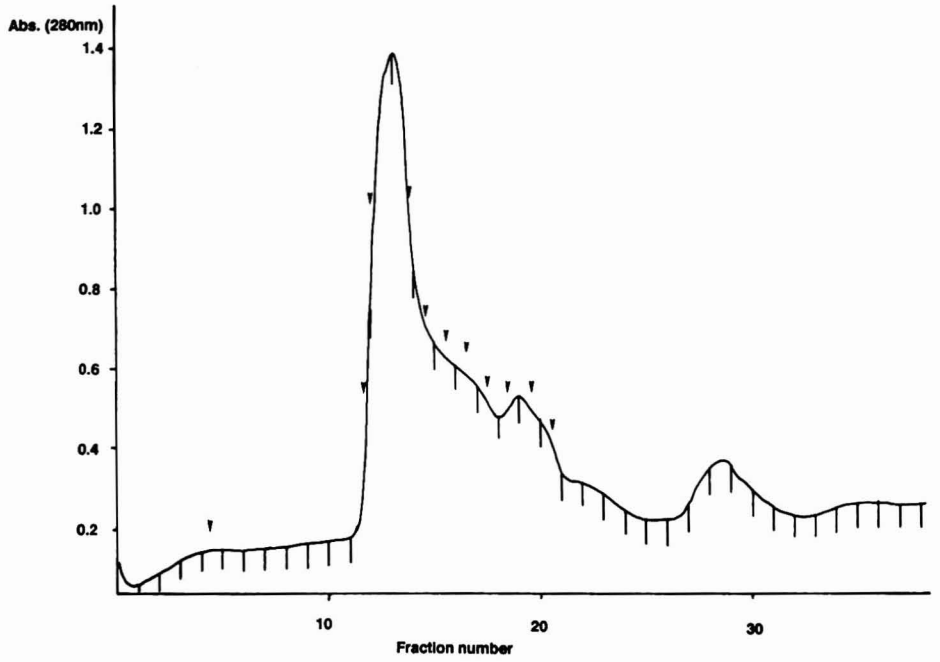

Fig. 2. Gel filtration chromatography of solubilized recombinant gp41 on an S-200 Sepharose column (100 cm × 1.6 cm I.D.) equilibrated with 8 *M* urea buffer [50 m*M* Tris–HCl (pH 8.5)–2 m*M* EDTA–2 m*M* DTT–0.1 m*M* PMSF]. Applied to the column were 6 ml of solubilized protein-containing solution (corresponding to 2 g wet weight of cells) and the proteins were eluted with 8 *M* urea buffer (flow-rate 0.5 ml/min). Fractions indicated with arrows were analysed by SDS-PAGE (Fig. 3).

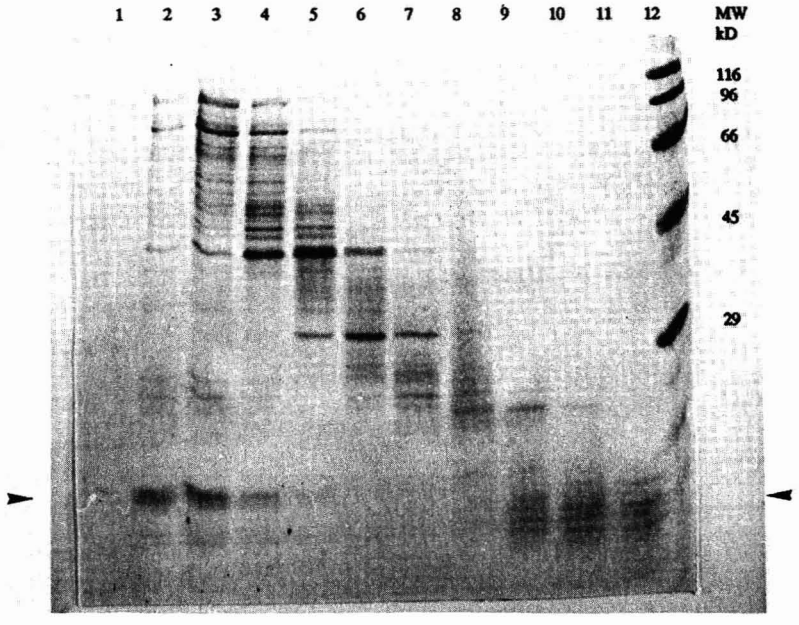

Fig. 3. 17% SDS-PAGE of fractions collected from the gel filtration run shown in Fig. 2. The molecular masses are indicated on the right. In lanes 1–11, the aliquots separated are those marked with arrows in Fig. 2 (from left to right). The position of the recombinant gp41 is marked with an arrow.

Impurities could be removed by washing the pellet with 6 *M* urea buffer. Finally, the resulting pellet was solubilized in 8 *M* urea or 6 *M* GHCl buffer by increasing the pH to 12.5 with 1 *M* NaOH. Some remaining impurities could be excluded by solubilization in 6 *M* GHCl buffer and dialysing extensively against 8 *M* urea buffer. Precipitated material was removed by centrifugation at 20 000 *g* for 45 min at 4°C. The solubilized antigen was chromatographed on a Sephacryl S-200 column. The protein was eluted with 8 *M* urea buffer [50 m*M* Tris–HCl (pH 8.5)–5 m*M* DDT–0.1% PMSF]. As shown in Figs. 2 and 3, the protein elutes mainly as a high-molecular-mass aggregate even in the presence of 8 *M* urea. A small part of the protein elutes as a low-molecular-mass protein as judged by SDS-PAGE and Western blot analysis (not shown). Neither the addition of 1% SDS nor running the column in 6 or 7 *M* GHCl buffer gave a better resolution. The reason for this anomalous behaviour could be due in part to the content of hydrophobic amino acids in this recombinant protein [15]. However, expression constructs omitting the entire transmembrane region and the hydrophobic amino-terminal fusion peptide behaved in nearly the same way. The presence of an extended amphipathic, α-helical fibre [7] might be responsible for the aggregation of gp41 by intermolecular bonding between these hydrophobic stretches. Gel filtration on a Superose 12 column in the above-described buffer resulted in a comparable elution profile.

Fractions containing the recombinant protein were further purified by reversed-phase chromatography on a ProRPC column. The recombinant protein eluted at >65% acetonitrile–isopropanol (2:1) (Fig. 4). This further emphasizes the hydro-

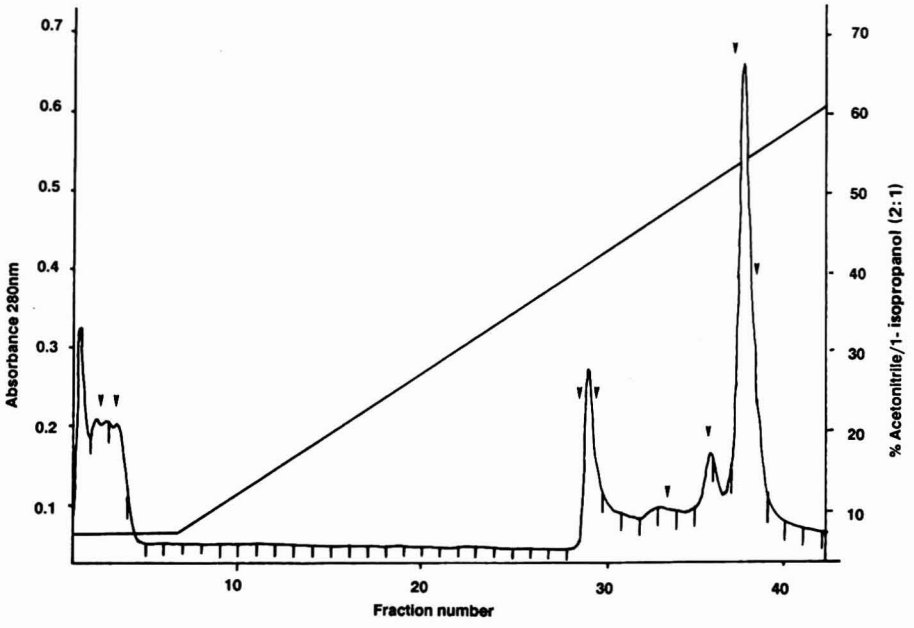

Fig. 4. Reversed-phase column run on a ProRPC column developed with a linear acetonitrile–isopropanol (2:1) gradient. A 1-ml aliquot of the high-molecular-mass fraction from the gel filtration was injected after acidifying with 0.1% TFA. The proteins were eluted at a flow-rate of 1 ml/min. Aliquots of fractions indicated with arrows were analysed by SDS-PAGE (Fig. 5).

phobic behaviour of this protein. In SDS-PAGE the protein appears as a homogeneous and pure band (lane 7, Fig. 5a). Western blot analysis with HIV pool serum demonstrated the immunoreactivity of this reversed-phase purified protein (Fig. 5b). A protein band at about 34 000 dalton could be recognized as a dimer (in lane 7), which is seen more or less in every preparation even under highly reducing conditions (β-mercaptoethanol or DTT and SDS). However, the incubation with the high-titered anti-*E. coli* serum revealed some impurities which could only be seen by overloading the gel with a concentrated purified protein and silver staining (not shown).

Another approach which resulted in a more enriched gp41 with respect to impurities (up to 90%) was to solubilize the antigen in 8 *M* urea buffer at pH 12.5 and to titrate back to pH 9.4, which resulted in precipitation of impurities, leaving the recombinant antigen in solution. After centrifugation of the suspension and filtration, the antigen-containing solution was applied to a Mono Q HR 5/5 column in 8 *M* urea (containing 20 m*M* ethanolamine) buffer (pH 9.4). The column was developed with 8 *M* urea (containing 20 m*M* ethanolamine) buffer (pH 9.4) using a linear NaCl gradient from 0.1 to 1 *M*. The protein eluted in a pure form at >80% buffer B (8 *M*

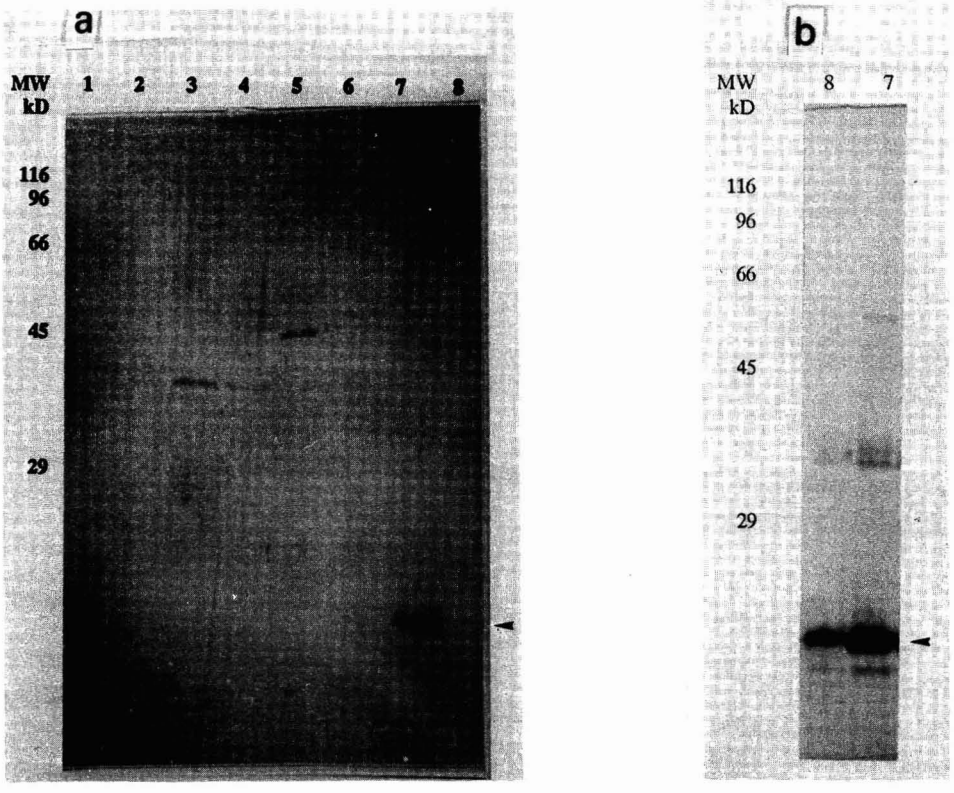

Fig. 5. (a) SDS-PAGE (17% gel) of fractions derived from the reversed-phase column run (Fig. 4). Molecular masses are indicated on the left. In lanes 1–8, the aliquots separated are those marked with arrows in Fig. 4 (from left to right). The recombinant gp41 is marked with an arrow. (b) Western blot analysis of the fractions shown in lanes 7 and 8 in (a) containing the recombinant gp41 protein.

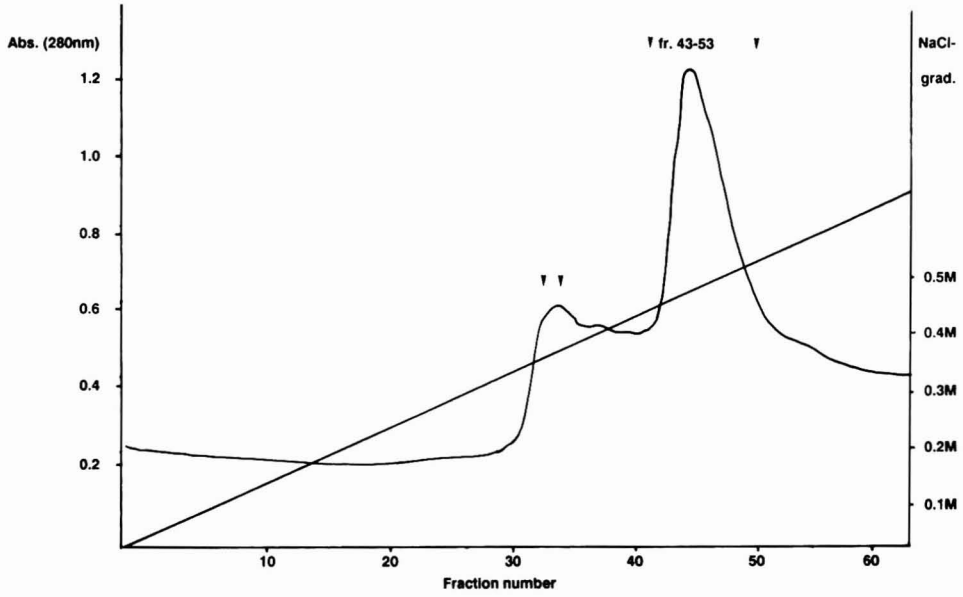

Fig. 6. Anion-exchange chromatography on a DEAE-Sepharose Fast Flow column (25 cm × 2.3 cm I.D.). The column was equilibrated with 20 m*M* ethanolamine buffer (pH 9.4) containing 2 m*M* EDTA, 5 m*M* DDT and 8 *M* urea. The solubilized protein fraction was applied in the same buffer. The proteins were eluted with a linear gradient from 0 to 1 *M* NaCl. The recombinant gp41 elutes at about 0.5 *M* NaCl. Fractions indicated with arrows were analysed by SDS-PAGE (Fig. 7).

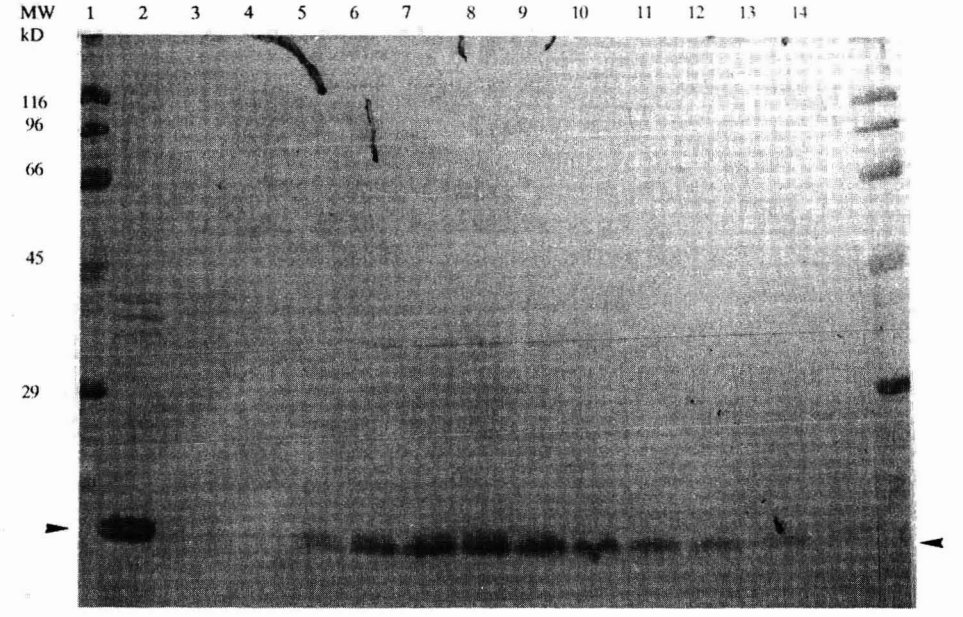

Fig. 7. SDS-PAGE of aliquots from the anion-exchange run on the DEAE-Sepharose Fast Flow column (Fig. 6). Molecular masses are indicated on the left. In lane 2 an aliquot of the filtered solution which was applied to the column is shown. In lanes 3–14, the aliquots separated are those marked with arrows in Fig. 6. The recombinant gp41 is marked with an arrow.

urea, pH 9.4, 20 m*M* ethanolamine, 1 *M* NaCl). Western blot analysis showed an immunoreactive antigen free of contaminants (similar as shown in Fig. 7).

For producing greater amounts of up to 10 mg per column run we finally used a DEAE-Sepharose Fast Flow column (25 × 2.6 cm I.D.) with 8 *M* urea buffer (20 m*M* ethanolamine–5 m*M* DTT–0.1% PMSF) and a linear NaCl gradient from 0 to 1 *M* (Figs. 6 and 7). The recombinant protein eluted at >0.5 *M* NaCl. Western blot analysis showed a pure and immunoreactive protein. No impurities could be detected by incubation with the anti-*E. coli* rabbit serum (Fig. 8). Depending on the amount of gp41 applied to the SDS gel, multiple banding and smearing occurs, together with a prominent band at the size of the dimer. These bands could only be seen in the more sensitive Western blot analysis and depend also on the serum dilution and the anti-gp41 antibody content. These minor bands are not seen after staining with Coomassie brilliant blue. The multiple banding could be due to the tendency of the gp41 protein to aggregate; even in the presence of SDS a minor portion appears in an aggregated form of variable size.

The purified antigen could now be used in a diagnostic assay system, based on the Western blot principle. For this, antigens such as recombinantly produced and purified gp41, p24 and reverse transcriptase from HIV I, and gp36 from HIV II were

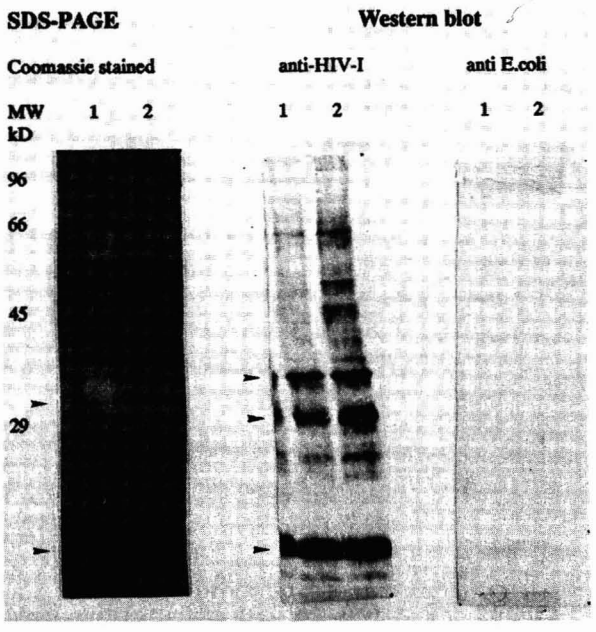

Fig. 8. SDS-PAGE and Western blot analysis of purified recombinant gp41. Molecular masses are indicated on the left. In lanes 1 and 2 purified antigen preparations of different batches are separated. The Coomassie brilliant blue-stained gel shows a homogeneous band at 16 000 dalton. There is also a band at about 32 000 dalton, which could be identified as a dimer of the recombinant protein. The Western blot incubated with HIV pool serum showed the immunoreactivity of the purified antigen. As can be seen, even under denaturing conditions in the SDS-PAGE aggregation of the protein occurs, which resulted in a smear over the whole lane. The incubation with anti-*E. coli* rabbit serum demonstrated the purity of the antigen preparations.

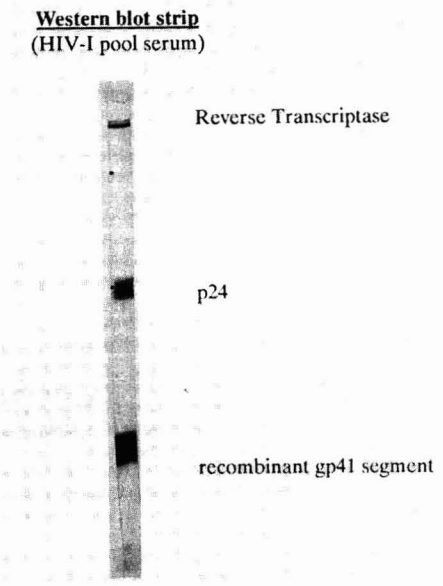

Fig. 9. Usage of the purified recombinant gp41 in a confirmation test based on the Western blot assay principle. A nitrocellulose strip was incubated with an HIV I-positive patient's serum and bound antibodies were made visible by immunodetection as described under Experimental. On the strip are indicated electrophoretically separated a recombinant-produced reverse transcriptase (p66), a p24 equivalent and the purified gp41 antigen.

applied to SDS-PAGE and transferred to nitrocellulose. After incubation with a patient's serum [diluted 1:100 with Tris-buffered saline containing Tween 20 (0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 7.5, 8.01% Tween 20)], as shown for example in Fig. 9, and immunological detection with a rabbit serum containing anti-human IgG antibodies conjugated with peroxidase, a clear test result confirming the seropositivity was obtained. In comparison to the conventional Western blot, using whole virus lysates, a more sensitive detection of anti-gp41 antibodies could be demonstrated. This is due to the absence of glycosylation of the recombinant gp41 and, therefore, sharp banding in SDS-PAGE and unrestricted availability, whereas especially the anti-gp41 reactivity is hampered for several reasons in the conventional Western blot assay system [9,10], including the small amount of gp41 present in every individual preparation, glycosylation, which resulted in diffuse banding in SDS-PAGE, and under-representation compared with other viral components.

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Isolation and partial characterization of angiotensinase A and aminopeptidase M from urine and human kidney by lectin affinity chromatography and high-performance liquid chromatography

J. E. SCHERBERICH*, J. WIEMER, C. HERZIG, P. FISCHER and W. SCHOEPPE
Department of Nephrology, Centre of Internal Medicine, J. W. Goethe University, Theodor Stern Kai 7, D-6000 Frankfurt am Main (F.R.G.)

ABSTRACT

Angiotensinase A (ATA) and aminopeptidase M (APM) were partially purified from human urine specimens and human kidney particles using wheat germ lectin affinity chromatography, anion-exchange Fast Protein Liquid Chromatography (FPLC) (Mono Q), chromatofocusing (Mono P, FPLC) and Superose 12 gel filtration. APM, a globular 5-nm glycoprotein, is localized in the brush border membrane of the proximal tubule; angiotensin II-degrading ATA is present on glomerular endothelia and podocytes and, to lesser extent, in the brush border. For the first time, both peaks of ATA and APM activity from urine samples were separated by the above-mentioned techniques with only slight overlap; ATA (146 000 dalton; p*I* 4.8) was enriched more than 20-fold and APM (153 000 dalton, p*I* 4.7) more than 50-fold compared with the activity of the starting material. Using similar separation steps, ATA and APM solubilized from kidney particles could not be resolved into two distinct peak fractions, however, except after hydrophobic interaction chromatography. Thus urine is a major source for the preparation of individual ATA and APM fractions, necessary to generate specific anti-enzyme antibodies for diagnostic purposes.

INTRODUCTION

Both angiotensinase A (ATA; E.C. 3.4.11.7) and aminopeptidase M {microsomal aminopeptidase (APM) = alanine aminopeptidase (AAP) [1]; E.C. 3.4.11.2} are membrane-bound enzymes with a characteristic distribution pattern in the kidney: whilst APM is a marker protein of the brush border of the proximal tubule, ATA {aminopeptidase A (APA) is angiotensinase A (ATA) [2,3]} is predominantly localized in glomerular endothelia and visceral epithelial cells (podocytes) and, to lesser extent, in the proximal tubule [4,5]. Previous studies suggest that ATA of human kidney is involved in the regulation of the intrarenal renin–angiotensin system [5].

Human kidney sections preincubated with 0.05 m*M* angiotensin II revealed nearly total inhibition of substrate splitting by ATA whereas APM activity was not significantly suppressed. Pretreatment of sections with angiotensin I and III did not alter ATA activity [6,7]. Further, ATA activity was competitively inhibited only by angiotensin II, but not by angiotensin I and III [7]. Hence it could be concluded that

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angiotensin II is specifically degraded into smaller peptide fragments by this enzyme [5].

Determinations of the molecular weight of ATA isolated from human serum gave values of 190 000 dalton [8] and, in the case of human maternal serum, of 260 000 [9]. The molecular weight of ATA isolated from pig kidney was 300 000 [10]. All determinations of the molecular weight were performed by Sephadex G-200 (Pharmacia) analytical gel filtration. In contrast, APM is a 240 000 exopeptidase of the brush border membrane which hydrolyses preferentially natural or synthetic substrates with an alanine residue. Other amino acids, especially leucine, may also be removed hydrolytically, with the exception of proline [6,11,12]. Physiological substrates of APM may include hormones such as glucagon, atrial natriuretic peptide, insulin B-chain, somatotropin and parathormone.

