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7th FPLC Seminar
Titisee, February 12–14, 1992

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INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

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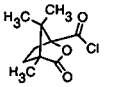

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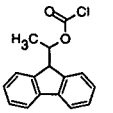

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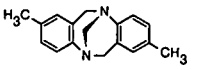

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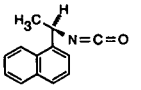

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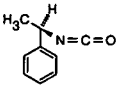

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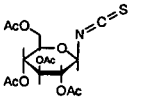

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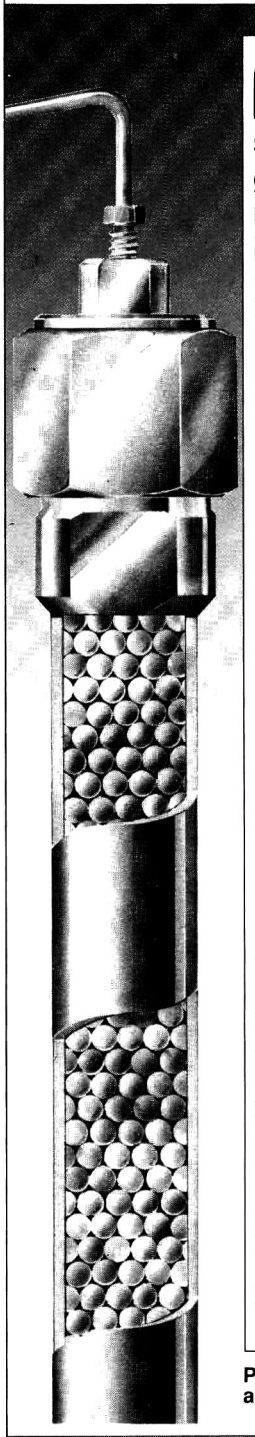

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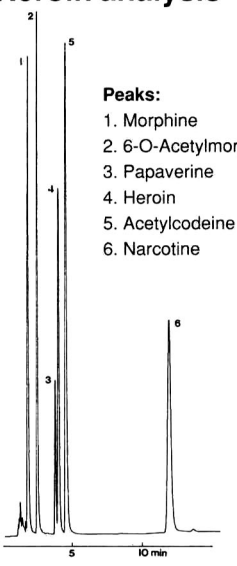

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VOL. 625 (1992)

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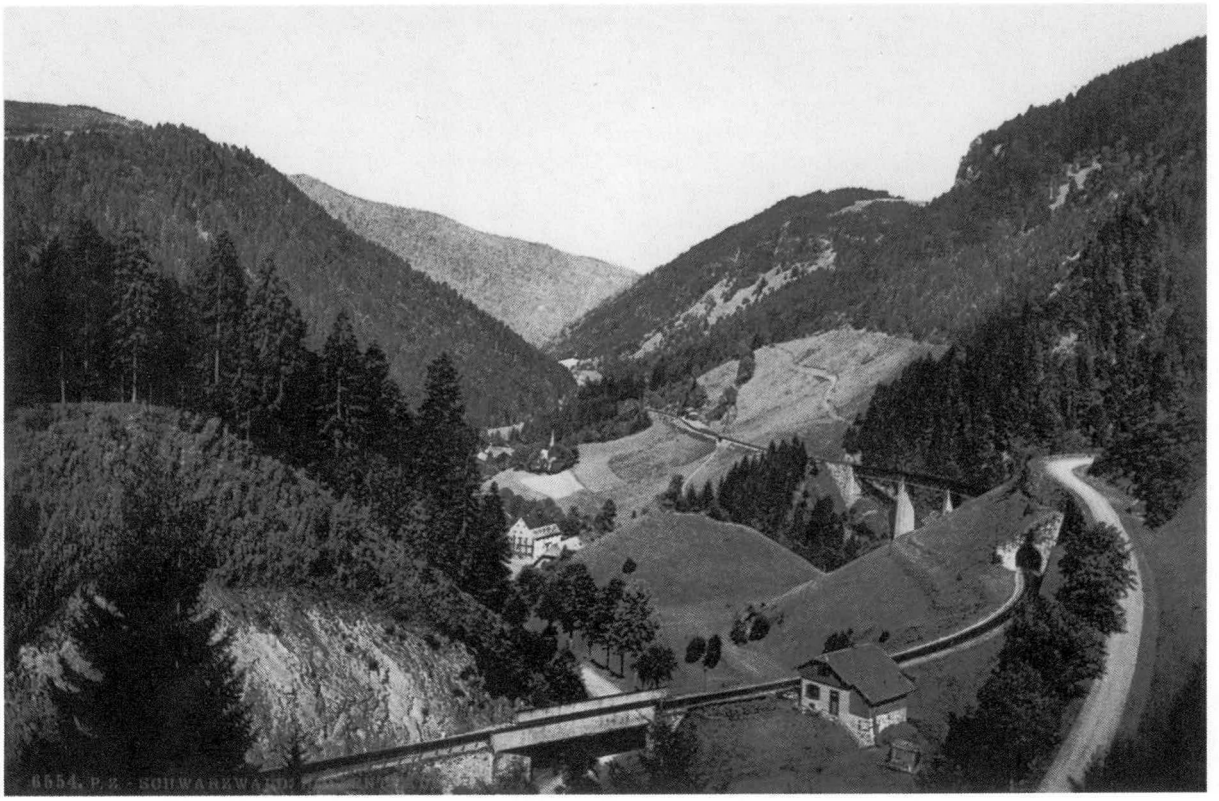

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Titisee (Germany), February 12–14, 1992

Guest Editors

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(Hamburg, Germany)

JOACHIM STADLER
(Freiburg, Germany)

Participants of the seminar.

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7TH FAST PROTEIN LIQUID CHROMATOGRAPHY SEMINAR, TITISEE, FEBRUARY 12–14, 1992

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CHROMSYMP. 2578

Foreword

The 7th FPLC Seminar at Titisee was held from February 12 to 14, 1992 at this internationally well-known resort in the Black Forest which is located at an elevation of 800 m, 30 km east of Freiburg. The FPLC Seminars, organized by Pharmacia Biosystems, Freiburg (Germany), have been held at Titisee since 1984. Their goal is to bring together scientists who primarily use chromatographic methods in their research, especially fast protein liquid chromatography, for the purification of proteins. In this context many other methods, used for the characterization, purification and production of proteins were presented during this seminar in order to obtain a complete description of all the techniques used by the researchers who presented their experimental work.

The lectures and discussions were almost freed from the limitations of the chairman's clock in order to promote the best possible understanding of the presented topics. The lively question-and-answer sessions permitted a lot of additional remarks to be made by the many experts present at Titisee. These remarks were stimulated either by the chairman or by the participants themselves in order to provide answers to methodological problems. The intensive discussions were augmented by the familiar atmosphere of this meeting and its location at Treschers Schwarzwald Hotel with its beautiful view to the