Both enzymes are excreted into urine at an increased rate in patients with renal damage [6,12]. Hence, in addition to kidney membrane fractions, in the case of tubular damage the urine is an excellent source for isolating and characterizing ATA and APM of human renal origin. However, attempts to purify ATA and leucine-aminopeptidase (apparently identical with APM) from human serum experienced extreme difficulty in the separation of the two enzymes [8]. This paper reports on the preparation of ATA and APM from kidney tissue fractions and from urine specimens of patients with renal diseases by applying wheat germ agglutinin (WGA) affinity chromatography, ion-exchange chromatography, chromatofocusing and Superose-12 gel filtration with fast protein liquid chromatographic (FPLC) equipment.

To our knowledge, this is the first investigation on ATA partially purified from urine specimens of patients with kidney diseases.

EXPERIMENTAL

Urine specimens with volumes between 1 and 3 l having high ATA and APM activities (ATA 5–22 U/l, APM 11–39 U/l), collected from patients with tubular damage, were centrifuged at 3000 *g* at 4°C for 10 min (GSA rotor) using a Sorvall RC2B centrifuge. The supernatants were dialysed with buffer containing 0.5 *M* Tris and 0.2 *M* NaCl (pH 7.6) and concentrated using a Millipore (Bedford, U.S.A.) tangential flow membrane unit (Minitan-System; sieving coefficient 30 000 dalton) or a Hemoflow HF-80 capillary (Fresenius) with a sieving coefficient of 50 000 dalton. The ATA activities in the concentrates were between 83 and 136 U/l and the APM activities between 141 and 624 U/l.

In addition, a crude ATA–APM fraction was prepared from normal human kidney by digestion of tissue homogenates with bromelain (kidney protein: bromelain ratio = 30:1, w/w) followed by fractionated ammonium sulphate precipitation. After treatment of the membranes with bromelain, the supernatant was decanted and aliquots were taken for protein and enzyme assays. A 492-g amount of solid ammonium sulphate was added to 1260 ml of the supernatant with continuous stirring (60% ammonium sulphate saturation). After centrifugation (10 000 *g*, 20 min, 4°C), ammonium sulphate was added to the supernatant (80% saturation) and centrifuged again. The precipitate was dissolved in 35 ml of 50 m*M* Tris buffer (pH 7.2) and loaded onto a phenyl-Sepharose CL 4B column (12.5 × 2.6 cm I.D.). Proteins were eluted using an ammonium sulphate gradient from 1.7 to 0 *M* with 50 m*M* Tris buffer (pH

7.2) at a flow-rate of 1.5 ml/min. Peak fractions of APM and ATA were pooled and rechromatographed.

Lectin affinity chromatography

Concentrated and native samples (enzyme activities as mentioned above, sample volumes between 175 and 450 ml) were loaded onto a wheat germ lectin–Sepharose column (15 cm × 1 cm I.D.) and eluted with 0.05 *M* Tris buffer containing 0.01 *M* $CaCl_2$ and 0.2 *M* NaCl (pH 7.6) (fraction size 5 ml, flow-rate 1 ml/min). Glycoproteins biospecifically adsorbed to the WGA–gel matrix were then desorbed with the same Tris–NaCl buffer containing 60 m*M* N-acetyl-D-glucosamine (same flow-rate and fraction size as during elution). Protein absorbance was measured at 280 nm (Uvicord S; Pharmacia, Uppsala, Sweden).

Conventional gel filtration

The following conditions were used: Sephacryl S-300 gel matrix; column, 120 cm × 5 cm I.D.; sample volume, 15 ml; elution buffer, 0.2 *M* Tris–0.5 *M* NaCl (pH 7.6); flow-rate, 0.75 ml/min; fraction size, 7 ml. The protein absorbance was measured at 280 nm (Uvicord III).

Fast protein liquid chromatography

The following FPLC system, manufactured by Pharmacia, was used: LCC-Controller-500 Plus (gradient programmer), two P-500 pumps, a UV-M monitor (280 nm), an REC 482 recorder, an MV-7 multi-position motorvalve, a FRAC 100 fraction collector, a flow-through pH electrode and a pH monitor. Buffers were degassed and ultrafiltered (Millipore filtration unit, 0.45-μm membrane pore size).

FPLC anion-exchange chromatography was performed with a Mono Q HR 5/5 column (Pharmacia) using the following buffer system: 20 m*M* Tris–HCl (pH 7.2 and 7.0) with an NaCl gradient from 0 to 1 *M*. The sample volume was 100–500 μl, the flow rate 1 and 2 ml/min and the fraction volume 1 ml.

FPLC chromatofocusing was performed using a Mono P HR 5/20 column. The sample was equilibrated with starting buffer following elution from a PD 10 column (Sephadex G-25-M) according to the manufacturer's instruction manual. Then 500 μl of the sample were applied to the Mono P column equilibrated with starting buffer [0.025 *M* methylpiperazine (pH 5.7)]; protein fractions were eluted with Polybuffer 74 (Pharmacia); the pH gradient ranged from 4 to 5. The flow-rate was 1 ml/min and the fraction volume was 1 ml.

FPLC gel filtration was carried out with a Superose 12 HR 10/30 column. The buffer was 0.15 *M* NaCl with 0.05 *M* Tris–HCl (pH 7.6), the sample volume was 100 μl, the flow-rate was 1 ml/min and the fraction size was 1 ml.

Enzyme determination

Individual peak fractions of the enzymes eluted from the columns were analysed in a kinetic assay (PM-4 spectrophotometer; Zeiss, Oberkochen, F.R.G.) at 405 nm and 37°C. For ATA activity, the assay medium contained 0.8 ml of 0.05 *M* Tris buffer (+ 62.5 m*M* Ca^{2+}) (pH 7.7), 0.1 ml of the sample and 0.1 ml of substrate solution (substrate: L-glutamic acid 4-nitroanilide), giving a final concentration of 1.5 m*M*. For APM activity, the assay medium contained 0.8 ml of 0.05 *M* Tris buffer (pH 8.0),

0.1 ml of sample solution and 0.1 ml of substrate solution (substrate: DL-alanine-*p*-nitranilide), giving a final concentration of 1.66 m*M*. All the activities were calculated in U/l. Screening for enzyme activity (ATA, APM) was performed in a colorimetric fast processing microtitre plate assay [enzyme-linked immunosorbent assay (ELISA) reader, Flow Labs.] at 405 nm and room temperature. The assay medium was the same as mentioned above. Further details, *e.g.*, the determination of dipeptidylaminopeptidase IV and γ-glutamyl transpeptidase, have been reported elsewhere [5,6,12–14].

Protein determination

Protein was determined by the method of Bradford (Bio-Rad Labs. assay No. M9610-15).

Electrophoretic analysis

Native polyacrylamide gel electrophoresis, isoelectric focusing and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) were performed using the PHAST system equipment (Pharmacia). PHASTGel gradient 8–25 gels and PHASTGel native buffer strips were applied for native PAGE (sample volume 1 μl). The electrophoresis was run at 400 V, 10 mA and 280 V h. SDS-PAGE was performed as described previously [13]. Isoelectric focusing was carried out using PHASTGel IEF 3–9 according to the Separation Technique File No. 100 (Pharmacia). Protein bands were developed using the sensitive silver staining method (Development Technique File No. 210). After PAGE, enzyme-specific staining of protein bands for ATA and APM activity was performed as reported previously [6,12,14]. In brief, Fast Blue B was used as a coupling salt and the following peptide-4-methoxy-2-naphthylamide (MNA) substrates were used: for APM, alanine-MNA; and for ATA, α-glutamyl-MNA. Gels were incubated for 20 min at 37°C, washed and dried. The high- (HMW) and low-molecular-weight (LMW) calibration kits (Pharmacia) were used as a standard for native PAGE and SDS-PAGE, and the Pharmacia broad p*I* calibration kit for analytical electrofocusing.

RESULTS AND DISCUSSION

Initial purification steps of ATA and APM from urine specimens of patients with tubular damage included a concentration step (ultrafiltration) and gel permeation chromatography (Sephacryl S-300) of the samples. Separation of urine concentrates on Sephacryl S-300, however, revealed that ATA was eluted in a similar elution profile to that for APM (Fig. 1). Hence removal of ATA from APM was not possible by this approach. However, most of the γ-glutamyl activity as well as slight activities of ATA, APM and DAP IV were found in fractions 90–110 (according to the exclusion volume determined with blue dextran). We assume that these large enzyme forms represent multi-enzyme complexes of "vacuolar blebs" derived from tubular epithelial membranes and shed into urine in patients with renal damage [11,12,15]. Similar data on the exfoliation of multi-enzyme complexes in the plasma derived from liver cell membranes were reported by other investigators [11].

A separation procedure including WGA affinity chromatography, anion-exchange and chromatofocusing and finally gel filtration with a Superose 12 HR 10/30

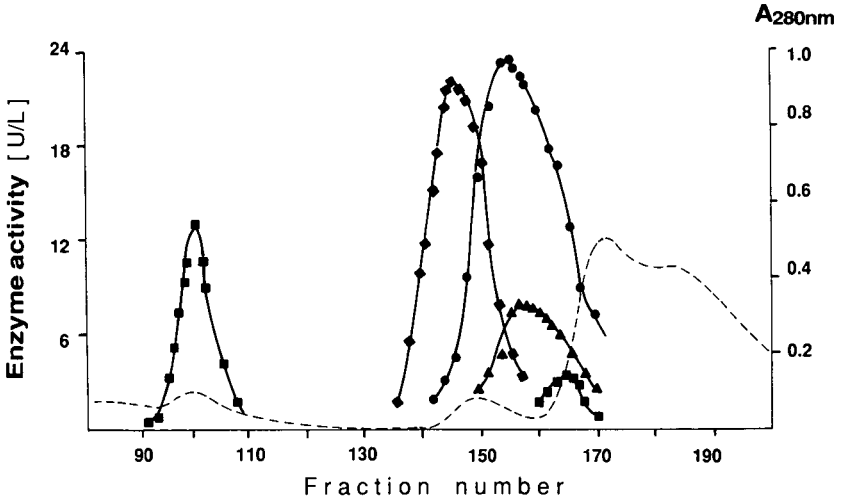

Fig. 1. Gel filtration (Sephacryl 300) of pooled urine concentrate from patients with tubular damage. Elution profiles of protein and kidney marker enzymes γ-glutamyl transpeptidase (γ-GT), dipeptidyl aminopeptidase IV (DAP IV), angiotensinase A (ATA) and aminopeptidase M (APM). Protein absorbance recorded at 280 nm. Column, 120 cm × 5 cm I.D.; sample volume, 15.0 ml; buffer, 0.2 *M* Tris–0.5 *M* NaCl (pH 7.6); flow-rate, 0.75 ml/min; fraction size, 7 ml. Note similar elution profiles of ATA and APM. ▲ = ATA; ● = APM; ◆ = DAP IV; ■ = γ-GT; dashed line = protein.

column turned out to be more successful for separating the two enzymes into two individual peak fractions: WGA affinity chromatography of urine specimens removed large amounts of non-glycosylated proteins, while ATA and APM were specifically

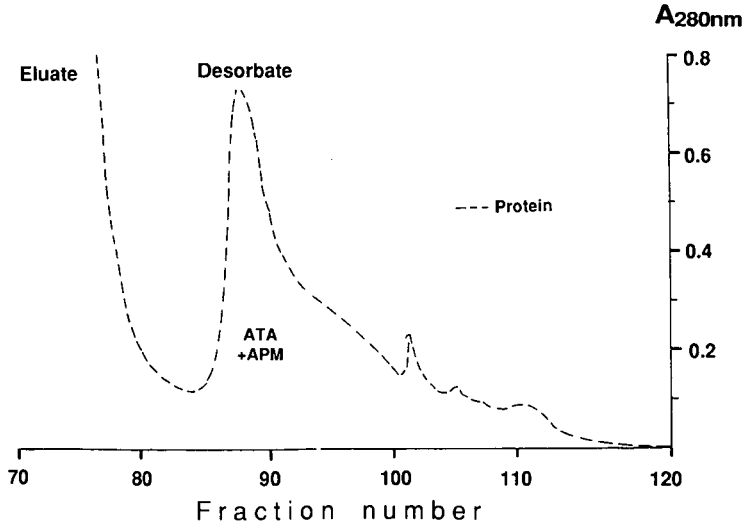

Fig. 2. Wheat germ lectin affinity chromatography of urine from patients with renal disease (tubular damage). ATA and APM excreted in urine were biospecifically bound to the gel matrix and then desorbed (desorbate) with 0.05 *M* Tris–0.2 *M* NaCl + 0.01 *M* $CaCl_2$ buffer containing 60 m*M* N-acetyl-D-glucosamine. Flow-rate, 1 ml/min; fraction size, 5 ml; eluate = unbound proteins; sample volume, 370 ml. Dashed line = protein.

TABLE I

COMPARISON OF SELECTED PROPERTIES OF ATA AND APM PREPARED FROM URINE SPECIMENS AND HUMAN KIDNEY PARTICLES, RESPECTIVELY

Source	Fraction	Specific activity (mU/mg)	Purification factor (fold)	Isoelectric point (pH)	Molecular mass (kilodalton)
Urine	*Angiotensinase A*				
	Pool (concentrate)	96	1	4.85	
	WGA desorbate	990	10.3	4.85	146[b]
	Mono Q FPLC	1446	15.1		
	Superose 12 FPLC	⩾2000[a]			
	Aminopeptidase M				
	Pool (concentrate)	92	1	4.7	
	WGA desorbate	2743	29.7	4.7	153[b]
	Mono Q FPLC	3716	40.3		
	Superose 12 FPLC	> 6000[a]			
Human kidney	*Angiotensinase A*				
	Supernatant (bromelain digest)	78	1	4.3–4.6	178–196[c]
	Ammonium sulphate precipitate	341	4.3	4.3–4.6	
	Mono Q/Mono P FPLC: insufficient separation of ATA and APM				
	Hydrophobic interaction chromatography (phenyl-Sepharose CL 4B)	6550	83.9		177[b]
	Aminopeptidase M				
	Supernatant (bromelain digest)	505	1	4.0–4.6	141–155[c]
	Ammonium sulphate precipitate	1684	3.3		145[b]
	Hydrophobic interaction chromatography (phenyl-Sepharose CL 4B)	16 680	33.0		

[a] No final data given; protein concentration under detection limit.

[b] Molecular weights as determined by gel filtration using Superose 12 HR 10/30 columns calibrated with Blue Dextran 2000, thyroglobulin, ferritin, catalase, aldolase, bovine serum albumin, ovalbumin chymotrypsinogen and ribonuclease; correlation coefficient of the standard line was 0.968.

[c] Molecular weights as determined by native PAGE.

adsorbed to the lectin matrix and, after changing the buffer system, were enriched 10–30-fold compared with the starting material (Table I, Fig. 2). Isoelectric focusing (PHASTGel IEF, pH 3–9) of WGA-bound ATA and APM revealed a p*I* of 4.85 for ATA and of 4.7 of APM; enzyme-specific staining showed sharp individual bands (Table I, Fig. 3). Based on these findings, further separation of ATA and APM was carried out using FPLC Mono Q and Mono P columns. Applying these techniques, peaks of both ATA and APM activity were separated with only slight over-lapping (Figs. 4 and 5). The peak fractions were pooled, concentrated and applied to a Superose 12 column. After FPLC gel permeation, other glycoproteins ("impurities") which were co-eluted during Mono Q/Mono P FPLC could now be removed from the ATA–APM fraction (Figs. 6 and 7). However, as the ATA fraction still contained some APM activity, we suggest that both enzymes have similar structural properties.

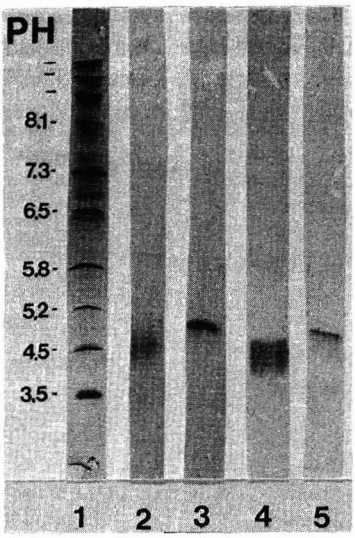

Fig. 3. Isoelectric focusing of urine protein fractions using PHASTGel p*I* 3–9. (1) Marker proteins for calibration of p*I*; (2) ATA solubilized from kidney tissue particles (p*I* 4.3–4.6); (3) ATA urine (p*I* 4.85); (4) APM solubilized from human kidney particles (p*I* 4.0–4.6); (5) APM urine (p*I* 4.7); enzyme-specific staining.

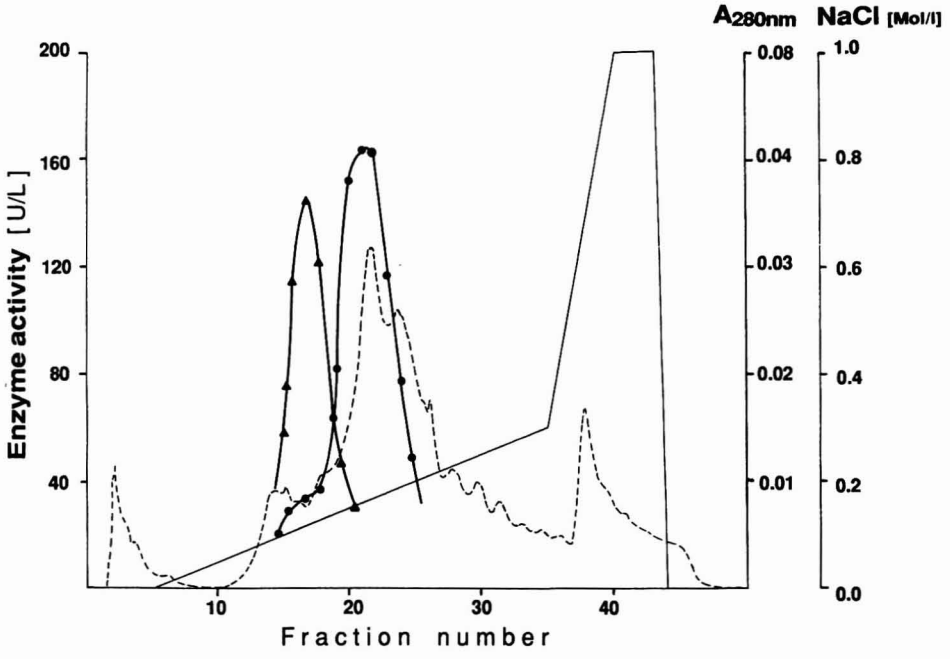

Fig. 4. Anion-exchange chromatography of urinary proteins (see above) using a Mono Q HR 5/5 column. FPLC: flow-rate, 2 ml/min; pressure, 2 MPa; sample volume, 100–500 μl; 20 m*M* Tris–HCl buffer (pH 7.2), fraction size, 1 ml. Using an NaCl gradient from 0 to 1 *M* ATA and APM activities are eluted in separate peak fractions. ▲ = ATA; ● = APM. Dashed line = protein; solid line = NaCl gradient.

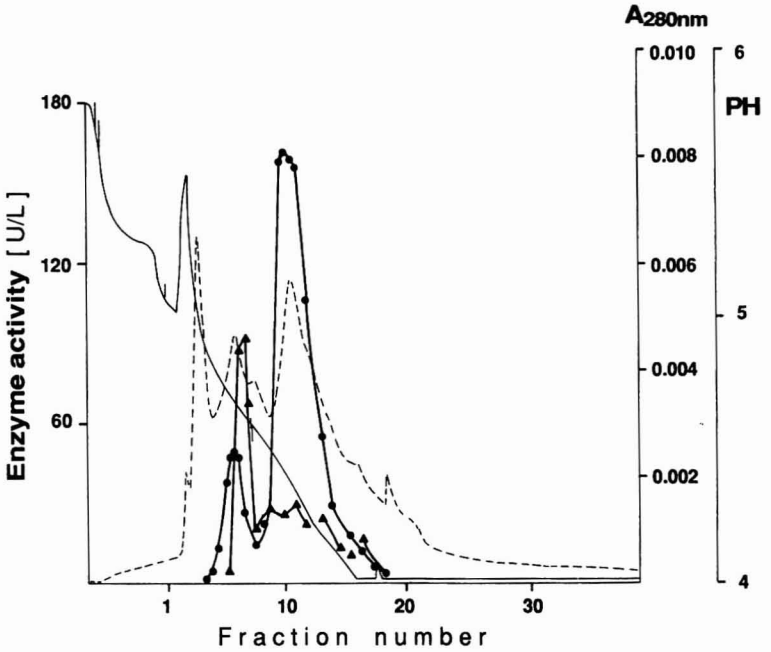

Fig. 5. FPLC chromatofocusing of urine proteins (see above) applying a Mono P HR 5/20 column. Precolumn procedure as described in the text (buffer equilibration/PD-10 Sephadex). Buffer A, 0.025 *M* methylpiperazin (pH 5.7); buffer B, Polybuffer 74; pH gradient from 5 to 4 (elution range of proteins); sample volume, 500 μl; flow-rate, 1.0 ml/min; pressure, 2.5 MPa; fraction size, 1 ml. Enzyme maxima of ATA and APM are eluted in different peak fractions. ▲ = ATA; ● = APM; dashed line = protein; solid line = pH gradient.

Both ATA and APM may constitute peripheral "microdomains" of the tubular plasmamembrane [12,16]. The APM protein is a globular 5–10-nm component of a glycoconjugate complex of the brush border membrane surface also containing γ-glutamyl transpeptidase, maltase and dipeptidylaminopeptidase IV [6,11–16].

In contrast to the enzymes from urine specimens, ATA–APM from human kidney tissue fractions were more heterogeneous (Fig. 3): the p*I* ranged from 4.33 to 4.62 (ATA, kidney) and from 4.03 to 4.59 (APM, kidney). These wide ranges might be the reason for difficulties in separating ATA from APM activity after solubilization from tissue fractions. ATA and APM from ammonium sulphate precipitation (bromelain digest of kidney particles) could not be prepared as single peak fractions following the separation steps used for the urinary enzymes (WGA affinity chromatography, Mono Q, Mono P, Superose 12 columns). Similar problems in removing leucyl-β-naphthylaminidase activity from aminopeptidase A (= ATA) of serum origin have been reported earlier [8]. Thus, a modified scheme was developed including ammonium sulphate precipitation, hydrophobic interactivity chromatography and chromatofocusing. Further studies are currently in progress to purify human kidney ATA with the help of these techniques (hydrophobic interaction chromatography), where preliminary data were more promising.

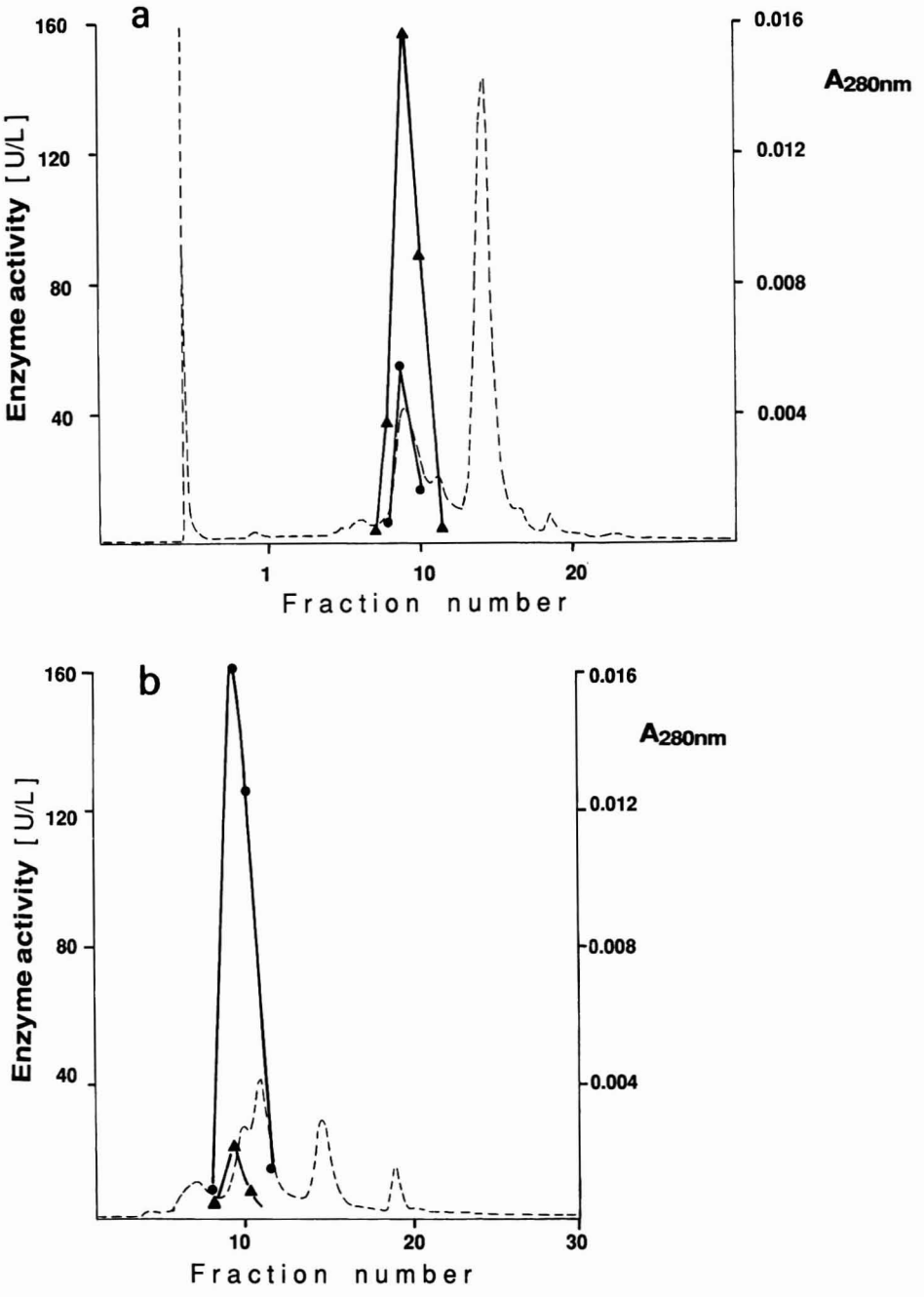

Fig. 6. FPLC gel permeation of urine proteins using a Superose 12 HR 10/30 column. Pooled peak fractions of either (a) ATA or (b) APM activity after anion-exchange FPLC (Mono Q, Fig. 3) were applied to the gel and eluted with 0.15 *M* NaCl–0.05 *M* Tris–HCl buffer (pH 7.6). Fraction size, 1 ml; sample volume, 100 μl; flow-rate, 1 ml/min; pressure, 2 MPa. Elution of (a) a major ATA and (b) a major APM peak; removal of non-enzymatic protein, especially as seen in elution profile (a). ▲ = ATA; ● = APM; dashed line = protein.

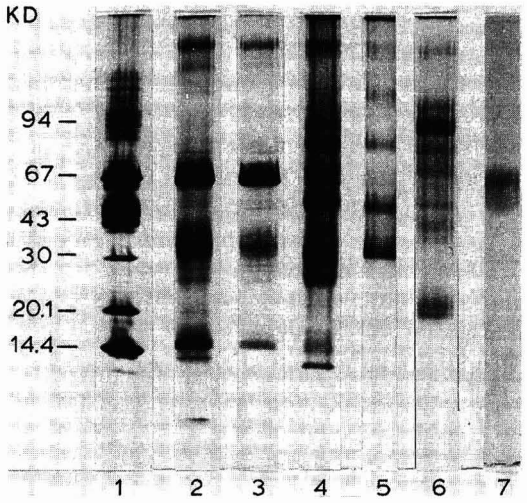

Fig. 7. SDS–PAGE of urinary protein fractions with a PHAST system. 1 = LMW marker; 2 = urine pool used for the separation of ATA and APM; 3 = WGA eluate (unbound proteins); 4 = WGA desorbate (adsorbed glycoproteins); 5 = APM peak fractions (Mono Q column); 6 = ATA peak fractions (Mono Q); 7 = native PAGE of APM peak fraction (enzyme-specific staining). 1–6, Silver staining; peak fractions from Fig. 5 gave one faint protein band after silver staining for both ATA and APM maxima (not shown). KD = Kilodalton.

CONCLUSIONS

ATA and APM from human urine specimens were partially purified using WGA affinity chromatography, anion-exchange FPLC (Mono Q), chromatofocusing (Mono P, FPLC) and Superose 12 gel filtration, whereby both marker enzymes could be sufficiently separated from each other. As ATA–APM solubilized from human kidney particles could not be resolved into two distinct peak fractions (except after hydrophobic interaction chromatography), urine appears to be a major source for the preparation of individual ATA and APM fractions. This result is an important initial step in the production of specific anti-enzyme antibodies, relevant for diagnostic and research purposes.

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Rapid purification of cytochrome *c* oxidase from *Paracoccus denitrificans*

GUY C. M. STEFFENS*, EVELYNE PASCUAL and GERHARD BUSE
Institut für Biochemie, Rheinisch-Westfälische Technische Hochschule Aachen, Klinikum, Pauwelstrasse 30, D-5100 Aachen (F.R.G.)

ABSTRACT

Two methods are described for the purification of cytochrome *c* oxidase from Triton X-100 extracts of the periplasma membrane of *Paracoccus denitrificans*. The first is a large-scale procedure for the preparation of 100–250 nmol of cytochrome *c* oxidase (10–20 mg) in 1 week. The second is a rapid procedure for isolating up to 25 nmol in 2–3 days. Owing to the high yields given by fast protein liquid chromatography (FPLC) on Mono Q columns, the overall yield is about 20%, whereas the yield in many other previously published procedures does not exceed 10%. The use of FPLC on Mono Q also offers a considerable saving of time.

INTRODUCTION

Oxidative phosphorylation in procaryotes is achieved through a variety of terminal oxidases [1]. Among them, cytochrome *o*(*bo*) appears to be the most widespread enzyme [2]; the best studied respiratory components, however, are the aa_3-type oxidases [1]. The first procedures for the isolation of this type of oxidase were described by Yamanaka and Fukumori [3], Ludwig and Schatz [4] and Fee *et al.* [5]. Since then, procedures for the isolation of aa_3-type oxidases from a large number of prokaryotes have been described [6–8]. All these procedures have in common that they are time consuming and that the yields are low. Therefore, in an attempt to increase the overall yield and to decrease the time requirement, we developed a procedure based on fast protein liquid chromatography (FPLC) on Mono Q columns. Two methods will be discussed: a large-scale procedure for the isolation of 100–250 nmol and a more rapid, small-scale procedure for amounts up to 25 nmol.

EXPERIMENTAL

Cells of *Paracoccus denitrificans* (ATCC 13543) are grown in large-scale fermenters (100–3000 l) at the Gesellschaft für Biotechnologische Forschung (Braunschweig, F.R.G.) with succininic acid as carbon source. After collection of the cells, the biomass is frozen at −70°C.

Membranes of *Paracoccus denitrificans* are prepared essentially as described in

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ref. 4. After thawing of 1 kg of wet biomass, the suspension obtained is diluted to about 2 l with 10 m*M* potassium phosphate (pH 7.4) and homogenized in a Dyno-Mill, using 0.3-mm glas beads and a rotation speed of 4500 rpm. The temperature is kept as low as possible, in any case below 10°C. The cell suspension is passed twice through the homogenizer and whole cells are sedimented by a 30-min centrifugation at 5000 rpm (Beckman J2-21 with JA-20 rotor). The supernatant is then centrifuged overnight to sediment the crude membranes at 8000 rpm and 4°C[a]. The supernatant of this centrifugation step is checked for haeme a and protein contents. About 10% of the total haeme a is lost in this step. As the haeme a-to-protein (h/p) ratio of this solution is below 0.1 nmol/mg, this fraction is discarded. The membrane is then pre-extracted with deoxycholate and KCl (final concentrations: 0.1% deoxycholate and 0.2 *M* KCl). After a 1-h stirring period, the suspension is centrifuged overnight at 8000 rpm[a]. The supernatant is checked again for haeme a and usually discarded. Only 2–3% of the haeme a is lost in this step. The membrane suspension is frozen in 100-ml portions.

Preparation of the Triton X-100 extract

Depending on the desired procedure, one or more portions of membrane suspension are thawed. The intrinsic membrane proteins are solubilized by addition of Triton X-100–potassium phosphate (pH 7.8)–EDTA to the final concentrations of 2%, 0.1 *M* and 0.1 m*M*, respectively. The suspension is homogenized in a Potter–Elvehjem homogenizer and centrifuged for 1 h using a Beckman JA-10 rotor. The supernatant is saved and the pellet is resuspended in 2% Triton X-100–0.1 *M* potassium phosphate (pH 7.8)–0.1 m*M* EDTA and treated as above. The combined supernatants are characterized and concentrated by ultrafiltration.

Large-scale protocol

About 100 ml of Triton X-100 extract, corresponding to *ca.* 2000 nmol of haeme a and *ca.* 6000 mg of protein, are applied to an Ultrogel AcA-34 column (90 × 10 cm I.D.) and chromatographed in 50 m*M* potassium phosphate (pH 7.6)–0.2% Triton X-100–20 m*M* KCl–0.1 m*M* EDTA. The eluate is monitored at 280 and 420 nm; the haeme a-to-protein (h/p) ratio of selected fractions are determined. Fractions in which the h/p ratio is clearly higher than the initial value are pooled for further purification. This solution is concentrated and freed from salts by ultrafiltration and applied to a Q-Sepharose column (20 × 5 cm I.D.), equilibrated with 10 m*M* potassium phosphate (pH 7.8)–1% Triton X-100–0.1 m*M* EDTA. A smaller part of the proteins, mostly cytochrome *b*, is not bound and appears in the effluent. All cytochromes with reduced minus oxidized difference absorption bands at 550, 590 and 605 nm are firmly bound to the anion exchanger under these conditions. Proteins are eluted by a linear gradient from 0 to 1 *M* NaCl. Again, the eluate is monitored at 280 and 420 nm, and fractions are checked for their specific h/p ratios. Fractions with twice the initial h/p ratio are pooled and used for further purification on Mono Q.

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[a] The centrifugation time can be reduced considerably when the preparation is performed on a smaller scale and ultracentrifuges are used.

Small-scale protocol

A 20-ml volume of Triton X-100 membrane extract containing *ca.* 200 nmol of haeme a and having an h/p ratio of *ca.* 0.3 nmol/mg are applied to a small Ultrogel AcA-34 column (30 × 4 cm I.D.) and chromatographed overnight in the same buffer as in the large-scale procedure. Fractions with a substantially improved h/p ratio are used for further purification steps on Mono Q. The crude cytochrome *c* oxidase solution is applied in two portions of about 60 nmol of haeme a to a Mono Q HR 10/10 column. In two consecutive runs cytochrome *c* oxidase is further purified and the cytochrome *c* oxidase fractions are again pooled and used for two or three subsequent runs on Mono Q.

Fast Protein Liquid Chromatography

FPLC is performed on a Mono Q HR 10/10 column (Pharmacia). This column is coupled to an LKB 2150 GTI HPLC pump, an LKB 2152 controller, a LKB 2158 Uvicord SD monitor with a titanium flow cell, an LKB 2212 Helirac fraction collector and an electromagnetic valve to form the gradient. The buffers used in these FPLC experiments were (A) 25 m*M* potassium phosphate (pH 7.8)–1% Triton X-100–0.1 m*M* EDTA and (B) A containing 1 mol/l NaCl. In some experiments potassium phosphate was replaced with 20 m*M* Tris–HCl (pH 7.8) in order to allow determinations of phophorus by inductively coupled plasma atomic emission spectrometry (ICP-AES). The Mono Q column was washed after every 4–8 runs according to the specifications of the manufacturer (Pharmacia). Prior to a series of runs on Mono Q, the crude cytochrome *c* oxidase solutions were filtered through a 0.2-μm filter (Sartorius 11107 25 N).

Ultrafiltration

This was carried out using a Minitan tangential flow ultrafiltration system (Millipore) with membranes having a cut-off of 30 kilodalton (Minitan PTTK, Millipore).

Haeme a

Haeme a was determined spectrophotometrically according to ref. 9. Haeme a concentrations were calculated using a molar absorption coefficient of 12000 l/mol · cm for the difference absorbance at 605 nm between dithionite-reduced and hexacyanoferrate(III)-oxidized cytochrome *c* oxidase. This value has been adopted from bovine heart cytochrome *c* oxidase [9] and has so far given satisfactory results. Cytochromes *b*, *c* and a_1 were identified by their reduced minus oxidized difference absorbance bands at 560, 550 and 590 nm, respectively [1].

Protein contents

Protein contents were determined with the bicinchoninic acid assay (BCA) of Pierce [10].

RESULTS

Two methods are described for the purification of cytochrome *c* oxidase from *Paracoccus denitrificans*. The use of these procedures is not restricted to the isolation of cytochrome *c* oxidase but may also be used for the purification of other intrinsic

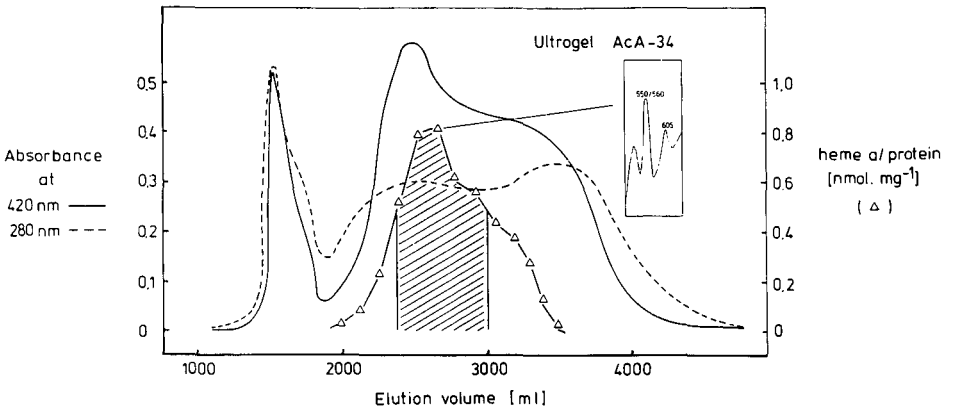

Fig. 1. Gel chromatography of Triton X-100 membrane extract of *Paracoccus denitrificans* on Ultrogel AcA-34. The fractions corresponding to the shaded area were used for subsequent purification on Q-Sepharose.

membrane proteins, such as cytochromes *b*, *c*, *o*(*bo*) and a_1. We have already successfully purified cytochrome *c* oxidase (caa_3) from *Thermus thermophilus* by the same procedure [11].

Large-scale purification

The large-scale procedure starts with the solubilization by Triton X-100 of an appropriate sample of membrane suspension, usually 200–300 ml, as described under Experimental. The h/p ratios of these membrane extracts vary from 0.2 to 0.6 nmol haeme a/mg. Fig. 1 shows the result of the first chromatography of concentrated membrane extract on Ultrogel AcA-34. As shown in Table I, this procedure leads to a loss of about 45% in terms of haeme a.

TABLE I

LARGE-SCALE PURIFICATION OF CYTOCHROME *c* OXIDASE FROM *PARACOCCUS DENITRIFICANS*

Step	Haeme a (nmol)[a]	Protein (mg)	Haeme a/protein (nmol/mg)	Yield (%)[b]	Overall yield (%)
Triton X-100 membrane extract	2.100	6.550	0.3	100	100
Ultrogel AcA-34	1.200	1.714	0.7	57	57
Q-Sepharose	715	477	1.5	60	34
	150	100[c]	1.5		
Mono Q (run a)	123	27	4.6	82	28
Mono Q (run b)	111	8.6	12.9	90	25
Mono Q (run c)	98	4.7	20.8	88	22

[a] 2 nmol of haeme a correspond to 1 nmol of cytochrome *c* oxidase.

[b] Yield as a percentage of haeme a in Triton X-100 extract.

[c] As the binding capacity of Mono Q HR 10/10 is limited, only 100-mg portions are used for further purification.

The fractions corresponding to the shaded area are pooled and concentrated by ultrafiltration. The salt concentration is reduced by alternating ultrafiltration and dilution steps prior to the next purification step on Q-Sepharose. When the specific conductivity of the sample has attained the value of the starting buffer [25 mM potassium phosphate (pH 7.8)–1% Triton X-100–0.1 m*M* EDTA], the material is bound to the anion exchanger and, after washing with 300 ml of starting buffer, eluted with a linear gradient from 0 to 1 *M* NaCl in the same buffer. The result of this experiment is shown in Fig. 2. The resolution in this step is already much improved in comparison with the gel chromatographic step on Ultrogel AcA-34. As judged from the reduced minus oxidized difference spectra (see insets in Fig. 2), several discrete fractions are observed, including cytochrome *b* or *o*(*bo*) (560 nm), cytochrome a_1 (590 nm), cytochrome *c* (550 nm) and cytochrome *a* (605 nm). After characterization, the fractions are pooled according to their specific h/p ratios. Again, a substantial loss is observed and the overall yield drops to 36% (Table I).

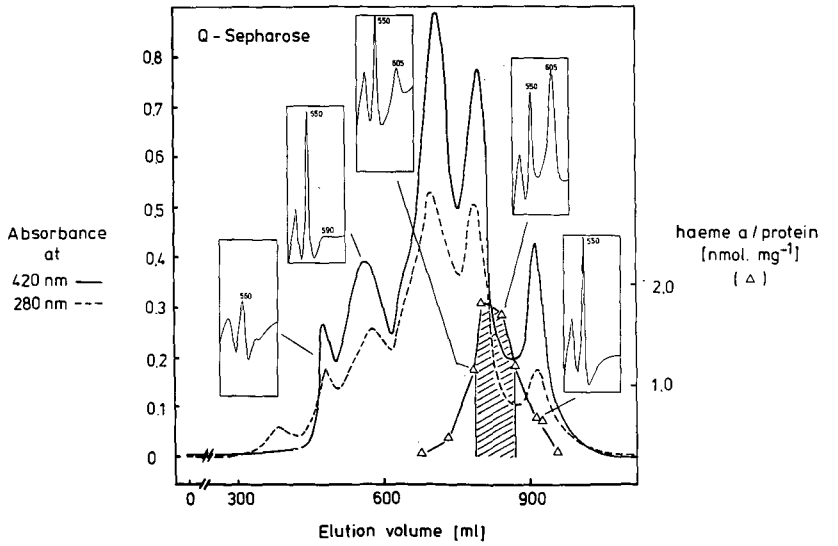

Fig. 2. Ion-exchange chromatography on Q-Sepharose. The reduced minus oxidized difference absorbance spectra in the insets indentify the cytochrome components (see Results). The material corresponding to the shaded area was used for further purification by FPLC on Mono Q.

The pooled fractions from the Q-Sepharose chromatography with an h/p ratio of 1.5 nmol/mg (Fig. 2 and Table I) are concentrated and dialysed by ultrafiltration and, in portions of 100 mg, corresponding to 150 nmol of haeme a, bound to the Mono Q column. Under the conditions used, all the protein material binds to the matrix. In the first Mono Q run (Fig. 3a), the gradient used is 0–5 min 0% B, 5–15 min 0–20% B, 15–65 min 20–60% B, 65–75 min 60–100% B, 75 min 100% B, 75–80 min 100–0% B and 80–90 min 0% B, at a flow-rate of 2 ml/min; in the subsequent runs (Fig. 3b and c) the same gradient is used.

In three consecutive Mono Q runs, which can be performed in the course of one day, pure cytochrome *c* oxidase (h/p ratio *ca.* 22 nmol/mg) is obtained. The molecular

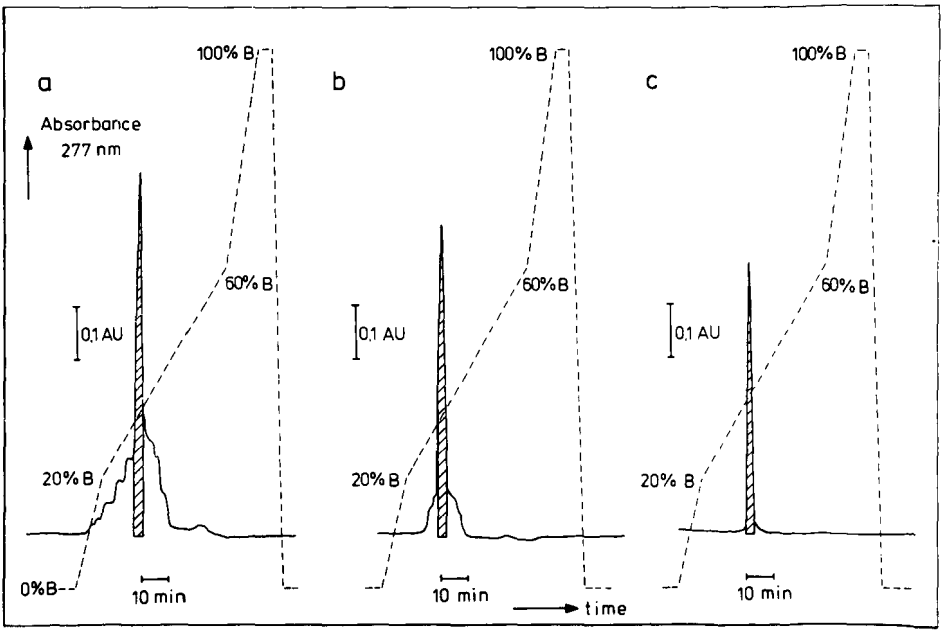

Fig. 3. Series of consecutive chromatographic steps on Mono Q. In experiment (a) the crude product from the Q-Sepharose with an h/p ratio of 1.5 nmol/mg is further purified; (b) and (c) show the chromatograms (absorbance monitored at 277 nm) of the subsequent experiments on Mono Q. The material corresponding to the shaded area was used for further purification and/or final characterization.

mass of the two-subunits complex is, on the basis of the sequence, calculated to be 90 kilodalton [12,13]; the theoretical h/p ratio is then 22.2 nmol/mg. The step-by-step yields of the Mono Q runs are considerably higher than those in the two preceding purification steps, ranging from 82 to 90%. Thus, according to this method, cytochrome *c* oxidase from *Paracoccus denitrificans* can be obtained in relatively high yields and with a substantial saving of time.

A possible explanation for the low purification rate and the low yield in the initial step on Ultrogel AcA-34 may be that cytochrome *c* oxidase, at least in part, shares the same micelles with other contaminating membrane proteins. Thus, in the early phase of the preparation, cytochrome *c* oxidase is eluted together with contaminating membrane proteins, giving rise to the observed low purification rate and low yield. Apparently, the strong binding of the proteins to the anion-exchange groups of both Q-Sepharose and Mono Q causes a more differentiated partition of the various membrane proteins over the micelles, leading to increased resolution between cytochrome *c* oxidase and contaminating proteins.

Small-scale purification

For the small-scale purification, *ca.* 20 ml of Triton X-100 membrane extract with *ca.* 200 nmol of haeme a and an h/p ratio of 0.3 nmol/mg is applied to a smaller Ultrogel AcA-34 column and chromatographed overnight. Fractions are monitored and checked for their individual h/p ratios. They are then pooled as described for the

TABLE II

RAPID, SMALL-SCALE PURIFICATION OF CYTOCHROME *c* OXIDASE FROM *PARACOCCUS DENITRIFICANS*

Step	Haeme a (nmol)	Protein (mg)	Haeme a/protein (nmol/mg)	Yield[a] (%)	Overall yield (%)
Triton X-100 membrane extract	200	670	0.3	100	100
AcA-34	118	197	0.6	59	59
Mono Q (run a)[b]	90	69	1.3	76	45
Mono Q (run b)	72	16	4.5	80	36
Mono Q (run c)[c]	51	3.9	13.1	71	25
Mono Q (run d)	35	1.6	21.9	69	17

[a] Yield as a percentage of haeme a in Triton X-100 extract.
[b] In two runs.
[c] Not shown in Fig. 4.

large-scale protocol. In order to speed up the purification procudure, the Q-sepharose chromatograhic steps are omitted in the small-scale protocol. Only fractions with substantially improved h/p ratio are used for subsequent purification steps on Mono Q. Thus, *ca*, 120 nmol of haeme a with an h/p ratio of *ca*. 0.7 nmol/mg is, after dialysis, applied to the Mono Q column (Table II). This crude cytochrome *c* oxidase solution still contains cytochrome *b*, most of which does not bind to the Mono Q column under these conditions and thus appears in the effluent together with the excess of Triton

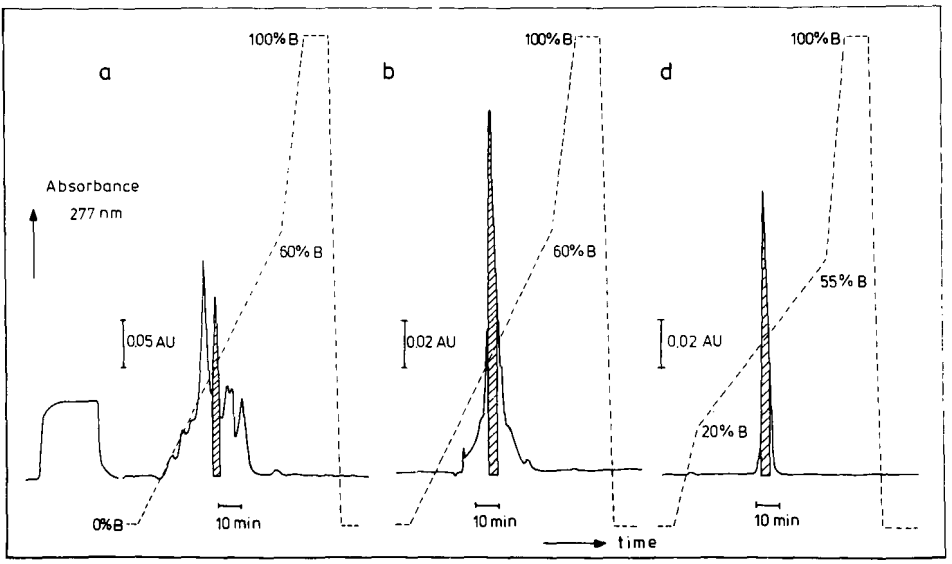

Fig. 4. Series of consecutive chromatographic steps on Mono Q. In (a) the crude product of the small-scale AcA-34 chromatographic step (not shown) with an h/p ratio of 0.6 nmol/mg is further purified; (b) and (d) show the chromatograms (absorbance monitored at 277 nm) of the subsequent experiments on Mono Q. The chromatogram of experiment (c) (see Table II) is omitted. The material corresponding to the shaded area was used for further purification and/or final characterization.

X-100 accumulated during the ultrafiltration process. The cytochrome *b* and the excess of Triton X-100 cause a shift of the baseline on loading of the Mono Q column (Fig. 4a). In the first experiment the membrane proteins are eluted with the following gradient: 0–5 min 0% B, 5–65 min 0–60% B, 65–70 min 60–100% B, 70–80 min 100% B, 80–85 min 100–0% B and 85–95 min 0% B, at a flow-rate of 2 ml/min. The second peak was identified by spectral investigation as cytochrome *c* oxidase. At least three other membrane-bound cytochrome *c* components are observed in this Mono Q run. They were, however, not investigated further. The yield amounts to 76% and a substantial purification result is obtained (Tabel II). The cytochrome *c* oxidase fraction (shaded area in Fig. 4a) is applied to the Mono Q column and rechromatographed under the same conditions (Fig. 4b). Again, a good yield is observed and the h/p ratio has increased to 4.5 nmol/mg. The next two chromatographic steps in which less steep gradients (0–5 min 0%B, 5–15 min 0–20% B, 16–65 min 20–55% B, 65–70 min 55–100% B, 70–80 min 100% B, 80–85 min 100–0% B and 85–150 min 0% B) are used, lead to pure cytochrome *c* oxidase with an h/p ratio of 22 nmol haeme a/mg.

Some characteristics of the purified cytochrome c oxidase

The purity of the membranous enzyme was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) [10]. In agreement with the results of Ludwig and Schatz [4], two bands are observed, corresponding to proteins with apparent molecular weights of $45 \cdot 10^3$ and $28 \cdot 10^3$ dalton. However, recently, Haltia *et al.* [14] have shown that cytochrome *c* oxidase from *Paracoccus denitrificans* can be isolated as a three-subunit complex by using detergents other than Triton X-100. This complex is far less stable than the two-subunit complex and therefore not suitable for the investigation of structure–function relationships. The genes for these three proteins have been cloned and sequenced [12,13].

Using ICP-AES, a technique which allows the simultaneous determination of a large number of elements, we could show that cytochrome *c* oxidase from *Paracoccus denitrificans*, as its mitochrondrial counterpart from bovine heart, is characterized by the stoichiometric presence of three copper ions per two haeme irons [15]. The presence of three copper ions per two haemes thus appears to be a general property of all aa_3-type cytochrome *c* oxidases, Whereas the Zn and Mg stoichiometries in bovine heart cytochrome *c* oxidase preparations are close to unity and vary only slightly from preparation to preparation, the same elements very considerably in the *Paracoccus* enzyme. In the absence of phosphate (use of Tris–HCl buffers), the stoichiometry of phosphorus in these preparations can be determined by ICP-AES and directly correlated with the number of bound phospholipids: a varying number from one to five phospholipid molecules remain bound in these preparations [15]. Thus, our preparations, although homogeneous in SDS-PAGE and pure in terms of its h/p ratio, clearly show inhomogeneities with respect to the bound metals and bound phospholipids. Future research will focus on the elimination of these inhomogeneities.

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Separation of the cellulolytic and xylanolytic enzymes of *Clostridium stercorarium*

K. BRONNENMEIER, C. EBENBICHLER and W. L. STAUDENBAUER*
Institute for Microbiology, Technical University Munich, Arcisstrasse 21, D-8000 München 2 (F.R.G.)

ABSTRACT

Exoenzymes of the thermophilic, anaerobic bacterium *Clostridium stercorarium* involved in cellulose and arabinoxylan degradation were resolved by a combination of fast protein liquid chromatographic (FPLC) chromatofocusing and FPLC anion-exchange chromatography. The purified enzymes include an *endo*-β-1,4-glucanase (Avicelase I), an *exo*-β-1,4-glucanase (Avicelase II), a β-D-glucosidase, a β-D-xylosidase, an α-L-arabinofuranosidase and multiple xylanases. Two celloxylanases capable of hydrolyzing both cellodextrins and xylans were also purified.

INTRODUCTION

Cellulose and xylans are the major polysaccharide components of plant biomass and constitute the most abundant renewable resources for the production of fermentable sugars by enzymatic saccharification. Cellulose is a linear polymer of D-glucose residues linked by β-1,4-glucosidic bonds. Its enzymatic hydrolysis involves at least three types of enzymes: *endo*-β-1,4-glucanases (1,4,-β-D-glucanohydrolase; E.C. 3.2.1.4), *exo*-β-1,4-glucanases (1,4,-β-D-glucan cellobiohydrolase; E.C. 3.2.1.91) and β-D-glucosidase (β-D-glucoside glucohydrolase; E.C. 3.2.1.21) [1]. A synergistic interaction of these enzymes is necessary for the complete hydrolysis of crystalline cellulose.

Xylans are branched heteroglycans with a backbone of β-1,4-linked D-xylopyranosyl residues. Branches consist commonly of α-1,3-linked L-arabinofuranose and α-1,2-linked D-glucuronic acid residues [2]. The frequency and composition of branches in xylans varies depending on the source. Thus, xylans from grasses and softwoods are generally arabinoxylans. Enzymatic hydrolysis of the xylan backbone involves *endo*-β-1,4-xylanases (1,4-β-D-xylan xylanohydrolase; E.C. 3.2.1.8) and β-D-xylosidases (1,4-β-D-xylan xylohydrolase; E.C. 3.2.1.37) [3]. With arabinoxylans, removal of L-arabinosyl side-groups is catalysed by α-L-arabinofuranosidases (E.C. 3.2.1.55).

Thermophilic bacteria have received considerable attention as sources of highly active and thermostable cellulolytic and xylanolytic enzymes. The cellulase complex of *Clostridium thermocellum* has been studied most extensively. This complex contains 14–18 different polypeptides forming a stable extracellular structure termed cellulo-

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some [4]. The thermophilic anaerobe *Clostridium stercorarium* differs from *C. thermocellum* by being able to ferment a wide variety of carbohydrates in addition to cellulose [5]. Growth on either cellulose or xylan was found to induce the production of both cellulotytic and xylanolytic enzymes [6]. The cellulase of *C. stercorarium* is of low complexity and consists of single *endo*-β-1,4-glucanase, *exo*-β-1,4-glucanase and β-D-glucosidase activities [7–11]. On the other hand three distinct endoxylanases [6] and two β-D-cellobiosidases of unknown function [9] have been purified from *C. stercorarium* culture supernatants.

In this paper we report the complete resolution of the various *C. stercorarium* exoenzymes involved in cellulose and arabinoxylan hydrolysis employing a combination of fast protein liquid chromatographic (FPLC) chromatofocusing and FPLC anion-exchange chromatography. It is shown that the purified β-D-cellosidases possess xylanase activity and might be considered as celloxylanases. In addition to the previously identified *C. stercorarium* enzymes, the isolation of a β-D-xylosidase and an α-L-arabinofuranosidase is described.

EXPERIMENTAL

Enzyme preparation

C. stercorarium NCIB 11745 was grown under anaerobic conditions at 60°C in prereduced GS-2 medium [12] with 1% xylan as carbon source. Culture supernatant (8.3 l) was concentrated by tangential flow ultrafiltration in a Minitan system (Millipore) using polysulphone filter sheets with a nominal molecular mass limit 10 000. The retentate was desalted by five cycles of ultrafiltration following a two-fold dilution with 20 m*M* Tris–HCl (pH 8.0). Protein concentration was determined by the method of Sedmark and Grossberg [13].

Chromatography and chromatofocusing

The chromatographic system consisted of a Pharmacia FPLC apparatus. Anion-exchange chromatography on Q Sepharose was carried out in a Pharmacia HR 16/10 column (10 × 1.6 cm I.D.) packed with Q Sepharose FF. Pooled fractions were concentrated and desalted by tangential flow ultrafiltration. FPLC chromatofocusing was performed on a Pharmacia Mono P HR 5/20 column (20 × 0.5 cm I.D.) with a pH gradient from 6.0 to 3.0 formed with Polybuffer 74. Pooled fractions were concentrated by Centriprep-30 ultrafiltration (Amicon). Change of buffer was effected by three cycles of dilution and ultrafiltration. Anion-exchange FPLC was carried out on a Pharmacia Mono Q HR 5/5 column (5 × 0.5 cm I.D.) or on an HR 10/10 column (10 × 1.0 cm I.D.). Gel filtration FPLC was performed on a Pharmacia Superose 12 HR 10/30 column as described previously [10].

Enzyme assays

Avicelase and carboxymethylcellulase (CMCase) activities were determined as described previously [9]. One unit of activity corresponds to the formation of 1 μmol of glucose equivalent per minute. Xylanase was assayed by incubation for 60 min at 60°C in a 1% (w/v) solution of oat spelts xylan in 0.1 *M* succinate buffer (pH 6.0). Reducing sugars released from the substrate were determined with 3,5-dinitrosalicylic acid reagent [14]. One enzyme unit corresponds to the release of 1 μmol of xylose equivalent per minute.

β-D-Glucosidase, β-D-cellobiosidase, β-D-xylosidase and α-L-arabinofuranosidase activities were determined by measuring the release of *p*-nitrophenol from the corresponding *p*-nitrophenylglycoside. Assay mixtures (1 ml) containing 2 m*M* substrate in 40 m*M* citrate–phosphate buffer (pH 6.0) were incubated for 30 min at 60°C. Reactions were stopped by addition of 2 volumes of 1 *M* sodium carbonate solution. The absorbance of the liberated *p*-nitrophenol was measured at 395 nm. One unit of activity is defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per minute.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate–Polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% polyacrylamide gels in the presence of 0.1% SDS according to Laemmli [15] using a Pharmacia Midget electrophoresis unit. Protein bands were detected by staining with Coomassie Brilliant Blue R-250. Xylanase activity was detected by *in situ* staining of polyacrylamide gels containing 0.1% xylan as described previously [16]. Glycosidases were identified by fluorescence on incubating the gels with the corresponding 4-methylumbelliferyl glycoside [16].

Chemicals

Avicel TG 104 and CMC (extent of carboxymethylation 0.7%) and chemicals for SDS-PAGE were obtained from Serva (Heidelberg, F.R.G.) and xylan (oat spelts), *p*-nitrophenylglycosides, 4-methylumbelliferyl glycosides and molecular mass markers for SDS-PAGE from Sigma (St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Fractionation by Q Sepharose chromatography

A prerequisite for the characterization of the *C. stercorarium* exoenzyme was the concentration of the culture supernatant with minimum loss of enzyme activities. This was achieved by ultrafiltration employing a Minitan tangential flow system equipped with polysulphone filters. The concentrated enzyme preparation was then fractionated on a Q Sepharose column connected to a Pharmacia FPLC system. The protein fractions were assayed for various enzymes involved in cellulose and hemicellulose degradation.

Fig. 1 shows the elution profile of the enzymes related to cellulose degradation. With microcrystalline cellulose (Avicel) as substrate, two peaks of activity were observed. The first peak eluting at 0.27 *M* NaCl also exhibited CMCase activity and corresponds to Avicelase I [9,11], which has been previously characterized as an *endo*-β-1,4-glucanase [7]. The smaller Avicelase peak eluting at 0.33 *M* NaCl has been designated Avicelase II [9] and is presumably identical with the *exo*-β-1,4-glucanase activity detected by Creuzet *et al.* [8].

With the substrate *p*-nitrophenyl-β-D-cellobioside three peaks of activity were detected (Fig. 1B). A distinct peak eluting at 0.16 *M* NaCl was also able to hydrolyse *p*-nitrophenyl-β-D-glucoside and has previously been characterized as β-D-glucosidase [10]. At higher salt concentration two broad and overlapping peaks of β-cellobiosidase activity were observed, which were devoid of β-glucosidase activity. The corresponding enzymes have been designated β-cellobiosidase I and β-cellobiosidase II, respectively [9].

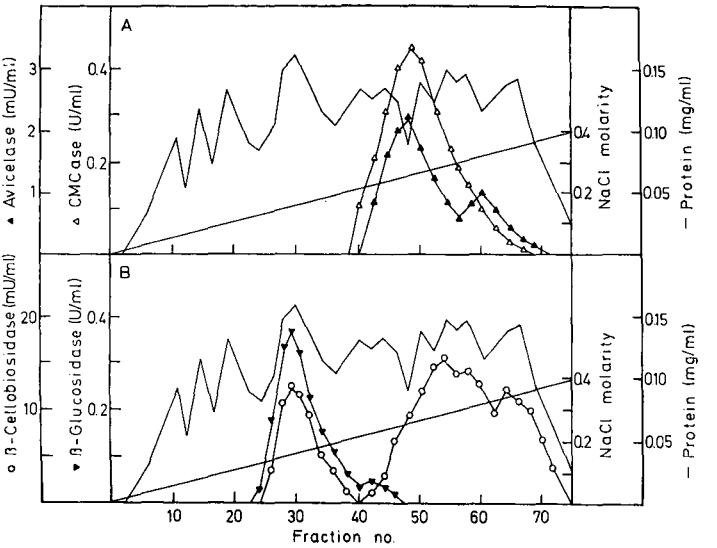

Fig. 1. Fractionation of *C. stercorarium* cellulolytic enzymes by Q Sepharose chromatography. Concentrated culture supernatant (90 mg of protein) was applied to a Q Sepharose Fast Flow HR 16/10 column equilibrated with 20 m*M* Tris–HCl (pH 8.0). Elution was performed with a 1000-ml linear gradient (0.0–0.6 *M* NaCl) in equilibration buffer at a flow-rate of 4 ml/min. Fractions (10 ml) were collected and assayed for (A) Avicelase and CMCase and (B) β-D-glucosidase and β-D-cellobiosidase.

The separation of enzymes involved in the hydrolysis of arabinoxylans is shown in Fig. 2. A major peak of xylanase activity was found to elute around 0.1 *M* NaCl (Fig. 2A). This peak corresponds to the xylanase activity described by Creuzet and Frixon

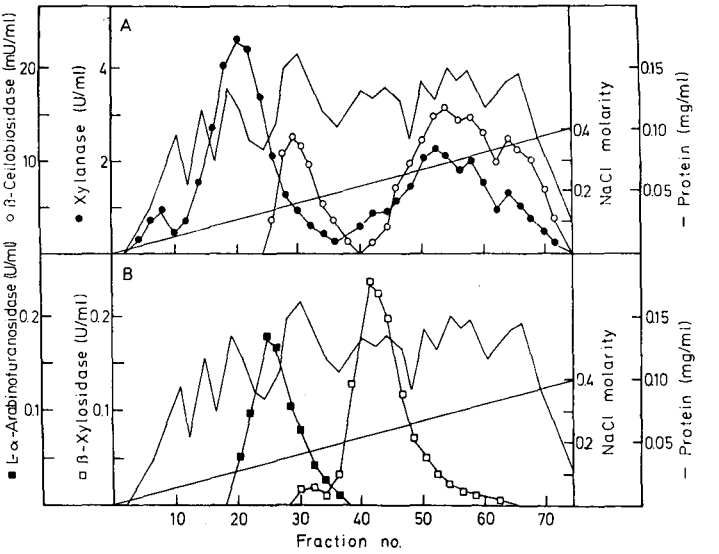

Fig. 2. Fractionation of *C. stercorarium* arabinoxylanolytic enzymes by Q Sepharose chromatography. Fractions from the Q Sepharose column described in Fig. 1 were assayed for (A) xylanase and β-D-cellobiosidase, and (B) β-D-xylosidase and α-L-arabinofuranosidase.

[7]. In contrast to the previous work, two additional xylanase peaks were detected, which coeluted with β-cellobiosidase I and II.

With *p*-nitrophenyl-α-L- arabinofuranoside as substrate one peak of activity was observed (Fig. 2B). The elution profile of the α-L-arabinofuranosidase overlapped with that of xylanase and β-D-glucosidase. Similarly, a single peak of β-D-xylosidase activity hydrolysing β-nitrophenyl-β-D-xyloside was detected. The β-D-xylosidase peak partially overlapped with that of Avicelase I (Fig. 1A).

Separation by FPLC chromatofocusing

A further separation of the individual enzymes was achieved by chromatofocusing. For that purpose, the fractions from the Q Sepharose column were combined in two pools. Pool I (fraction 5–36) contained the major xylanase activity in addition to the β-D-glucosidase and α-L-arabinofuranosidase activities. Pool II (fraction 38–70) included the β-D-xylosidase, Avicelase and β-D-cellobiosidase activities. Chromatofocusing was performed on a Pharmacia Mono P column with a pH gradient from 6.0 to 3.0.

The α-L-arabinofuranosidase eluted at pH 5.2 and could be completely separated from β-D-glucosidase and xylanase activities eluting at pH 4.8 and 4.2, respectively (Fig. 3). During gel filtration on Superose 12 this enzyme migrated as a single peak with an apparent molecular mass of 180 000 (data not shown). Analyis of the purified enzyme by SDS-PAGE revealed two proteins bands with molecular masses of 45 000 and 55 000, indicating an oligomeric structure of the enzyme.

The β-D-glucosidase was freed from residual xylanase activity and purified to homogeneity by a combination of Mono Q FPLC and gel filtration [10]. It is a monomeric protein with a molecular mass of 85 000 as determined by SDS-PAGE. Similarly, the major peak of xylanase activity was further resolved by Mono Q chromatography as described below.

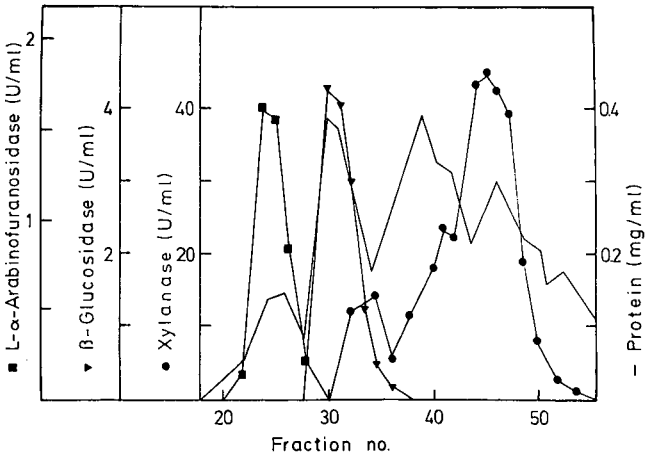

Fig. 3. Separation of α-L-arabinofuranosidase, β-D-glucosidase and xylanase by chromatofocusing. Fractions 5–36 from the Q Sepharose column (12 mg of protein) were applied to a Mono P HR 5/20 column equilibrated with 20 m*M* histidine–HCl (pH 6.0). Elution was performed with 10 ml of starting buffer followed by 45 ml of 1:10 diluted Polybuffer 74 (pH 3.0) at a flow-rate of 1 ml/min. Fractions (0.5 ml) were assayed for protein and enzyme activities.

Chromatofocusing clearly resolved the β-D-xylosidase (elution pH 5.0) from other cellulolytic and xylanolytic enzyme activites present in pool II (Fig. 4). SDS-PAGE revealed a single protein band with a molecular mass of 85000. The identity of this protein band with β-D-xylosidase was established by activity staining with 4-methylumbelliferyl-β-D-xyloside. The β-cellobiosidase activity (elution pH 4.7) again copurified with xylanase activity (Fig. 4A), indicating that both activities are properties of the same enzyme(s). This was shown by further purification as described below.

It should be noted that chromatofocusing did not result in a complete separation of the β-D-cellobiosidase and xylanase activities from Avicelase I, which eluted around pH 4.5 (Fig. 4B). However, as proteins elute from the Mono P column at a pH close to their p*I*, the Avicelase showed a strong tendency to precipitate during overnight storage at 4°C and could be removed by centrifugation. The combined Avicelase fractions could be resolved into Avicelase I and II by subsequent gel filtration on a Superose 12 column [11]. They represent monomeric proteins with molecular masses of 100000 (Avicelase I) and 87000 (Avicelase II).

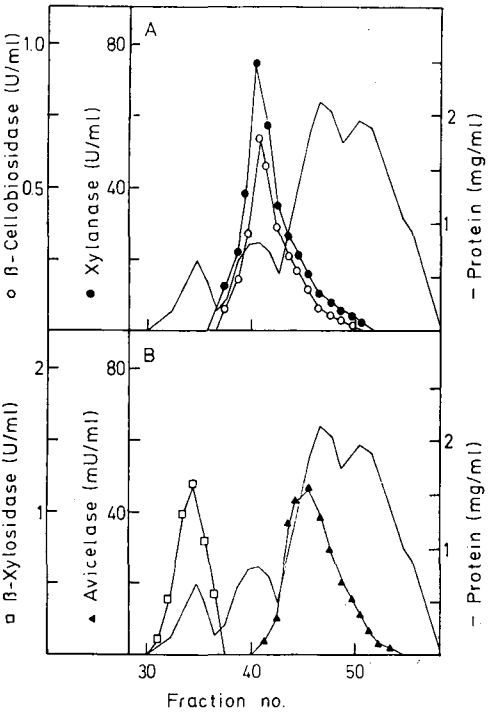

Fig. 4. Separation of β-D-xylosidase, β-D-cellobiosidase and Avicelase by chromatofocusing. Fractions 38–70 from the Q Sepharose column (19 mg of protein) were applied to a Mono P HR 5/20 column equilibrated with 20 m*M* histidine–HCl (pH 6.0). Elution was performed as described in Fig. 3. Fractions (0.5 ml) were assayed for (A) xylanase and β-D-cellobiosidase activity and (B) β-D-xylosidase and Avicelase activity.

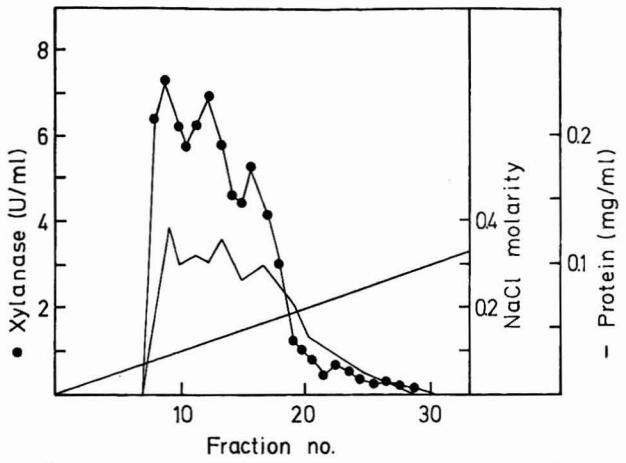

Fig. 5. Resolution of xylanases by Mono Q anion exchange chromatography. Fractions 38–50 from the Mono P column (Fig. 3) were applied to a Mono Q HR 5/5 column equilibrated with 20 m*M* Tris–HCl (pH 8.0). Proteins were eluted with a 20-ml linear gradient (0.0–0.40 *M* NaCl) in equilibration buffer at a flow-rate of 1 ml/min. Fractions (0.5 ml) were assayed for xylanase activity.

Resolution of xylanases and celloxylanases by Mono Q FPLC

The pooled xylanase fractions from the gradient shown in Fig. 3 were further fractionated by FPLC on a Mono Q column at pH 8.0 (Fig. 5). Three peaks of xylanase

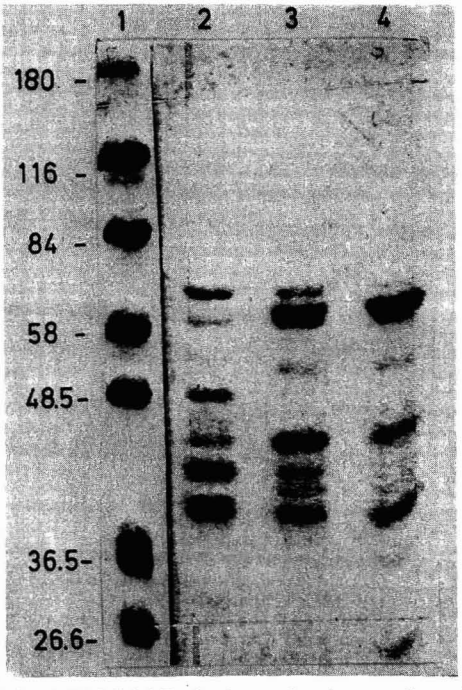

Fig. 6. SDS-PAGE of xylanase fractions, performed in a 10% polyacrylamide slab gel in the presence of 0.1% SDS [15]. Lanes: 1 = molecular mass markers; 2 = fraction 8; 3 = fraction 12; 4 = fraction 16. The numbers on the left are the molecular masses ($\times 10^{-3}$) of marker proteins.

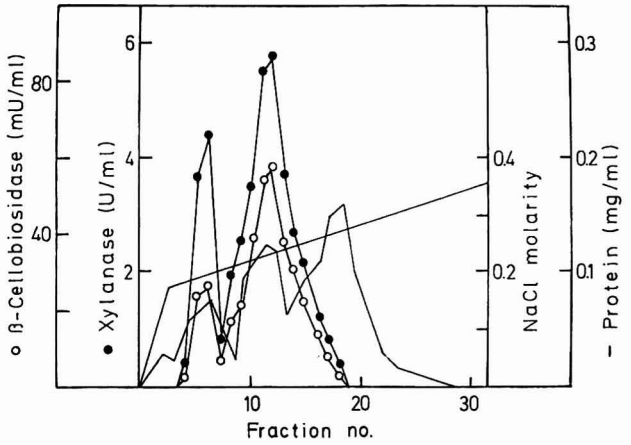

Fig. 7. Resolution of celloxylanases by Mono Q anion exchange chromatography. Fractions 38–43 from the Mono P column (Fig. 4) were applied to a Mono Q HR 10/10 column equilibrated with 20 m*M* Tris–HCl (pH 6.0). Elution was effected with an 8-ml linear gradient (0.0–0.17 *M* NaCl) followed by a 168-ml linear gradient (0.17–0.40 *M* NaCl) in equilibration buffer at a flow-rate of 4 ml/min. Fractions (4 ml) were assayed for enzyme activities.

activity could be partially resolved. Analysis of the peaks fractions by SDS-PAGE (Fig. 6) revealed the presence of several protein bands in each fraction. *In situ* activity staining for xylanase activity showed that all protein bands were enzymatically active. Prominent activity bands were detected at positions corresponding to molecular masses of 70 000, 62 000 and 42 000. The molecular masses of these isoenzymes are nearly identical with those reported previously for xylanase A, B and C of *C.*

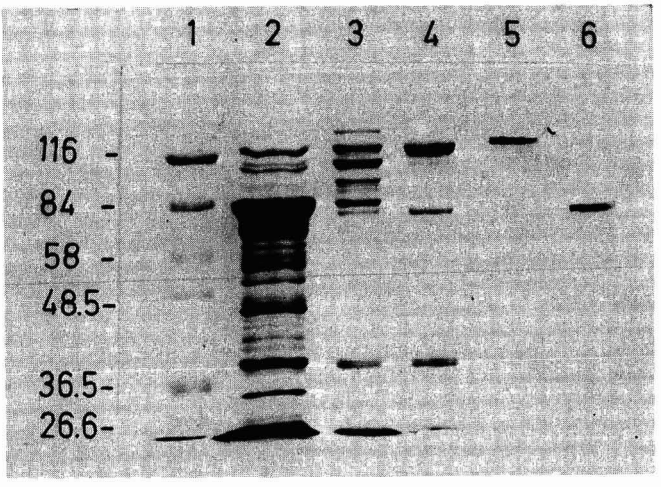

Fig. 8. SDS-PAGE of celloxylanase fractions, carried out as described in Fig. 6. Lane 1, molecular mass markers; lane 2, concentrated culture supernatant; lane 3, pooled fractions from the Q Sepharose column; lane 4, pooled and centrifuged fractions from the Mono P column; lane 5, pooled fractions 4–5 from the Mono Q column (celloxylanase II); lane 6, pooled fractions 10–13 from the Mono Q column (celloxylanase I). The numbers on the left are the molecular masses ($\times 10^{-3}$) of marker proteins.

stercorarium by Berenger *et al.* [6]. The immunological cross-reactivity of these enzymes [6] suggests that the different isoenzymes arose by partial proteolysis of a common enzyme precursor. This notion was confirmed by analysis of the gene products produced by expression of the cloned *C. stercorarium* xylanase gene *xynA* in *E. coli* [17].

The fractions from the gradient shown in Fig. 4 expressing both β-D-cellobiosidase and xylanase activity were subjected to Mono Q chromatography at pH 6.0 (Fig. 7). It can be seen that the xylanase activity copurified again with the β-cellobiosidase activity. SDS-PAGE (Fig 8) showed single protein bands with molecular masses of *ca.* 80 000 (cellobiosidase I) and 120 000 (cellobiosidase II). Activity staining for xylanase and β-cellobiosidase activity revealed that both activities reside in the same protein bands (data not shown). Both enzymes were able to hydrolyse cellodextrins and β-glucans in addition to xylan and aryl-β-cellobiosides. Enzymes displaying activity with either cellodextrins, β-glucans or xylans as substrates have been isolated from rumen bacteria [18] and *C. acetobutylicum* [19]. This novel type of enzyme has been termed celloxylanase [18]. The *C. stercorarium* β-D-cellobiosidases are therefore more appropriately designated as celloxylanase I and II, respectively.

The properties of the *C. stercorarium celX* gene product cloned in *E. coli* [20] closely resemble those of celloxylanase II with respect to substrate specificity, temperature optimum and pH profile (unpublished results). Moreover, the DNA sequence of the *celX* gene contains an open reading frame coding for a protein of $11.54 \cdot 10^5$ dalton, which is in good agreement with the molecular mass of celloxylanase II determined by SDS-PAGE (Fig. 8). These data strongly suggest that celloxylanase II is the product of the *celX* gene. It remains to be determined whether celloxylanase I arises by proteolytic processing of celloxylanase II or whether it is expressed from a different gene.

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Isolation and characterization of proteoglycans from different tissues

G. STÖCKER* and D.-Ch. FISCHER
Department of Clinical Chemistry and Pathobiochemistry, Medical Faculty, University of Technology, Pauwelsstrasse, D-5100 Aachen (F.R.G.)

ABSTRACT

Two chromatographic procedures for the isolation and purification of proteoglycans (PG) and their related glycosaminoglycan (GAG) peptides are described. PG from human aorta were isolated from tissue extract by sequential ion-exchange, size-exclusion and hydroxyapatite chromatography. Final purification of samples was achieved by chromatography on Mono Q. Homogeneity of samples was demonstrated by Western blot analysis of biotin-labelled compounds prior to and after enzymatic digestion and dual-wavelength detection in size-exclusion chromatography. The purity of samples obtained by the procedure described was sufficient for protein sequence analysis. GAG preparations of bovine trachea cartilage were purified by the sequential use of strong anion-exchange supports. Molecular weight distribution and sensitivity to treatment with glycan-specific enzymes was shown by size-exclusion chromatography.

INTRODUCTION

Proteoglycans (PG) play an important role in fundamental biological processes such as cell–cell interaction, proliferation and differentiation and in stabilizing the structure of tissue matrices [1–3]. They represent a family of macromolecules consisting of a core protein with at least one glycosaminoglycan (GAG) chain covalently attached. Physico-chemical properties, *e.g.*, polydispersity and charge density, are mainly given by the length of and charge distribution within the GAG chain. The negative charges are due to sulphate ester moieties and/or carboxyl functions in the disaccharide units of the GAG chains. These features may be used for the separation of different PG or GAG peptides from complex mixtures [4]. Here we report on the use of several chromatographic procedures for the isolation and purification of different PG or their related GAG peptides from tissue extracts.

EXPERIMENTAL

Sample materials

The preparation of human aortae and extraction of PG were performed as described previously [5]. Bovine tracheal cartilage was a generous gift from Professor H. W. Stuhlsatz (Aachen, F.R.G.).

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Chemicals

Sepharose Q, Sepharose CL-4B, Mono Q HR 10/10, Mono Q HR 5/5, TSK G 3000 SW, TSK G 4000 SW, TSK G SWP, preformed gels for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (8–25% gradient) and low-molecular-weight standards were obtained from Pharmacia–LKB (Freiburg, F.R.G.). Hydroxyapatite (Bio-Gel HTP) and Zeta-Probe blotting membranes were obtained from Bio-Rad Labs. (Munich, F.R.G.). High-molecular-weight standards and 3-[3-(cholamidopropyl)dimethylamino]-1-propanesulphonate (CHAPS) were purchased from Sigma (Deisenhofen, F.R.G.). Chondroitin sulphate lyase AC (E.C. 4.2.2.5), chondroitin sulphate lyase ABC (E.C. 4.2.2.4), heparinase [heparin lyase (E.C. 4.2.2.7)], heparitinase [heparan sulphate lyase (E.C. 4.2.2.8)] and keratanase (E.C. 3.2.1.103) were purchased from Seikagaku (Tokyo, Japan). Sulphosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin) was obtained from Pierce (Munich, F.R.G.). All remaining reagents (Merck, Darmstadt, F.R.G.) were of analytical-reagent grade.

Apparatus

Chromatography was performed either on an LC 31 A chromatography system from Bruker (Bremen, F.R.G.) or on a fast protein liquid chromatography (FPLC) system from Pharmacia (Freiburg, F.R.G.). For multi-wavelength detection, a diode-array UV–VIS spectrophotometer (Waters Assoc., Eschborn, F.R.G.) was used. The radioactivity was measured with an LKB-Wallach scintillation counter (Type 1217 Rack Beta) using Lumagel (Lumac, Basle, Switserland) as scintillator. Ultrafiltration was performed by using YM-2 or YM-5 membranes (Amicon, Witten, F.R.G.). Amino acid and amino sugar analysis were performed after hydrolysis of sample with 3 *M* hydrochloric acid at 105°C for 15 h using an LKB Alphaplus amino acid analyser.

Isolation and purification of GAG

Papain digestion was performed as described [6]. Briefly, enzyme treatment of cartilage preparation was carried out in 10 m*M* sodium phosphate buffer containing 0.15 *M* sodium chloride and 5 m*M* cysteinium chloride (pH 6.2).

A Sepharose Q column (15 cm × 2.6 cm I.D.) was prepared according to the manufacturer's instructions. Column equilibration was performed with 10 m*M* sodium phosphate buffer containing 0.15 *M* sodium chloride (pH 6.2). The papain-treated sample was applied and absorption of the eluted compounds was monitored at 214 nm. Bound material was eluted with three linear gradients (flow-rate 1 ml/min) performed from 150 mM to 3 M NaCl in starting buffer (80 ml of 0.15–0.5 *M* NaCl, 80 ml of 0.5 *M* NaCl, 120 ml of 0.5 *M* NaCl to 3 *M* NaCl). Buffer exchange of GAG-containing fractions was performed by ultrafiltration (YM-2 membrane). For further purification, sample was applied to a Mono Q HR5/5 column, equilibrated with 40 m*M* sodium phosphate (pH 6.5). Bound compounds were eluted with three linear gradients (*i.e.*, 16.5 ml of 0 to 0.45 *M* NaCl, 20 ml of 0.45 to 1.2 *M* NaCl and 20 ml of 1.2 to 3 *M* NaCl; flow-rate 0.5 ml/min). The eluted compounds were detected by measuring their absorbance at 214 nm. Homogeneity of the samples was checked by size-exclusion chromatography on a TSK G 3000 SW column (600 mm × 7.5 mm I.D.), equipped with a TSK G SWP precolumn (75 mm × 7.5 mm I.D.). The column was equilibrated with 0.3 *M* NaCl in 10 m*M* NaH_2PO_4–Na_2HPO_4 and eluted compounds were detected by measuring their absorbance at 206 nm.

Isolation and purification of proteoglycans

The 4 *M* guanidinium chloride-containing extract of human aorta intima/media preparations was dialysed against equilibration buffer as described below. The sample was applied to a Sepharose Q anion-exchange column (20 cm × 4.6 cm I.D.) equilibrated with 7 *M* urea in 0.05 *M* acetate buffer (pH 5.2) containing 0.05 *M* NaCl–0.05 *M* LiCl–0.002 *M* benzamidine hydrochloride–0.001 *M* phenylmethylsulphonyl fluoride–0.001 *M* N-ethylmaleimide–0.2% (w/v) CHAPS. The column was washed with equilibration buffer using 1.5 times the column volume. The bound material was eluted by a gradient to 4 *M* NaCl (equilibration buffer made up to 4 *M* NaCl). Gradient elution was performed at a flow-rate of 4 ml/min with two linear NaCl gradients (0.05 to 1.1 *M*, 450 ml, and 1.1 to 4.0 *M*, 450 ml). The elution of ^{35}S-labelled (sulphated) sugar compounds was monitored by scintillation counting.

Pooled fractions from the Sepharose Q chromatography were concentrated on YM-5 membranes and submitted to size-exclusion chromatography on Sepharose CL-4B. For this purpose a Sepharose CL-4B column (125 cm × 3.5 cm I.D.) was equilibrated with 7 *M* urea in 0.05 *M* acetate buffer (pH 5.8) containing 0.3 *M* NaCl–0.002 *M* benzamidine hydrochloride–0.001 *M* phenylmethylsulphonyl fluoride–0.01 *M* EDTA–0.2% (w/v) CHAPS. Chromatography was performed at a flow-rate of 0.9 ml/min. ^{35}S-containing fractions with the exception of those of the void volume were submitted to hydroxyapatite chromatography.

Hydroxyapatite chromatography was performed on a column (12 cm × 4.6 cm I.D.) equilibrated with 0.01 *M* sodium phosphate buffer (pH 6.8). After sample application the column was washed with equilibration buffer (1.2 times the column volume). Elution was performed at a flow-rate of 4 ml/min. Gradient elution was done by increasing the phosphate concentration to 0.7 *M* (pH 7.2). Two linear gradients (0.01 to 0.48 *M* phosphate, 320 ml, and 0.48 to 0.7 *M* phosphate, 100 ml) were applied. Elution of ^{35}S-labelled compounds was monitored by scintillation counting.

Final purification of PG preparations was achieved by Mono Q anion-exchange chromatography. A Mono Q column (Type HR 10/10) was equilibrated with 0.02 *M* sodium phosphate buffer (pH 4.5) containing 0.1 *M* NaCl–0.1 *M* LiCl–0.05% (w/v) CHAPS. Samples obtained after hydroxyapatite chromatography were diluted with water to a final phosphate concentration below 0.05 *M* and applied to the column. The column was washed with 45 ml of equilibration buffer. Gradient elution was performed at a flow-rate of 3 ml/min using three linear NaCl gradients (0.1 to 1.2 *M*, 240 ml, 1.2 to 2.0 *M*, 80 ml, and 2.0 to 4.0 *M*, 40 ml). Eluted compounds were detected by measuring the absorbance at 214 nm.

Homogeneity of purified PG samples was controlled by size-exclusion chromatography on TSK G 4000 SW. A column (300 mm × 7.5 mm I.D.) equipped with a TSK G SWP precolumn (75 mm × 7.5 mm I.D.) was equilibrated with 0.01 *M* sodium phosphate buffer containing 0.3 *M* NaCl (pH 6.0). Samples were applied to the column and eluted at a flow-rate of 0.6 ml/min. Absorbance was monitored at 210 and 280 nm.

Characterization of PG and GAG

Characterization of GAG samples was performed by glycan-specific digestion with either chondroitin sulphate lyase AC/ABC or keratanase as described [7,8]. GAG degradation was detected by the M_r shift using size-exclusion chromatography (TSK G 3000 SW) before and after enzymatic treatment of the sample.

PG preparations were analysed by treating biotin-labelled samples with either chondroitinase AC/ABC and/or heparinase–heparitinase [5]. After SDS–PAGE and blotting to nylon membrane the untreated and enzyme-treated samples were detected by using the avidin–peroxidase conjugate–diaminobenzidine system.

RESULTS AND DISCUSSION

Isolation and purification of glycosaminoglycan peptides from bovine trachea cartilage

For analysis of GAG chains cartilage was digested with papain; isolation of GAG peptides was performed by ion-exchange chromatography. Sepharose Q chromatography resulted in two partially separated peaks eluting between 2.1 and 3 *M* NaCl. The relative amount of total amino sugars compared with the total amount of amino acid increased with increasing ion strength in the gradient (data not shown). The first peak contained mainly peptides and only a minor part of GAG. Components eluting between 2.6 and 2.8 *M* NaCl contained GalN, indicating for chondroitin sulphate and/or dermatan sulphate, in addition to GlcN, indicative of keratan sulphate and/or oligosaccharides. Sample eluting above 2.8 *M* NaCl contained mainly GalN. In order to investigate KS-containing GAG, GlcN-containing fractions were pooled (see bar, Fig. 1). For identification of GAG chains, digestion by glycan-specific enzymes is a common procedure [9]. The presence of a special GAG can be demonstrated as a decrease in molecular weight after treatment with the respective enzyme. Size-exclusion chromatography on TSK G 3000 SW (Fig. 2) of sample 1 obtained by Sepharose Q chromatography showed the presence of at least two components of different molecular weight distribution.

In cartilage most GAG are chondroitin sulphate and keratan sulphate [3,10].

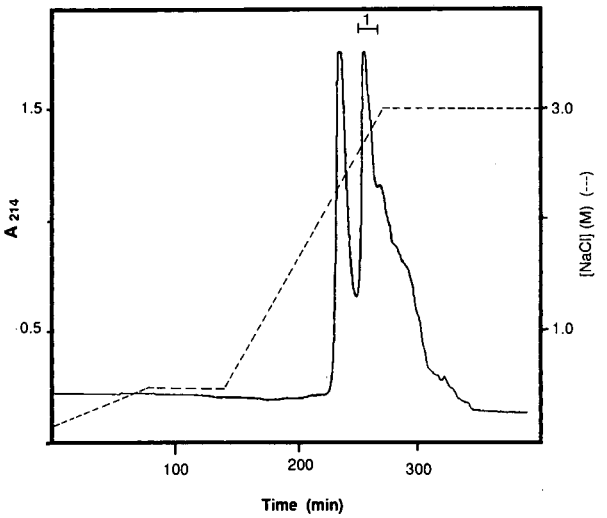

Fig. 1. Ion-exchange chromatography on Sepharose Q of GAG peptide preparation from bovine trachea cartilage. 25 ml of sample, equivalent to 2.5 g of papain-digested cartilage, were applied to a column (15 cm × 2.6 cm I.D.), equilibrated with 10 m*M* sodium phosphate buffer containing 0.15 *M* NaCl (pH 6.2). Elution was performed with linear gradients from 0.15 to 0.5 *M* NaCl and from 0.5 to 3 *M* NaCl; absorbance of eluted compounds was measured at 214 nm. Fractions were pooled as indicated (sample 1). Chromatography was carried out at room temperature.

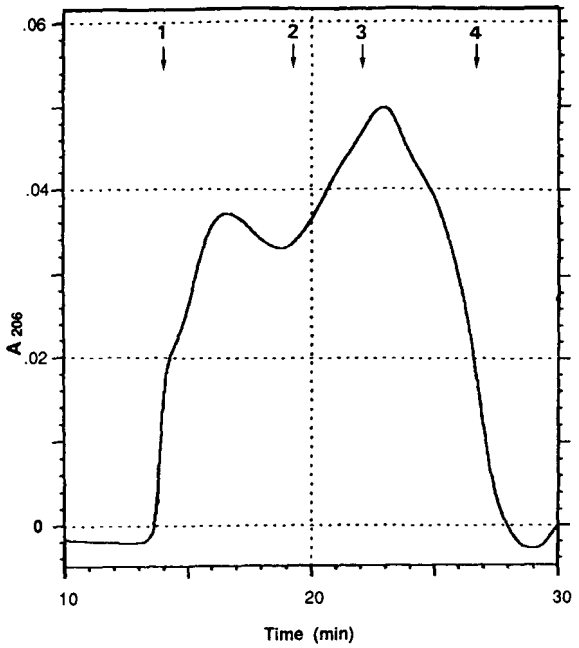

Fig. 2. Gel-permeation chromatography on TSK G 3000 SW of sample 1 prior to digestion with glycan-specific enzymes. Chromatography was performed with 0.3 *M* NaCl in 10 m*M* NaH_2PO_4–Na_2HPO_4 buffer at a flow-rate of 0.8 ml/min; elution of sample was monitored at 206 nm. Column calibration was performed by chromatography of marker proteins: 1 = thyroglobulin (669 000 dalton); 2 = immunoglobulin G (IgG) (160 000); 3 = bovine serum albumin (67 000); 4 = ovalbumin (43 000).

Aliquots of the sample were digested with either chondroitin sulphate lyase AC/ABC, keratanase or a mixture of them. The molecular weight shift obtained by enzyme treatment of the sample was determined by size-exclusion chromatography. The high-molecular-weight portion of sample is sensitive to chondroitin sulphate lyase AC/ABC (Fig. 3a), whereas the component of lower molecular weight is keratanase digestible (Fig. 3b). Incubation of the sample with both the keratanase and the chondroitin sulphate lyase AC/ABC affected the high- and low-molecular-weight components of the preparation (Fig. 3c). The peak at 22 min is due to bovine serum albumin, which is present in the enzyme preparation (Fig. 3d). Further purification was performed by high-performance anion-exchange chromatography on Mono Q (Fig. 4). The well separated major peak, eluting below 30 min, was associated with components from the initial papain digestion procedure. An extended peak, eluting in the range from 0.6 to 1.2 *M* NaCl was baseline separated from a peak eluting in the range from 1.2 to 2 *M* NaCl. Fractions were analysed for hexosamine and amino acid contents (Table I) and pooled in accordance with the molar ratio of GlcN to GalN (see bars, Fig. 4). A low GlcN/GalN ratio indicates chondroitin sulphate whereas a high ratio is indicative of keratan sulphate moieties. Aliquots of samples obtained by rechromatography on Mono Q were subjected to molcular weight distribution analysis on TSK G 3000 SW. The elution patterns were compared with those obtained from the sample prior to rechromatography on Mono Q. The second purification step resulted

in the separation of four GAG populations showing a lower diversity in molecular weight distribution (Fig. 5a and b). The purity of GAG preparations was controlled by differential enzyme digestion as described above (data not shown); no remaining contamination could be detected.

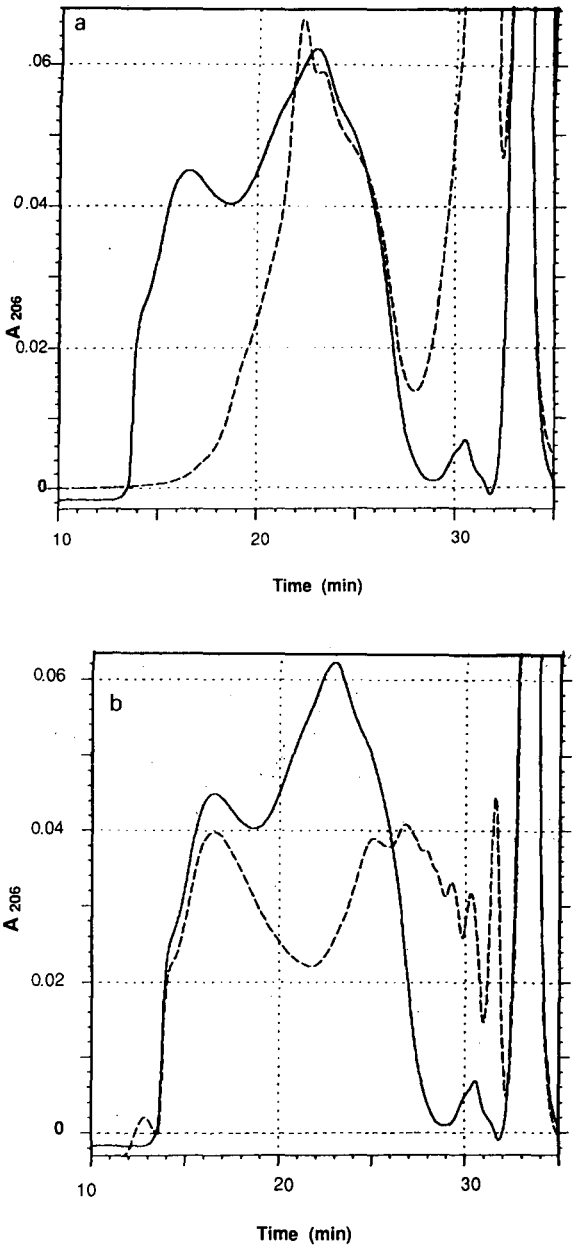

Fig. 3.

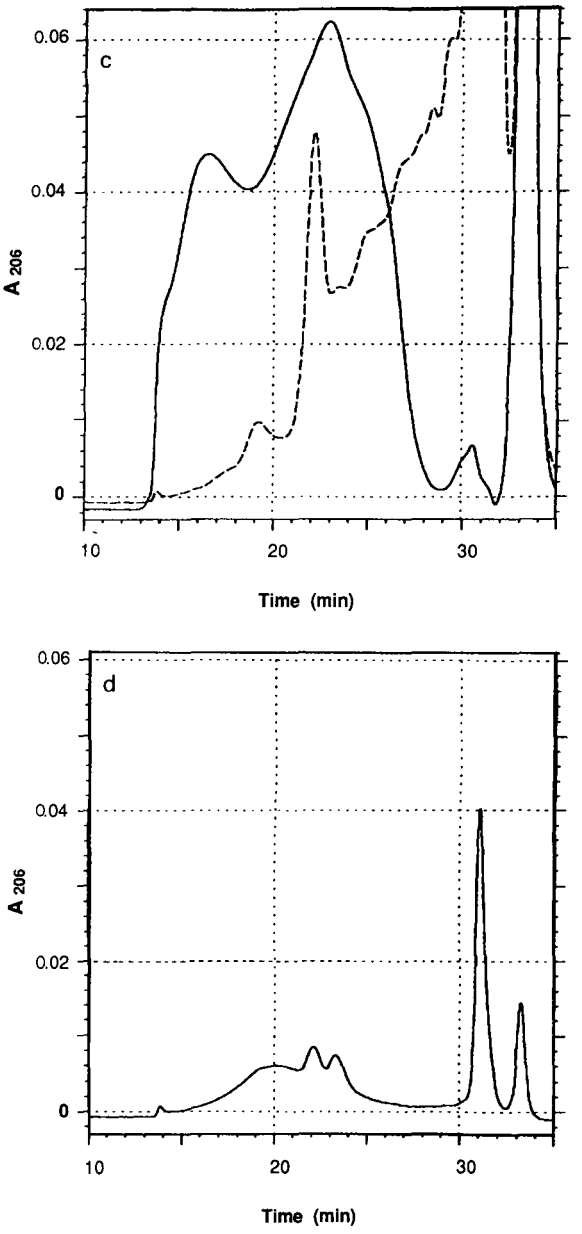

Fig. 3. Gel-permeation chromatography on TSK G 3000 SW of sample 1 prior and after treatment with glycan-specific enzymes. Conditions as in Fig. 2; in each instance 100 μl of sample (1 mg/ml) were injected. (a) Chondroitinase AC/ABC-digested (dashed line) and undigested (solid line) sample 1; (b) keratanase-digested (dashed line) and undigested sample 1 (solid line); (c) chondroitin sulphate lyase AC/ABC- and keratanase (dashed line)-treated and untreated (solid line) sample 1; (d) gel-permeation chromatography on TSK G 3000 SW of chondroitin sulphate lyase AC/ABC and keratanase preparation without addition of sample.

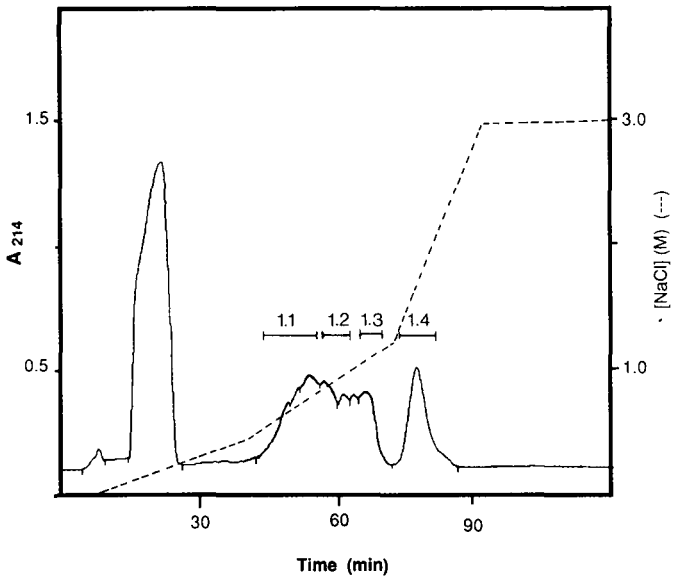

Fig. 4. Ion-exchange chromatography of sample 1 on Mono Q. 2 mg of GAG-peptide from sample 1, obtained after ion-exchange chromatography on Sepharose Q, were dissolved in 1 ml of equilibration buffer and submitted to Mono Q chromatography. The column was equilibrated with 40 m*M* sodium phosphate buffer (pH 6.5) prior to sample application. Elution was performed with three linear NaCl gradients (16.5 ml of 0 to 0.45 *M* NaCl, 20 ml of 0.45 to 1.2 *M* NaCl and 20 ml of 1.2 to 3 *M* NaCl) at a flow-rate of 0.5 ml/min; absorbance was monitored at 214 nm. Fractions were pooled as indicated by the bars (samples 1.1, 1.2, 1.3 and 1.4).

Isolation and purification of PG from human aorta

PG were extracted from human intima/media preparations in the presence of 4 *M* guanidinium chloride, protease inhibitors and CHAPS [5,11]. After changing the extraction buffer to 7 *M* urea, protease inhibitors and CHAPS anion-exchange chromatography was performed on Sepharose Q. Three distinct peaks could be separated by gradient elution (Fig. 6). The indicated peaks were submitted to size-exclusion chromatography for further purification. For none of the samples could a clear separation into different peaks be achieved (not shown); therefore, all fractions

TABLE I

MOLAR RATIO OF TOTAL AMOUNT OF AMINO ACID (aa_{tot}) AND AMINO SUGAR AND MOLAR RATIO OF GlcN TO GalN IN SAMPLE 1 AND THE DERIVED SUBFRACTIONS AFTER RECHROMATOGRAPHY ON MONO Q

Ratio	Sample				
	1	1.1	1.2	1.3	1.4
GlcN/GalN	0.40	1.20	0.25	0.04	0.06
aa_{tot}/HexN	0.59	1.03	1.26	0.51	0.46

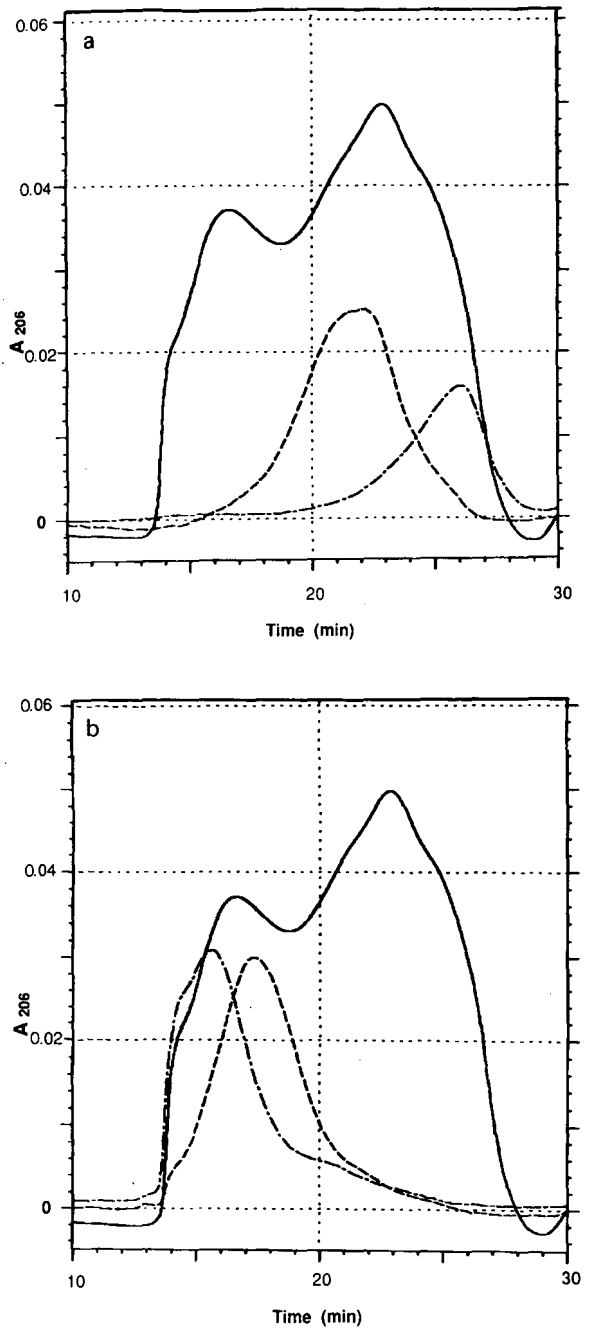

Fig. 5. Gel permeation chromatography on TSK G 3000 SW of samples obtained by rechromatography on Mono Q. Chromatograms are compared with that from sample 1 (———) of Sepharose Q chromatography. 50 µl of sample (0.4–0.8 mg/ml) were injected. Conditions of size-exclusion chromatography in Fig. 2. (a) Sample 1.1 (–·–) and sample 1.2 (– – –) in comparison with sample 1 (———). (b) Sample 1.3 (– – –) and sample 1.4 (–·–) in comparison with sample 1 (———).

with the exception of those of the void volume were pooled. Selection of the next chromatographic step was based on the finding that different GAG types show different affinities to Ca^{2+} ions [12]; in addition, hydroxyapatite has been shown to be useful for the separation of Ca^{2+}-binding proteins [13]. Therefore, PG separation of samples isolated after Sepharose CL-4B chromatography was performed on a hydroxyapatite column using phosphate gradient elution. For example, an elution pattern obtained from hydroxyapatite chromatography of a sample (Fig. 6, peak 3 after ion-exchange and Sepharose CL-4B chromatography) is shown in Fig. 7. A partially resolved peak (fractions 32–42, Fig. 7) was baseline separated from a peak eluting at high phosphate concentrations (fractions 55–58). Fractions 35–38 were pooled and submitted to high-performance anion-exchange chromatography on Mono Q.

By applying an NaCl gradient, elution of four distinct peaks could be achieved (Fig. 8). The major peak (PG-1), indicated by the bar, was selected for purity control by gel-permeation chromatography on TSK G 4000 SW. In addition to a signal in the void volumn, resulting from the high-molecular-weight portion of the PG preparation, a well defined peak was obtained. Homogeneity of the PG was demonstrated by two-wavelength detection, where a synchronous signal for absorbance at 210 and 280 nm was observed (Fig. 9). The same purification procedure was applied to another sample obtained after Sepharose Q chromatography (peak 1, Fig. 6). The result of the final purification step on Mono Q is shown in Fig. 10. Despite the compounds eluting after sample application, several baseline-separated peaks were obtained. One of them, indicated by the bar, was submitted to gel-permeation chromatography as described above (Fig. 11). Purity of the sample (PG-2) eluting near the void volume could be

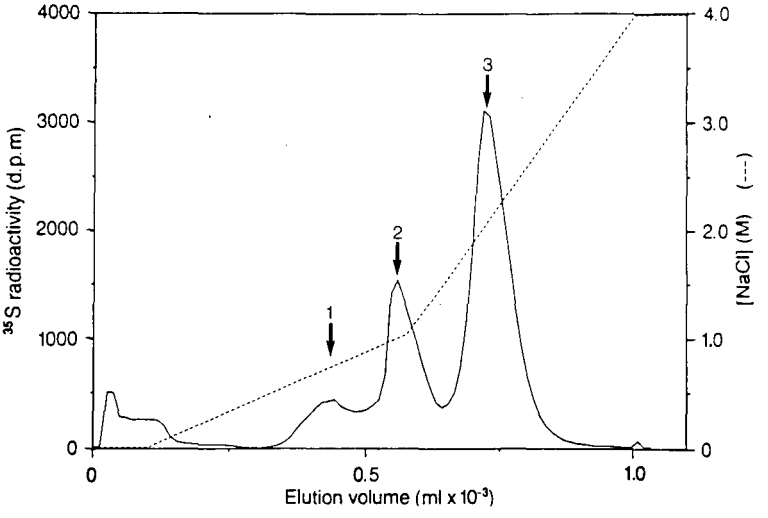

Fig. 6. Ion-exchange chromatography on Sepharose Q of PG extracted from human aorta (intima/media preparations). Prior to sample application the extraction buffer was changed to 0.05 *M* acetate buffer containing 7 *M* urea in addition to NaCl–LiCl–CHAPS and proteinase inhibitors (see Experimental). Sample application was followed by washing the column with 1.5 column volumes of starting buffer. Bound material was eluted at a flow-rate of 3 ml/min by applying two linear NaCl gradients (0.05 to 1.1 *M* and 1.1 to 4 *M* NaCl). Eluted compounds were monitored for ^{35}S radioactivity and indicated peaks were submitted to further purification procedures; the recovery of the applied activity was greater than 95%.

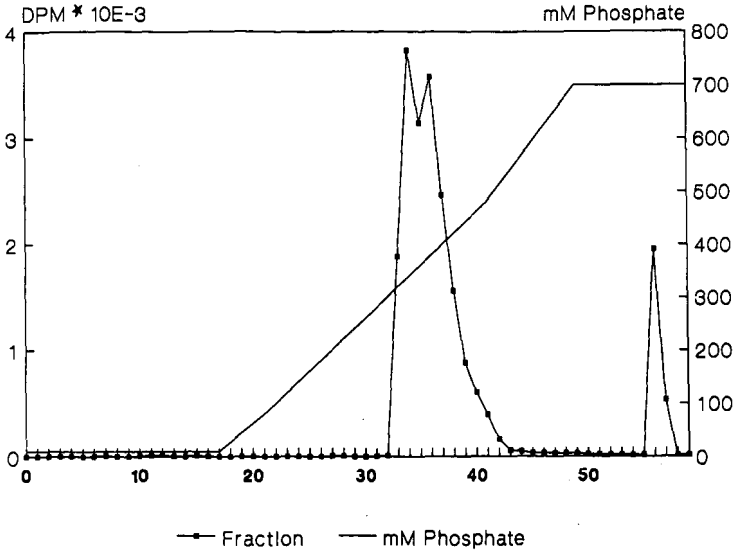

Fig. 7. Hydroxyapatite chromatography of peak 3 (Fig. 6) obtained by ion-exchange chromatography. Detailed conditions of hydroxyapatite chromatography are given under Experimental. Briefly, elution of the sample was performed by two linear gradients. Fractions (10 ml) were analysed for ^{35}S radioactivity. The recovery of the applied radioactivity was greater than 98%.

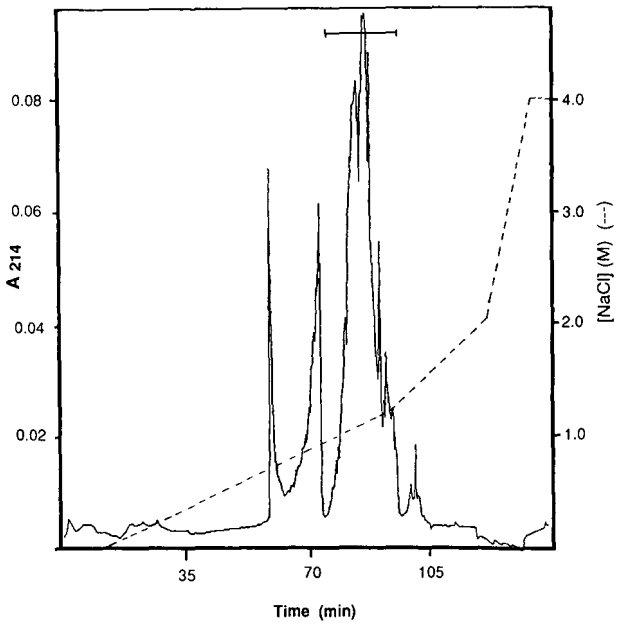

Fig. 8. Final ion-exchange HPLC on Mono Q of PG preparation purified from peak 3 (Fig. 6) of ion-exchange chromatography. Sample, isolated from peak 3 of initial ion-exchange chromatography and further purified by gel-permeation and hydroxyapatite chromatography, was applied to the column; unbound components were washed off with 45 ml of equilibration buffer before gradient elution of bound material was performed. Elution conditions are given under Experimental. Absorbance was monitored at 214 nm. Fractions were pooled as indicated by the bar (PG-1).

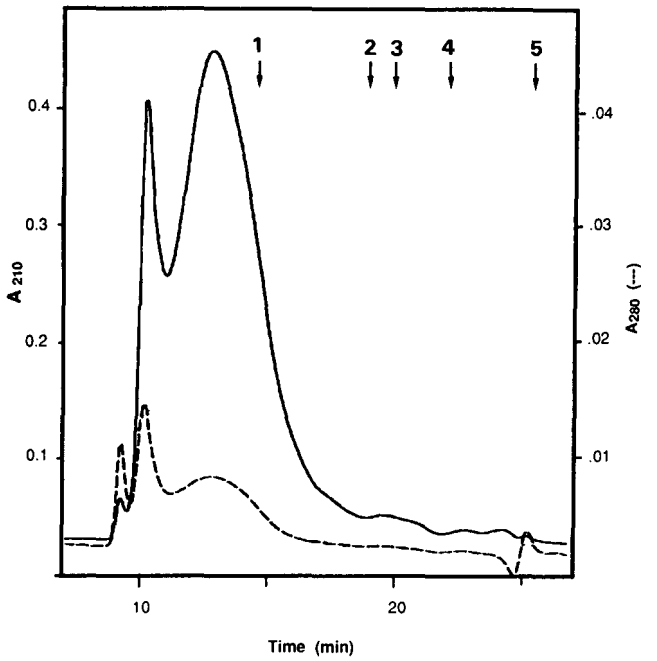

Fig. 9. Gel-permeation chromatography on TSK G 4000 SW of PG-1. 20 µl of sample (2 mg/ml) were analysed for molecular weight distribution and purity; absorbance at 210 nm and 280 nm were monitored. Elution conditions are given under Experimental. Column calibration was performed by chromatography of marker proteins: 1 = thyroglobulin (669 000 dalton); 2 = IgG (160 000); 3 = ovalbumin (43 000); 4 = myoglobin (17 000); 5 = vitamin B 12 (1300).

demonstrated. In addition, the identity and purity of the sample were evaluated by sensitive Western blot analysis (Fig. 12). The biotin-labelled sample (lane 2) was digested with glycan-specific enzymes (heparinase–heparitinase). The broad signal, ranging from the entry of the separating gel to about 180 000 dalton, disappeared after enzyme treatment, while a core protein-related signal in the region of 130 000 dalton showed up (lane 3). Amino sugar analysis showed the presence of GlcN only (data not shown), indicating KS- or HS-PG. Treatment of the sample with keratanase did not affect the staining pattern, hence the sample was identified as pure HS-PG.

CONCLUSIONS

PG and GAG were isolated from different tissues. Well established procedures use ultracentrifugation under different conditions and subsequent chromatography on a DEAE-based anion exchanger [4,9]. As these methods are time consuming, especially for preparative isolation, an alternative method was established, based on chromatographic steps only. Separation conditions were chosen with respect to physico-chemical properties. In accordance with the high charge density within the GAG moieties and the large molecular size of PG, strong anion exchangers based on wide-pore supports were used for the separation of PG. These materials have been

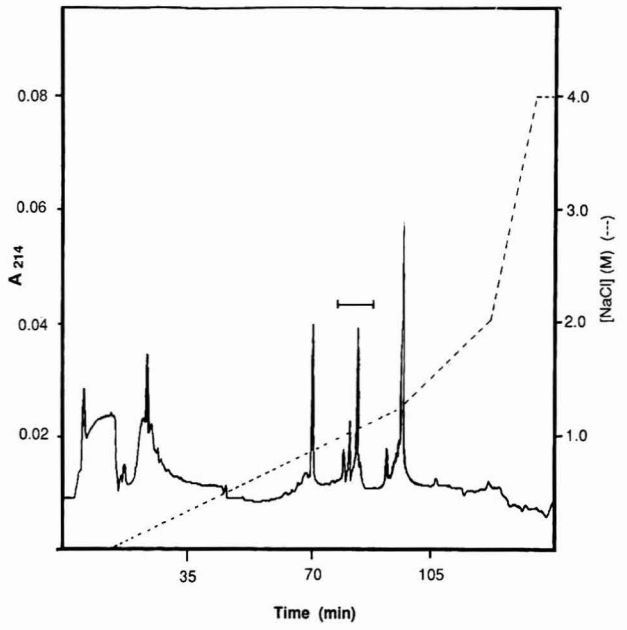

Fig. 10. Final ion-exchange HPLC on Mono Q of PG preparation isolated from peak 1 (Fig. 6) of initial ion-exchange chromatography. Sample, isolated from peak 1 of initial ion-exchange chromatography and further purified by gel-permeation and hydroxyapatite chromatography, was applied to the column; unbound components were washed off with 45 ml of equilibration buffer before gradient elution of bound material was performed. Elution conditions are given under Experimental. Absorbance was monitored at 214 nm. Fractions were pooled as indicated by the bar (PG-2).

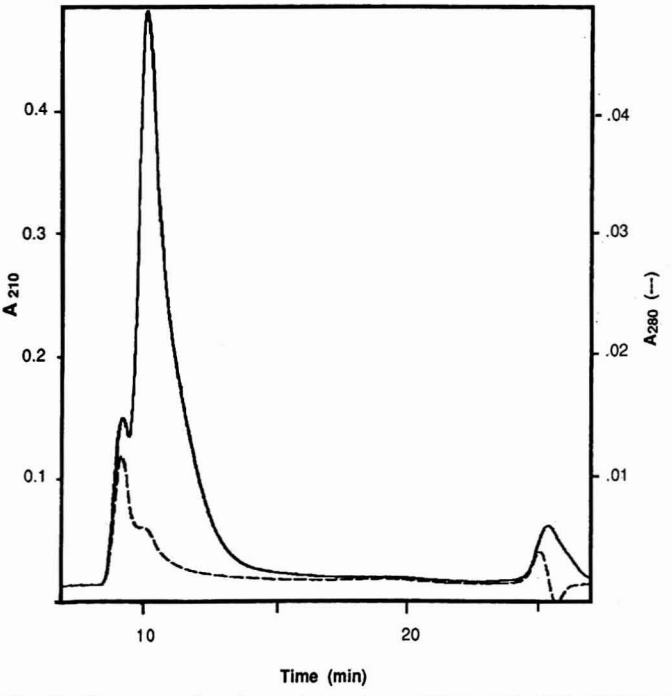

Fig. 11. Gel-permeation chromatography on TSK G 4000 SW of PG-2. 20 μl of sample (1 mg/ml) were analysed for molecular weight distribution and purity; absorbance at 210 nm and 280 nm were monitored. Elution conditions are given under Experimental.

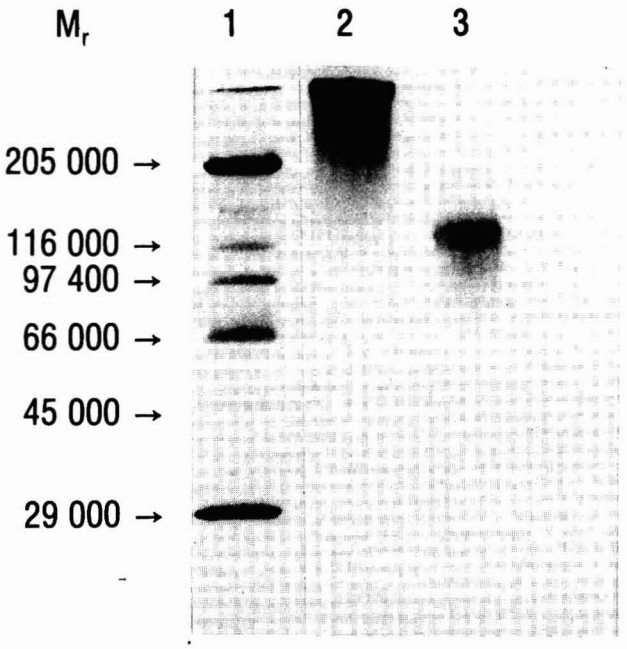

Fig. 12. Western blot of biotin-labelled PG-2 prior to and after heparinase–heparitinase digestion. Heparinase–heparitinase-digested and undigested biotin-labelled PG-2 were submitted to SDS-PAGE (8–25% gel, reducing conditions) prior to Western blotting. Lanes: 1 = molecular weight (M_r) standard; 2 = PG-2; 3 = PG-2 after heparinase–heparitinase digestion.

shown to be valuable also for the purification of GAG. Based on the Ca^{2+}-binding properties of GAG moieties, a hydroxyapatite chromatographic procedure was established. Using the protocol described, PG were isolated from complex mixtures. The purity of the isolated PG was sufficient for protein sequence analysis [14].

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Isolation of an outer membrane protein complex from *Borrelia burgdorferi* by *n*-butanol extraction and high-performance ion-exchange chromatography

KLAUS B. GONDOLF*, STEPHEN R. BATSFORD and ARNOLD VOGT
Department of Immunology, Institute for Medical Microbiology, Hermann-Herder Strasse 11, D-7800 Freiburg (F.R.G.)

ABSTRACT

Borrelia burgdorferi, the causative agent of Lyme disease, expresses two major membrane proteins, designated outer surface proteins A and B, which are of antigenic relevance, especially in the chronic phase of Lyme disease. Both proteins exhibit strain-related molecular weight variation. A method is described for obtaining these proteins from the bacterial membrane, without the use of detergents, by a combination of *n*-butanol extraction and cation-exchange chromatography on a Mono S fast protein liquid chromatographic column. This method yields up to five times larger amounts of the proteins in aqueous solution than previously described protocols, which applied ionic or non-ionic detergents. A comparison of extracts obtained by this method from different *Borrelia burgdorferi* strains is reported.

INTRODUCTION

The spirochete *Borrelia burgdorferi* is the causative agent of tick-borne Lyme disease [1,2]. This complex disorder, the most common vector-borne infection in the U.S.A. [3] and probably also Europe, encompasses a number of clinical symptoms. The primary manifestation is a skin efflorescence called erythema chronicum migrans (ECM). Weeks to months later neurological, dermatological and rheumatoid syndromes may develop (for a clinical review, see ref. 4).

Our interest has focused on two major borrelia proteins, outer surface proteins A and B (OspA and OspB), which vary in their electrophoretic mobility in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) between 30 000 and 32 000 dalton and 34 000 and 36 000 dalton, respectively, depending on the isolate examined [4]. They are surface-exposed membrane proteins [5,6] with a cationic charge [7]. Both proteins are encoded on a linear, extra-chromosomal plasmid [8] and their nucleic acid sequences have been published [9,10].

Because of the hydrophobic character of the Osps, the isolation procedures reported to date used detergents for membrane dissociation [11,12]. These techniques are subject to limitations because they gave a low yield in our hands and it is difficult to remove detergents from the protein solutions, restricting their use under *in vivo* and *in vitro* experimental conditions.

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We were able to avoid detergents by employing a combination of *n*-butanol as extraction medium and cation-exchange chromatography on a Mono S column for single-step purification and concentration.

EXPERIMENTAL

Spirochetes

The following strains were used: B31 (American type culture collection No. 35210), the original tick-isolate from Shelter Island, New York [1]; GeHo, Bo23, P-Er and Pko (the last was a kind gift from Dr. Preac-Mursic, Munich, F.R.G.), all isolated from ECM skin lesions; and Z37, Z118 and Z136, tick-isolates from the Freiburg area (these three strains were a kind gift from Dr. Pelz, Freiburg, F.R.G.). Cultures were grown in modified Barbour–Stoenner–Kelly (BSK II) medium [13] at 35°C for 4–5 days until the late logarithmic phase was reached.

The bacteria were harvested by centrifugation at 10 000 *g* for 20 min at 25°C and washed twice in phosphate-buffered saline (PBS) (pH 7.4) at 4°C. The pellet was resuspended in PBS and sonicated using a Branson (Danbury, U.S.A.) sonifier for 15 min in an ice-bath. The resulting suspension is refered to as "whole-cell lysate" in the following text.

n-Butanol extraction

The whole-cell lysate was centrifuged at 27 000 *g* for 90 min at 4°C and the resulting pellet was resuspended in PBS (1/50th of the original volume of the culture medium) and sonicated again for 15 s to aid suspension. Four parts of cold *n*-butanol (analytical-reagent grade; Merck, Darmstadt, F.R.G.) were added to five parts of the suspension and the mixture was stirred in an ice-bath. After 1 h the mixture was centrifuged at 27 000 *g* for 90 min at 4°C and a three-phase system resulted: an upper butanol phase, followed by a waxy interphase and finally an aqueous phase. The aqueous phase was carefully removed and dialysed extensively against 5 m*M* 2-(N-morpholino)ethanesulphonic acid (MES) (Sigma, Deisenhofen, F.R.G.), pH 6.0.

Ion-exchange chromatography

All chromatographic procedures were carried out on a fast protein liquid chromatography (FPLC) system (Pharmacia LKB, Freiburg, F.R.G.).

A Mono S HR5/5 cation-exchange column (50 mm × 5 mm I.D.) was equilibrated with 5 m*M* MES buffer (pH 6.0) (starting buffer). The dialysed aqueous phase from the *n*-butanol extraction (see above; volume 20–50 ml with a total protein content of 2–15 mg) was applied to the column with a Superloop application system (Pharmacia LKB). The column was then washed with starting buffer until the absorbance at 280 nm returned to the baseline. The bound proteins were eluted with linear NaCl gradients in 5 m*M* MES (pH 6.0) (from 0 to 0.6 *M* in 25 ml and from 0.6 to 2 *M* in 10 ml) at a flow-rate of 1 ml/min.

Size-exclusion chromatography

This was performed on a Superose 12 HR10/30 column (300 mm × 10 mm I.D.), equilibrated with PBS or 8 *M* urea (Ultrapure; BRL, Gaithersburg, MD, U.S.A.) in PBS. A volume of 100–300 μl of protein solution (total protein content 300 μg) was

applied and chromatography was performed at a flow-rate of 0.5 ml/min with PBS and 0.3 ml/min when equilibrated with urea.

SDS-PAGE

The protein preparations were analysed by SDS-PAGE according to the procedure of Laemmli and Favre [14] at a constant current of 20 mA (separating gel T12.6%/C2.7%, stacking gel T5%/C2.7%[a]). Molecular mass standards (Pharmacia LKB) included the following: α-lactalbumin (14 100 dalton), soybean trypsin inhibitor (20 100 dalton), carbonic anhydrase (30 000 dalton), ovalbumin (43 000 dalton), bovine serum albumin (BSA) (67 000 dalton) and phosphorylase B (94 000 dalton). The gels were stained with Coomassie Briliant Blue R-250 (Sigma).

RESULTS

The protein patterns (whole-cell lysates) of the *Borrelia burgdorferi* strains chosen in this study are shown in Fig. 1. Strain B31, GeHo, Z37 and Z118 showed major bands, known to represent OspA and OspB [15], with electrophoretic mobilities of about 31 000 and 34 000 dalton, respectively, whereas the Osps of strains Bo23 and P-Er were in the range 32 000–35 000 dalton in SDS-PAGE. We found no prominent bands in the 31 000–35 000 dalton range in strains Pko and Z 136. The main bands of these strain were in the range 22 000 and 24 000 dalton, respectively, a region where all

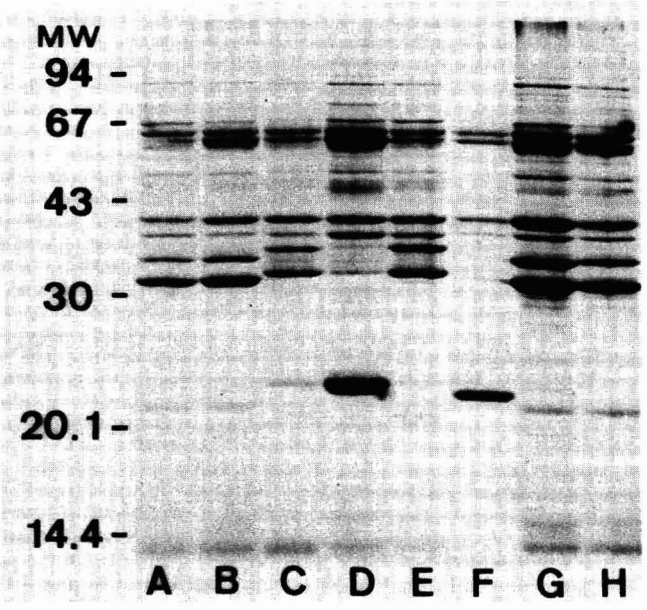

Fig. 1. SDS-PAGE of whole-cell lysates of different strains of *Borrelia burgdorferi* under non-reducing conditions. The protein load per lane was 5 μg. Lanes: A = B31; B = GeHo; C = Bo23; D = Pko; E = P-Er; F = Z136; G = Z37; H = Z118; MW = molecular mass markers (kilodalton, kD).

[a] C = g N,N'-Methylenebisacrylamide (Bis)/%T; T = (g acrylamide + g Bis)/100 ml solution.

the other strains tested showed only minor bands. Two other significant bands, 41 000 dalton (flagellin) and 65 000 dalton were uniform in all strains examined.

Extraction of 700 mg of borrelia (wet weight from 1 l of culture medium) with butanol resulted in an aqueous phase with a protein content between 0.3 and 0.6 mg/ml (total volume 20 ml), depending on the strain examined. The butanol phase did not contain protein when tested in SDS-PAGE and we did not examine the lipid components of this compartment further. SDS-PAGE of the interphase (not shown) revealed a protein pattern similar to that found in the whole-cell lysate (Fig. 1), apart from the proteins present in the aqueous phase, which were markedly reduced in this fraction.

Fig. 2 shows the content of the aqueous phase after dialysis against 5 m*M* MES (pH 6.0). As already seen when analysing the whole-cell lyates, the electrophoretic mobility of both OspA and OspB also varied in the *n*-butanol extraction within the different strains and was between 31 000–32 000 and 34 000–35 000 dalton, respectively. We found an identical pattern and size distribution of the Osps in the *n*-butanol extracts and the whole lysate in six of the eight strains examined. Strain Pko differed from all the others tested, as analysis of the whole-cell lysate (Fig. 1, lane D) and the *n*-butanol extract (Fig. 2, lane D) revealed at most only traces of Osps, the main band being a protein in the region of 22 000 dalton.

In contrast to the findings with strain Pko, we could extract both Osps (31 000 and 34 000 dalton) from strain Z136, which, like Pko, showed only faint bands in the Osp range and a major band at 21 000 dalton on analysis of whole-cell lysates (Figs. 1 and 2, lane F). Interestingly, the extract of this latter strain contained only minor amounts of the 21 000 dalton protein (Fig. 2, lane F).

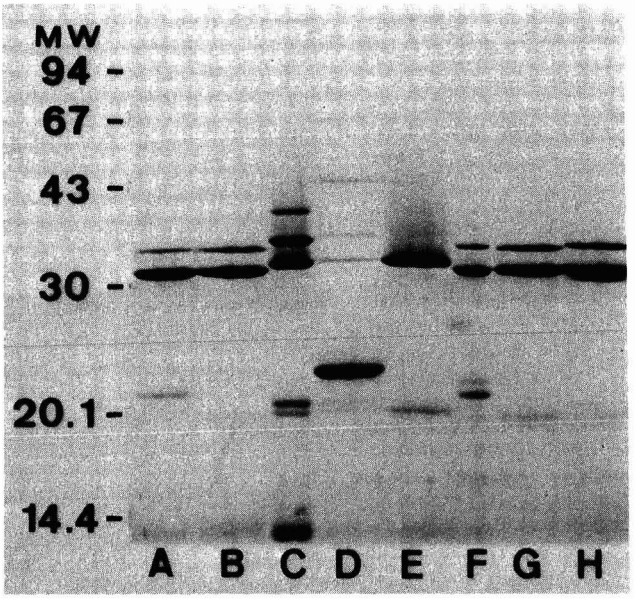

Fig. 2. SDS-PAGE of fractions obtained from *n*-butanol extraction of different strains of *Borrelia burgdorferi*. Running conditions, protein load and strains analysed as in Fig. 1.

In strain P-Er the upper band (interpreted as OspB), which was clearly visible in the whole-cell lysate, was not extractable by *n*-butanol (Figs. 1 and 2, lane E). Strain Bo23 differed from the others in that butanol extraction yielded three additional proteins (41 000 dalton, a double band at 20 000 dalton and a 12 000 dalton protein; Fig. 2, lane C).

The crude aqueous phase was heavily contaminated with nucleic acids and with minor amounts of higher-molecular-weight proteins (60 000, >100 000 dalton). For further purification and simultaneous concentration, the preparation was applied to a Mono S cation-exchange column after dialysis against the starting buffer (5 m*M* MES, pH 6.0). If the preparations were applied to the ion-exchange column without dialysis or without removing all the *n*-butanol, the proteins would not bind to the Mono S matrix.

Fig. 3 shows an example of the elution profile obtained by ion-exchange chromatography. The first, broad peak of non-binding material (fractions 0–15) contained mainly nucleic acids ($A_{280}/A_{260} \approx 0.55$) and also some contaminating proteins (Fig. 4, lane NB).

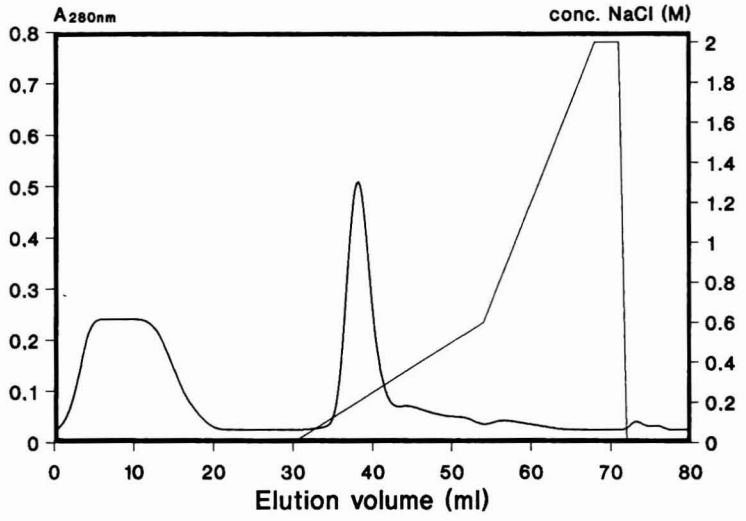

Fig. 3. Cation-exchange FPLC of *n*-butanol extract of strain GeHo on a Mono S cation-exchange column; 10 mg of protein in a volume of 20 ml were applied. Starting buffer, 5 m*M* MES (pH 6.0); gradient, 0–2 *M* NaCl; flow-rate, 1 ml/min; chart speed, 1 cm/ml.

The main protein peak was eluted from the column at about 0.2 *M* NaCl when a linear salt gradient was applied (Fig. 3). As an example of the protein pattern of this peak, the SDS-PAGE of the eluted fractions from ion-exchange chromatography of strain GeHo is shown in Fig. 4.

To elucidate the aggregation status of the fractions obtained from ion-exchange chromatography, we applied the 0.2 *M* peak fraction (after dialysis) to a Superose 12 size-exclusion chromatographic column, which had been calibrated with immunoglobulin (Ig) M (900 000 dalton), horse spleen ferritin (450 000 dalton), IgG (150 000

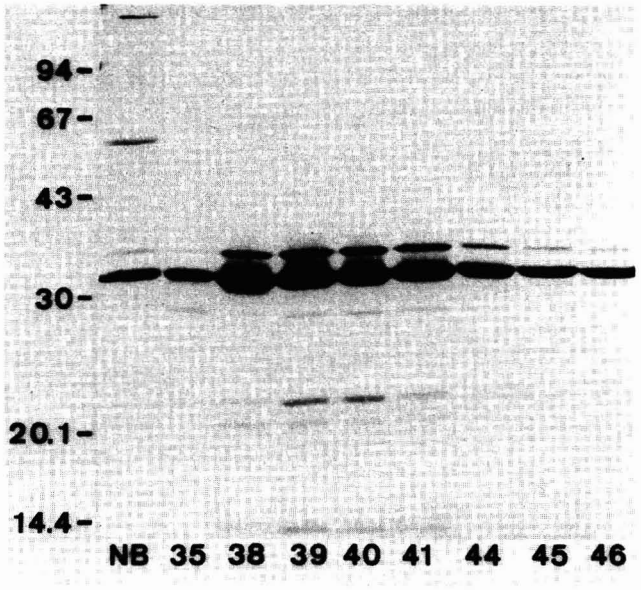

Fig. 4. SDS-PAGE of fractions from ion-exchange chromatography (Fig. 3; strain GeHo). Running conditions as in Fig. 1. The lane numbers refer to the eluted fractions from ion-exchange chromatography (Fig. 3). NB = material that did not bind to the matrix of the cation exchanger. The protein load was between 2.5 (lane NB) and 20 μg (lane 38) per lane.

dalton) and BSA (67 000 dalton). The fraction eluted as one peak shortly after IgM (Fig. 5). The same result was obtained when size-exclusion chromatography was conducted with 8 *M* urea in PBS as the buffer.

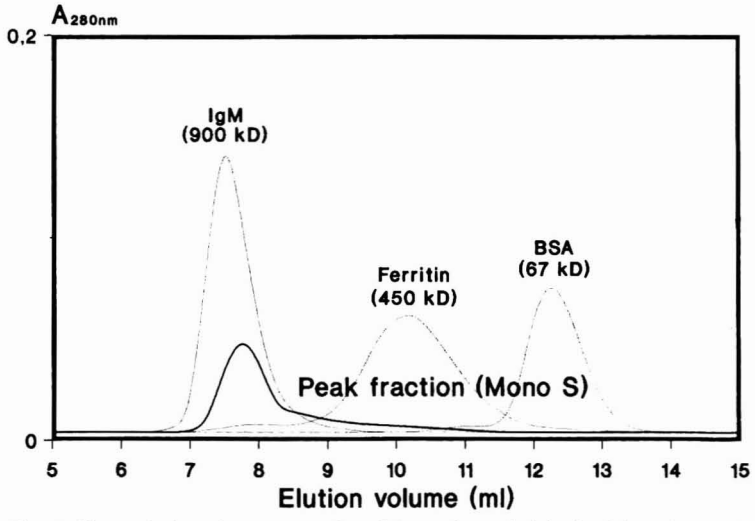

Fig. 5. Size-exclusion chromatography of the main peak (obtained from ion-exchange chromatography; fraction 29, Fig. 3) of strain GeHo. The buffer was PBS at a flow-rate of 0.5 ml/min. The superimposed curves are human IgM (900 000 dalton), horse spleen ferritin (450 000 dalton) and BSA (67 000 dalton).

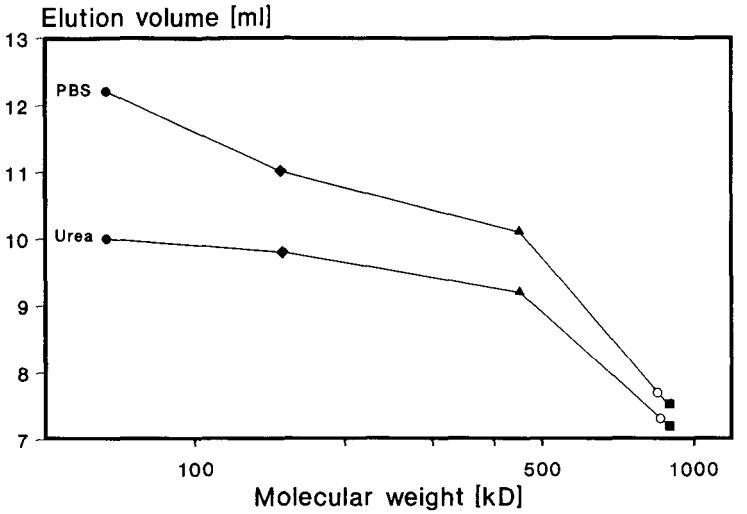

Fig. 6. Plot of elution volume against molecular mass for size-exclusion chromatography on Superose 12 using PBS or urea as buffers. Marker substances were (■) IgM (900 000 dalton), (▲) horse spleen ferritin (450 000 dalton), (◆) IgG (150 000 dalton) and (●) BSA (67 000 dalton). The open circles represent the elution volumes of *n*-butanol extracts, obtained by ion-exchange chromatography (fraction 29 in Fig. 3).

In Fig. 6 the results of the size-exclusion chromatographic runs are presented as elution volume of the marker substances *versus* the logarithm of their molecular mass. The elution behaviour of the 0.2 *M* peak (Mono S) on a Superose 12 column was similar in both PBS and urea and allows the assignment of a molecular mass of about 850 000 dalton.

SDS-PAGE of the eluted peaks from size-exclusion chromatography showed the same protein profile in both experiments as in the fractions applied (data not shown). These results indicate that the proteins extracted by *n*-butanol form a stable aggregate under physiological conditions and also when denaturated by urea.

DISCUSSION

The use of *n*-butanol for the extraction of proteins from microsomes and mitochondrial membranes was introduced by Morton in 1950 [16]. The procedure was subsequently used by other workers to allow the separation of a number of enzymes and other proteins from lipoprotein complexes. The method has also been used successfully for the purification of water-soluble proteins (for a review, see ref. 17).

The ability of organic solvents to disrupt lipoprotein complexes in aqueous media and to release undenatured protein may be ranked as follows: *n*- and isobutanol are most effective, followed by 2-butanol, cyclohexanol and 2-methyl-2-butanol; all other organic solvents tested were ineffective [17].

The unique effect of *n*-butanol may be attributed to a marked lipophilic property concomitant with hydrophilic characteristics. This combination causes the *n*-butanol molecules to orientate between lipid and water, produces a detergent-like action and allows the *n*-butanol molecules to compete with membrane phospholipids for the

hydrophobic domains of proteins. It has been reported that many of the enzymes extracted with *n*-butanol were water soluble and active after the procedure, indicating that this method causes only slight denaturation of proteins (for a more detailed description of the principle and references, see ref. 17).

Efforts have been made to develop a selective extraction method for the outer surface proteins of *Borrelia burgdorferi* and other spirochetes [11,12,18,19]. In all the published methods either ionic or non-ionic detergents were applied to disrupt the lipid layer of the outer membrane of the organism and to bring the proteins of interest into solution. In the preparation of *Borrelia burgdorferi* outer membrane proteins, the concentration of the detergent is a critical parameter: a modest increase drastically diminishes the selectivity of the method by solubilizing increasing amounts of other proteins [20].

In our hands, the methods described [11,12,18,19] gave a yield of 1–3 mg of Osps from about 700 mg of bacteria (wet weight from 1 l of BSK II medium) and rapid precipitation occurred on reduction of the detergent concentration, so that the preparations were not suitable for many experimental purposes. *n*-Butanol extraction is a highly reproducible alternative, yielding up to five times higher amounts of Osps under similar culture conditions.

The purification step on the FPLC cation-exchange column removed nucleic acids, which were a major contaminant and which accounted for most of the first peak in Fig. 3. Lane NB in Fig. 4 shows some protein contaminants that were also removed by this step. The amount of these contaminants is not readily assessable in Fig. 4, as lane NB contains only about one tenth of the amount of protein applied to lanes 38 and 39. Another important effect of the chromatographic procedure was concentration of the protein (from 0.3–0.6 mg/ml total protein in the crude *n*-butanol extract to 6–9 mg/ml in the major protein peak after chromatography). Hence it was possible to circumvent concentration, *e.g.*, by ultrafiltration, which in the case of cationic proteins often leads to undesirable losses of proteins.

It is well known that different strains of *Borrelia burgdorferi* species vary in their expression of Osps [21,22] and that even during cultivation shifts in protein expression may occur [23,24]. We selected some strains with distinct Osp profiles and examined whether the patterns found in SDS-PAGE of whole-cell lysates were identical with those seen in *n*-butanol extracts.

This was the case with most of the strains tested, but in two instances (P-Er and Z136) we obtained a protein profile in *n*-butanol extracts that differed considerably from that expected from whole cell analysis in SDS-PAGE. The most probable explanation for the apparent difference between whole-cell lysate and the *n*-butanol extract of strain Z136 (Figs. 1 and 2, lane F) is the presence of only minor amounts of Osps in this strain, so that the bands are barely visible in whole-cell lysate analysis, but are readily demonstrable when enriched by *n*-butanol extraction. A strong argument for this hypothesis is the fact that in immunoblotting it is possible to demonstrate binding of a monoclonal antibody (raised against OspA of strain B31) when analysing whole-cell lysates of Z136 (data not shown).

In strain P-Er, which did not reveal an OspB in *n*-butanol extracts (Fig. 2, lane E), either the 34 000 dalton protein seen in SDS-PAGE of whole-cell lysates (Fig. 1, lane E) could represent an OspB with an altered structure that resisted extraction, or the band seen in the SDS-PAGE analysis of the whole-cell lysate could represent an unrelated protein of similar size.

Our results suggest that *n*-butanol may be a strong inducer of protein aggregation, probably by hydrophobic interaction and Van der Waals forces. Hence another possible explanation for the fact that the 35 000 dalton protein is not found in the *n*-butanol extract of strain P-Er and for the low yield of the 21 000 dalton protein in strain Z136 is aggregation to other proteins and subsequent separation into the interphase.

Several workers have reported that OspA and OspB [7,25] and the 22 000 dalton protein [25] are cationic when analysed by two-dimensional electrophoresis. The isoelectric points of OspA and OspB, as calculated from the amino acid sequences, are 9.5 and 9.6, respectively [9]. These findings are strongly supported by our results with ion-exchange chromatography, although the extent to which the individual proteins contribute to the positive charge of the complex found after *n*-butanol extraction cannot be decided.

There is no information about the native aggregation status of the Osp available. As prepared here, the outer surface proteins of *Borrelia burgdorferi* occur as a stable complex of about 800 000–900 000 dalton. The fact that the aggregate is not disintegrated by urea strongly suggests that the complexes are formed by hydrophobic interactions. So far an enhanced aggregation tendency of proteins after *n*-butanol extraction has not been reported; the question of whether the complex reflects the native aggregation state of the proteins or whether it is the result of the extraction method remains unanswered.

The major components which were co-extracted with the Osps were proteins in the range 20 000–23 000 dalton. It is not known whether these proteins are membrane associated. Bundoc and Barbour [24] reported recently that after cloning of a single strain of *Borrelia burgdorferi* by limiting dilution, they obtained clones which produced proteins of 18 500 or 21 000 dalton. These proteins were surface exposed and their production was coincidental with a decreased or absent production of OspB. Further, the smaller proteins shared a cross-reacting epitope with outer surface protein B when tested with OspB-reactive monoclonal antibodies.

The 22 000 dalton protein that we could extract from strain Pko was designated "pC" by Wilske *et al.* [21] and has been found to be a preferential target of the immune response in patients with Lyme disease. Other workers [25] have reported that a protein of 22 000 dalton is surface exposed and that the N-terminal amino acid sequence bears a significant homology to the deduced sequence of OspA, reported by Bergström *et al.* [9]. These data could indicate that the 22 000 dalton protein is a member of the Osp family and is expressed on the surface, although at present it is not known whether all the proteins in the range 20 000–23 000 dalton found in different strains are variants of one protein or members of one protein family.

The extraction procedure presented in this paper could provide a useful tool for the further study of antigenic variations in *Borrelia burgdorferi* and perhaps facilitate the classification of Borrelia strains. Studies with soluble outer surface proteins may allow new insights into the pathogenesis of Lyme disease, particularly the late manifestations.

ACKNOWLEDGEMENT

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CHROMSYMP. 1963

Analysis, purification and properties of a 50 000-dalton membrane-associated phosphoprotein from human platelets

MARIA HALBRÜGGE* and ULRICH WALTER
Medizinische Universitätsklinik, Labor für Klinische Biochemie, Josef Schneider Strasse 2, 8700 Würzburg (F.R.G.)

ABSTRACT

Recently, the development of a monospecific antiserum against a 46 000/50 000-dalton membrane protein from human platelets which was stoichiometrically and reversibly phosphorylated in intact human platelets in response to vasodilators was reported. Using this antiserum, the subcellular distribution and the purification of this vasodilator-stimulated phosphoprotein (VASP) from human platelets has now been analysed. The VASP of human platelets is primarily a membrane-associated protein and can be purified to apparent homogeneity by salt extraction and sequential ion-exchange and dye-ligand chromatography with a purification factor of 1200 and a yield of 13%. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis under reducing and non-reducing conditions indicated that purified monomers of this VASP are linked by interchain disulphide bonding.

INTRODUCTION

Vasodilators which elevate the intracellular concentration of cGMP (*e.g.*, sodium nitroprusside, endothelium-derived relaxing factor, nitroglycerine) or cAMP (*e.g.*, prostacycline, prostaglandin E_1) inhibit the aggregation of platelets by an unknown mechanism [1,2]. In intact human platelets, these cyclic nucleotide-elevating vasodilators stimulate the phosphorylation of certain proteins, which is mediated by the activation of cGMP- and cAMP-dependent protein kinase (cGK and cAK, respectively) [3,4]. A 50 000-dalton membrane protein was identified which was phosphorylated in intact human platelets in response to both cGMP- and cAMP-elevating vasodilators and in platelet membranes by endogenous cGK and cAK [3,4]. Because of the potential importance of this vasodilator-stimulated phoshoprotein (VASP) for the mechanism of action of vasodilators, VASP was recently purified from human platelets by analysing its phosphate incorporation during the purification procedure [5]. VASP was purified as a dephospho-protein with an apparent molecular mass of 46 000 dalton and can be converted to a 50 000-dalton protein by stoichiometric phosphorylation [5]. In addition, a monospecific antiserum was developed which can be used to determine the level of VASP and the phosphorylation of this protein in intact human platelets [6]. Because of the limitation of the phosphate incorporation procedure for the determination of VASP in crude cell fractions [5], we

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now report an immunological analysis of the purification procedure and some additional biochemical properties of VASP.

EXPERIMENTAL

Materials

Cyclic nucleotides and protein markers were purchased from Boehringer (Mannheim, F.R.G.) and [γ-^{32}P]ATP (3000 Ci/mmol) and [^{125}I]protein A (30 mCi/mg) from Amersham Buchler (Braunschweig, F.R.G.). cGK from bovine lung and subunits of cAK from bovine heart were purified as described previously [7]. Q-Sepharose FF and the Mono S HR 5/5 columns were obtained from Pharmacia (Freiburg, F.R.G.), CM-cellulose (CM 52; Whatman) from Kontron (St. Leon Rot, F.R.G.) and Orange A (covalently linked to agarose) from Amicon (Witten, F.R.G.). Nitrocellulose paper was purchased from Schleicher & Schüll (Dassel, F.R.G.) and the Bio-Rad protein assay dye reagent concentrate from Bio-Rad Labs. (Munich, F.R.G.). All other chemicals were of highest purity commercially available.

Preparation of homogenates, cytosol and membranes of human platelets

Human platelets were prepared from freshly obtained blood as described [8] and resuspended in 10 m*M* NaH_2PO_4 (pH 7.6) containing 2 m*M* EDTA, 2 m*M* EGTA, 1 m*M* phenylmethylsulphonyl fluoride (PMSF) and 100 U aprotinin/ml. The platelets ($5 \cdot 10^9$/ml) were lysed by freezing in liquid nitrogen and thawing. Cytosol and membrane fragments were separated by centrifugation for 3 h at 100 000 *g* at 4°C. The membrane fragments were resuspended in 20 m*M* NaH_2PO_4 (pH 7.6) containing 2 m*M* EDTA, 2 m*M* EGTA, 1 m*M* PMSF and 100 U aprotinin/ml (one fifth of the lysis volume). Homogenates, cytosol and membranes were diluted with half the volume of 200 m*M* Tris–HCl (pH 6.7) containing 15% (v/v) glycerol, 6% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) β-mercaptoethanol and a trace of bromophenol blue, immediately boiled and then analysed by Western blotting (Fig. 1).

Radioimmunolabelling of proteins on nitrocellulose (Western blot)

Proteins of platelet extracts, of the pooled fractions after chromatography and purified VASP were separated by 9% SDS–polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose and radioimmunolabelled using a monospecific rabbit antiserum against VASP (diluted 1:800) as described in detail previously [6]. VASP was localized by autoradiography [6] and radioactivity bound to the 46 000- and 50 000-dalton forms was determined by cutting out and measuring the bands in a gamma-counter. Under the conditions used (purified VASP up to 500 ng; platelet homogenate or extract protein up to 200 μg), the radioactivity bound to VASP was proportional to the amount of purified VASP or total protein analysed.

Purification of VASP from platelet membranes

Purification was performed at 4°C as described in detail previously [5]. Platelet membranes were prepared from $3 \cdot 10^{12}$ platelets and incubated with 250 m*M* NaCl in buffer A [20 m*M* NaH_2PO_4 (pH 7.6)–2 m*M* EDTA–2 m*M* EGTA–100 U aprotinin/ml–1 m*M* PMSF) for 30 min. After centrifugation at 100 000 *g* for 1 h, the supernatant was separated and dialysed for 4 h against buffer A (pH 8.0) to remove the

salt and change the pH. The dialysed extract was applied to 30 g of Q-Sepharose FF, pre-equilibrated with buffer A (pH 8.0), stirred for 30 min and the anion exchanger was removed by filtration. The pH of the filtrate was then adjusted to 5.5 by the slow addition of 0.5 *M* HCl with constant stirring. Subsequently the filtrate containing VASP was applied to the cation exchanger CM-cellulose (15 g) that had been pre-equilibrated with buffer A (pH 5.5) and the mixture was stirred for 30 min. Unbound protein was removed by filtration and, after washing, the adsorbent was placed in a column (18 cm × 2.0 cm^2). Elution was performed with 0–1 *M* NaCl in 105 ml buffer A (pH 5.5) using a flow-rate of 1 ml/min. VASP was detected by incorporation of ^{32}P (see the next section). VASP-containing fractions (*ca.* 24 ml) were pooled and dialysed overnight against buffer A (pH 7.5). The dialysed fractions were applied at a flow-rate of 0.35 ml/min to an Orange A column (8 cm × 1.3 cm^2) that had been pre-equilibrated with buffer A (pH 7.5). After washing the gel, elution was performed with a gradient of 0–1 *M* KCl in 70 ml of buffer A (pH 7.5) using a flow-rate of 0.5 ml/min. VASP-containing fractions (*ca.* 35 ml) were dialysed overnight against buffer A (pH 6.3) and subsequently applied to a Mono S HR 5/5 column that had been pre-equilibrated with buffer A (pH 6.3). After washing the column, bound protein was eluted using a gradient of 0–1 *M* NaCl in 48 ml of buffer A (pH 6.3) and a flow-rate of 0.75 ml/min as indicated in Fig. 2. Fractions of 1 ml were collected and the absorbance at 280 nm was recorded. VASP was detected by Coomassie brilliant blue staining and incorporation of ^{32}P. Fractions 15–32 were concentrated by dialysis against buffer A (pH 7.5) containing 50% glycerol. The concentrated protein was stored at −20°C.

Phosphorylation experiments with partially purified fractions

For detection of VASP in the partially purified fractions, 10 μl of the fractions collected after chromatography on CM-cellulose, Orange A and Mono S were phosphorylated using the conditions described elsewhere [9], except that cGK (50 ng) was added to the reaction mixture (total volume 25 μl). Proteins were separated by 9% SDS-PAGE and stained with Coomassie brilliant blue. Incorporation of ^{32}P was detected by autoradiography.

SDS-PAGE under reducing and non-reducing conditions

Reducing and non-reducing SDS-PAGE were performed as described previously [10]. For non-reducing SDS-PAGE, 200 m*M* Tris–HCl (pH 6.7) containing 15% (v/v) glycerol, 6% (w/v) SDS, a trace of bromphenol blue and no β-mercaptoethanol was added to the purified preparation. Samples were heated for 5 min at 100°C and 3 min at 80°C for reducing and non-reducing SDS-PAGE, respectively. Proteins were separated by 9% SDS-PAGE and stained with Coomasie brilliant blue.

As standards cGK (monomer = 74000 dalton; dimer = 148000 dalton), RI (monomer = 47000 dalton; dimer = 95000 dalton) and RII (monomer = 56000 dalton) were analysed [11,12].

Measurement of protein

The protein content was measured according to the method described by Bradford [13] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The recent purification of the 46 000/50 000-dalton VASP from human platelets and the development of a monospecific polyclonal antibody against VASP [5,6] made this study of the subcellular distribution and determination of the purification procedure for VASP possible. Previously, VASP determination was based on the amount of ^{32}P incorporated from [γ-^{32}P]ATP into the 50 000-dalton protein catalysed by cGK. However, determination of VASP by this method was difficult and unsatisfactory, especially for crude cellular fractions, for a number of technical reasons, including interference by ATPases, phosphatases and other phosphoproteins with an apparent molecular mass of 50 000 dalton [5]. Further, the phosphorylation procedure could obviously measure only dephospho-VASP. In contrast, a radio-immunolabelling procedure using the antiserum and purified VASP standards was developed which could measure both the dephospho- (46 000 dalton protein) and phospho (50 000-dalton protein) forms of VASP in addition to the phosphorylation of VASP due to the conversion of the 46 000- to the 50 000-dalton protein [6].

With this procedure, the subcellular distribution of VASP in homogenate, membranes (particulate fraction) and cytosol (soluble fraction) prepared from human platelets was measured (Fig. 1). This Western blot analysis demonstrates that VASP of untreated human platelets is primarily present as the dephospho-form (46 000 dalton) and recovered in the membrane fraction (>95%) after cell lysis. A previous study showed that VASP of intact human platelets can be quantitatively converted to the

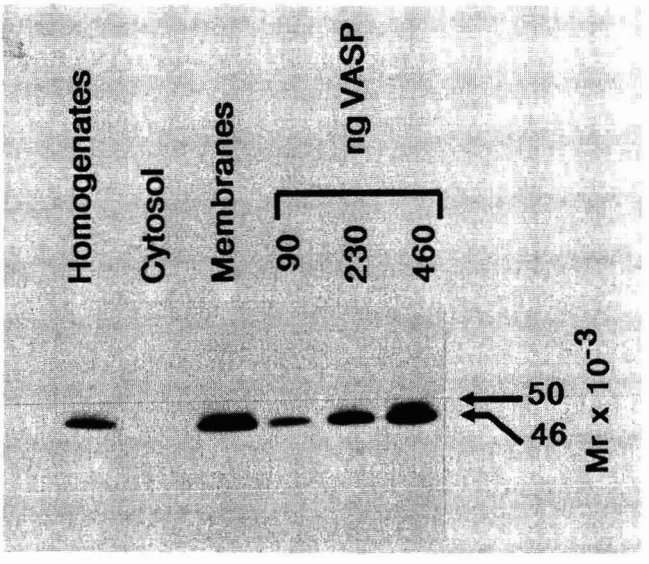

Fig. 1. Autoradiography showing the subcellular distribution of VASP in human platelets determined by an immunological analysis. Homogenates, cytosol and membranes (each 100 μg of protein) and 90–460 ng of purified VASP (lanes as indicated from left to right) were analysed by Western blotting using a monospecific antiserum against VASP (see Experimental). Arrows indicate the 46 000- and 50 000-dalton forms of VASP. Mr = Molecular mass.

50 000-dalton phospho-form by treatment with cyclic nucleotide-elevating vasodilators [6]. In this study, the radioactivity bound to both the 46 000- and 50 000-dalton protein was used for the determination of VASP (see Experimental). From experiments shown in Fig. 1 using various amounts of purified VASP standards, it could be estimated that platelet homogenates contain 2.45 μg of VASP per milligram of homogenate protein. This corresponds to an intracellular concentration of about 20 μM VASP, assuming an M_r of 46 000 for VASP and a mean platelet volume of 5.2 fl [14]. As more than 95% of platelet VASP was recovered in the membrane fraction (Fig. 1), this fraction was used as the starting material for the purification of VASP. About 65% of this membrane-bound VASP could be extracted with a buffer containing 250 mM NaCl, resulting in a 3.3-fold purification of VASP (Table I).

TABLE I

IMMUNOLOGICAL ANALYSIS OF THE PURIFICATION OF VASP FROM HUMAN PLATELET MEMBRANES

In this experiment, membranes of $3 \cdot 10^{12}$ platelets (corresponding to 10–15 l of human blood) were used. For the determination of specific and total activity of VASP, aliquots of the various purification fractions (membranes, NaCl extract and the pooled fractions obtained after chromatography on Q-Sepharose FF, CM-cellulose and Mono S) were analysed by Western blotting using a monospecific antiserum against VASP and [^{125}I]protein A (see Experimental and Fig. 1). VASP activity (determined by the binding of [^{125}I]protein A) is indicated here as specific activity (cpm/mg protein) or total activity (cpm) for the pooled fractions of the various purification steps.

Purification step	Total protein (mg)	Specific activity $\times 10^{-3}$ (cpm/mg)	Total activity $\times 10^{-3}$ (cpm)	Purification (-fold)	Yield (%)
Membranes	2268.00	233	528444	0	100.0
NaCl extract	448.00	771	345408	3.3	65.4
Q-Sepharose FF	70.40	2607	183533	11.2	34.7
CM-Cellulose	4.31	17659	75934	75.8	14.4
Orange A + Mono S	0.25	278830	69708	1196.7	13.2

Solubilized VASP was further purified by adsorbing unwanted proteins of the NaCl extract on the anion exchanger Q-Sepharose FF followed by cation-exchange chromatography on CM-cellulose, resulting in 76-fold purification with a yield of 14% (Table I). Final purification of VASP was obtained by sequential chromatography on the dye-ligand Orange A and the cation exchanger Mono S (Table I). Orange A chromatography was chosen because we found in preliminary experiments that certain phosphoproteins including VASP bind to this dye and that this chromatographic step separated VASP from other proteins. After Orange A chromatography, determination of the specific acitivity and purification factor for VASP was not possible because the VASP-containing fractions were too dilute to allow an accurate protein measurement. However, during the last step of the purification procedure, chromatography on Mono S at pH 6.3, VASP was concentrated and could be eluted as a sharp peak (Fig. 2) at about 120–150 mM NaCl. Analysis of an aliquot obtained from the peak fractions by SDS-PAGE and Coomassie brilliant blue staining showed that VASP was purified primarily as the dephospho-form (46 000-dalton protein) and

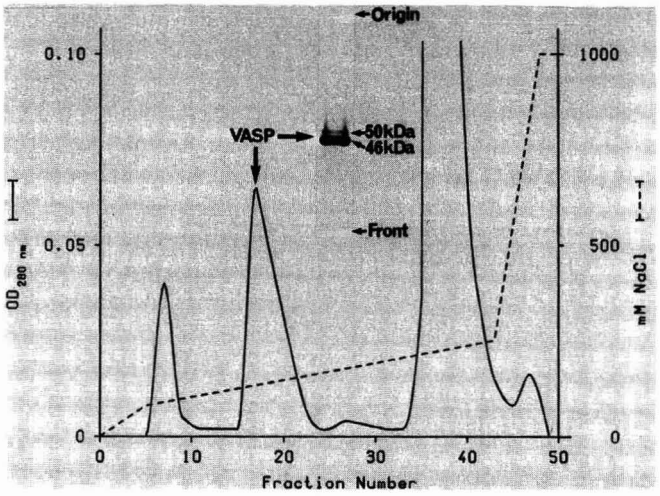

Fig. 2. Mono S chromatography of VASP. VASP was partially purified from human platelet membranes by salt extraction and chromatography on Q-Sepharose FF, CM-cellulose and Orange A. In the final purification step, VASP was chromatographed on a Mono S HR 5/5 column at pH 6.3. Elution was performed with a gradient of 0–1 *M* NaCl in buffer A (dashed line) as described under Experimental. Absorbance at 280 nm was measured (solid line) and 1-ml fractions were collected. Fractions 15–23 were pooled, dialysed and concentrated. Purified VASP (1.5 μg of protein) was analysed by SDS-PAGE and stained with Coomassie brilliant blue (inset). The proteins of this purified preparation migrated primarily as a 46 000-dalton (46 kDa) form and to a minor extent as a 50 000-dalton (50 kDa) form.

to a small extent as the phosho-form (50 000-dalton protein), as demonstrated in the inset in Fig. 2.

Aliquots of the pooled fractions of the various purification steps were then analysed for protein content and for VASP content by Western blotting (Fig. 3). In this experiment, an amount of protein from each purification step was selected that gave a roughly equal signal for VASP in the radioimmunolabelling procedure (Fig. 3). The autoradiogram demonstrates that the purifiation procedure removes some proteolysis products with lower molecular mass than intact VASP and that 0.1 μg of purified VASP gives about the same signal as VASP present in 100 μg of platelet membranes. This suggests that an enrichment of about 1000-fold is required in order to purify VASP from human platelet membranes to apparent homogeneity. This was indeed observed when the entire purification procedure was quantitatively analysed by Western blotting, which indicated a 1200-fold purification of VASP with a yield of 13% (Table I). This contrasts with the purification analysis by the phosphate incorporation method, which required a 200-fold enrichment to obtain VASP of apparent homogeneity with a yield of 3% [5]. The difference between the two methods is probably due to the fact that crude platelet fractions contain 50 000-dalton phosphoproteins in addition to VASP. This results in an overestimation of VASP in crude extracts and subsequent underestimation of the final yield and purification factor with the phosphorylation method. Using the more specific and reliable Western blot method, it can now be estimated that VASP represents about 0.1–0.2% of platelet protein, indicating that VASP is not a rare protein in human platelets. The

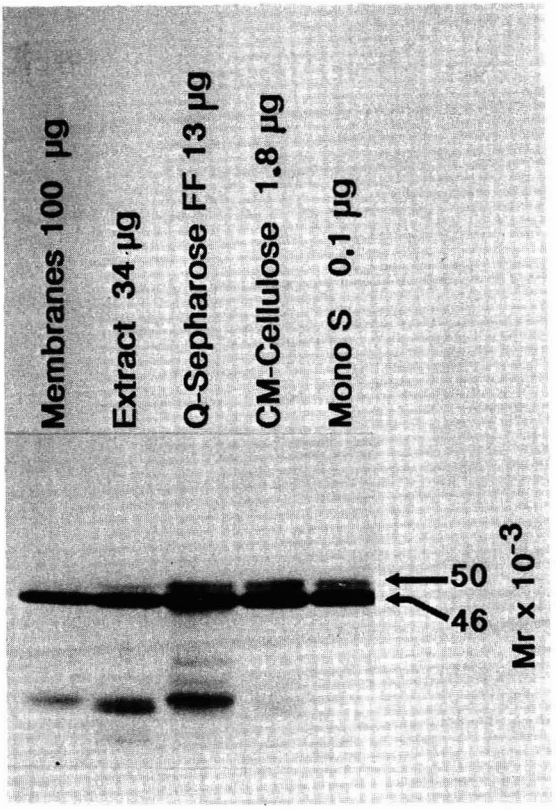

Fig. 3. Autoradiography showing the immunological analysis of VASP purification. Platelet membranes (100 μg of protein), the NaCl extract (34 μg of protein) and the pooled fractions after chromatography on Q-Sepharose FF (13 μg of protein), CM-cellulose (1.8 μg of protein) and Mono S (0.1 μg of protein) were analysed by SDS-PAGE (lanes as indicated from left to right) followed by Western blotting using a monospecific antiserum against VASP. The 46 000- and 50 000-dalton forms of VASP are indicated by arrows.

phosphorylation of VASP in intact platelets by the cytosolic cAK and cGK and the extractability of VASP from platelet membranes by salt strongly suggest that VASP of human platelets is a membrane-associated protein of the cytosolic compartment. Surprisingly, SDS-PAGE of VASP under reducing and non-reducing conditions indicated the presence of at least one interchain disulphide bonding in VASP (Fig. 4).

In the presence of β-mercaptoethanol VASP migrated as 46 000-dalton protein and in its absence 92 000-dalton protein, consistent with the occurrence of a disulphide bond-linked dimer of VASP under non-reducing conditions. This behaviour as a monomer or dimer under these two different experimental conditions was also observed with the regulatory subunit RI of the cAK (monomer 47 000 dalton, dimer 94 000 dalton) and with cGK (monomer 74 000 dalton, dimer 148 000 dalton), two proteins with established interchain disulphide bonding [11,12]. In contrast, the regulatory subunit RII of the cAK, a protein without interchain disulphide bonding [12], migrated as a monomer (56 000 dalton) in the presence and absence of

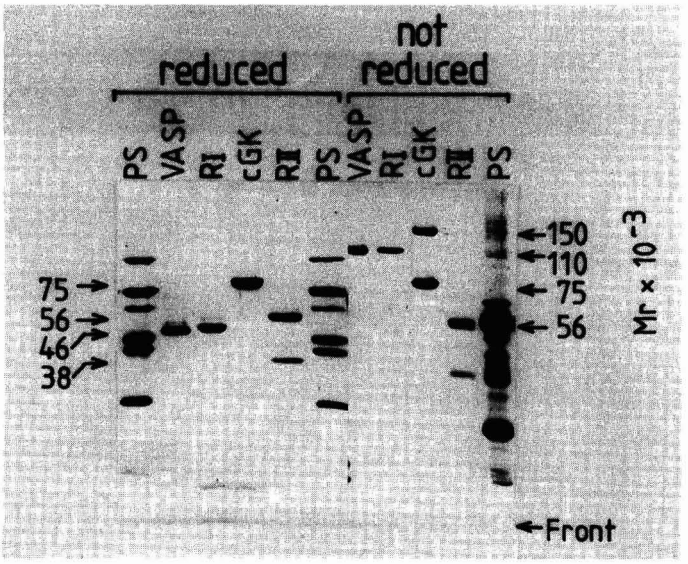

Fig. 4. SDS-PAGE of purified VASP and protein kinases under reducing and non-reducing conditions. Purified preparations of VASP, cAK regulatory subunit type I (RI), cGK and cAK regulatory subunit type II (RII) were heated for 5 min at 100°C in the presence (reduced) and for 3 min at 80°C in the absence (not reduced) of β-mercaptoethanol (see Experimental). The proteins (1.5 μg of each protein) were separated by SDS-PAGE (lanes as indicated from left to right) and stained with Coomassie brilliant blue. Arrows indicate the positions and the molecular masses of the apparent proteins and their subunits. PS = Protein standards.

β-mercaptoethanol (Fig. 4). The 38 000-dalton protein present in purified RII subunit is an RII fragment due to partial proteolysis. Although the occurrence of disulphide bonding is highly unusual for an intracellular protein owing to the high reducing capacity of the intracellular compartment, interchain disulphide bonding has been observed with some cytosolic enzymes such as RI, cGK and some other proteins [11,12]. Whether the interchain disulphide bonding of VASP indeed occurs *in vivo* and whether this is of physiological significance remains to be established. Preliminary gel filtration experiments [15] suggest that partially purified and homogeneous VASP under non-denaturing conditions is significantly larger than a dimer. Therefore, gel filtration and ultracentrifugation analysis of VASP before and after phosphorylation are required in order to determine the size and structure of VASP.

The availability of VASP as purified protein, the development of the specific antiserum and the increasing information about the biochemical properties of VASP should be helpful in elucidating the structure and function of VASP.

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Radionuclide X-Ray Fluorescence Analysis with Environmental Application

by **J. Tölgyessy,** *Slovak Technical University, Bratislava, Czechoslovakia,* **E. Havránek** *and* **E. Dejmková,** *Comenius University, Bratislava, Czecholovakia*

(Wilson & Wilson's Comprehensive Analytical Chemistry Vol. XXVI)

G. Svehla *(editor)*

Nuclear analytical methods are being used increasingly for solving analytical problems and, of these methods, radionuclide X-ray fluorescence analysis (radionuclide XRFA) is becoming more important. The purpose of this work is to present a comprehensive, instructive analysis of the basis of radionuclide XRFA, to describe methods of sample preparation for environmental analysis and to make the reader more familiar with the procedures, methods and instrumentation of radionuclide XRFA used in this field. This book discusses the use of radionuclide XRFA for solving analytical problems of the environment and information is presented concerning the current state of research and use of radionuclide XRFA in this significant area. The present volume will serve as a basic source of data and also as a laboratory handbook.

Contents. **1. Nuclear analytical methods and protection of the environment.** The importance of checking the quality of environmental components. Radionuclide X-ray fluorescence analysis (radionuclide XRFA) and the nuclear analytical methods. The origin and development of radionuclide X-ray fluorescence analysis. **2. The physical basis and method of radionuclide X-ray fluorescence analysis.** Physical basis of radionuclide XRFA. The radionuclide X-ray fluorescence analysis methods. **3. Source of environmental pollution.** Sources of atmospheric pollution. Sources of water pollution. Sources of soil pollution. The transport of pollutants from the atmosphere, water and soil into biological materials. The health hazard from the presence of harmful substances in the human environment. **4. Sampling procedures.** Atmospheric sampling. Water sampling. Soil sampling. Sampling of sludges, sediments and solid wastes. Sampling of biological materials. **5. The preparation of samples for the determination of pollutants in components of the environment.** Reference materials and the laboratory preparation of standards. Preparation of samples for determining impurities in the atmosphere. The preparation of samples for determining impurities in water. The preparation of samples of biological materials for analysis. The preparation of samples for the determination of impurities in soil, sludges, sediments and solid wastes. **6. A review of the use of radionuclide X-ray fluorescence analysis of environmental components.** Radionuclide XRFA of air samples. Radionuclide XRFA of air samples. Radionuclide XRFA of water samples. Radionuclide XRFA of soil and sediment samples. Radionuclide XRFA of samples of biological materials. **7. The use of radionuclide X-ray fluorescence analysis in other branches of science and technology.** The mining industry. The production of building materials. Metallurgy. The fuel and petroleum industries. The chemical, photographic and paper industries. Pharmacy. Space Research. Archaeology and art. The measurement of the thickness of thin coating. **8. References.** Subject index.

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INFORMATION FOR AUTHORS

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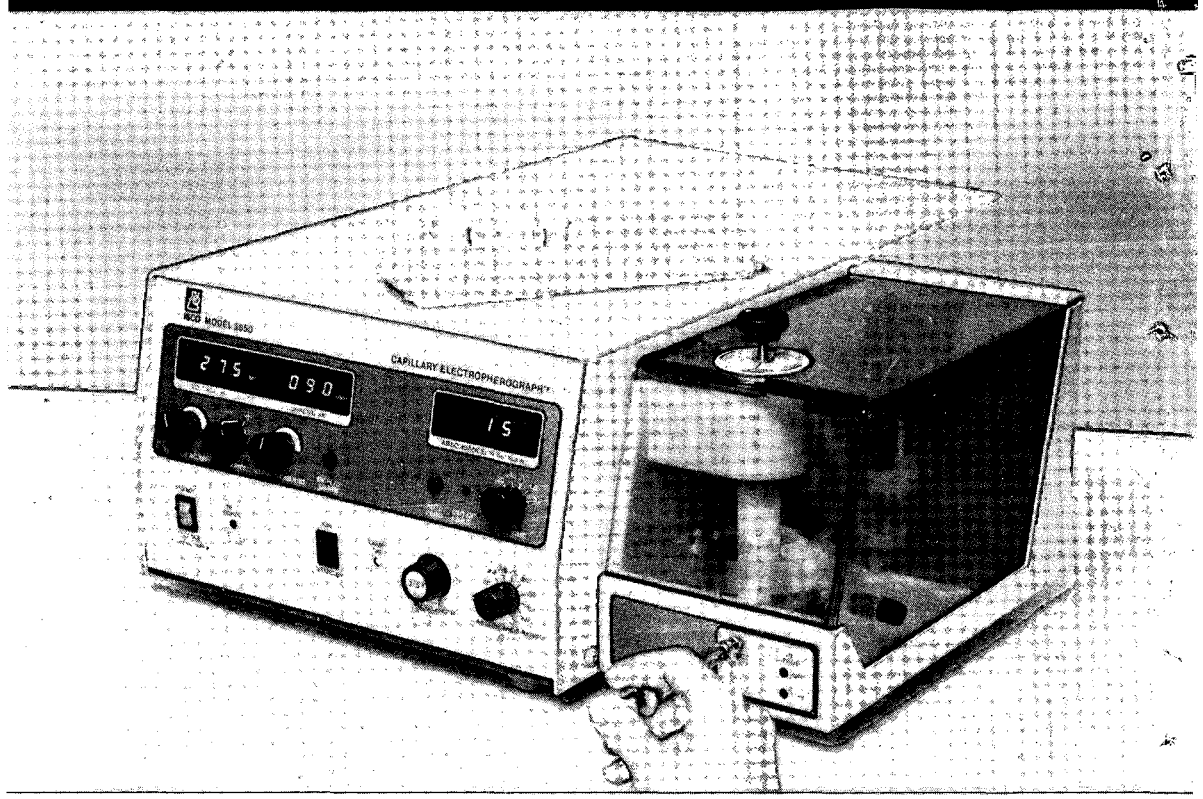

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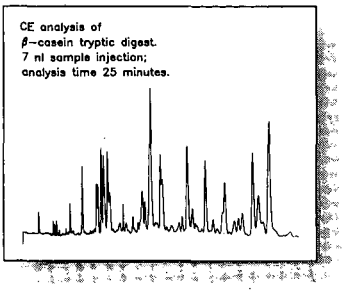

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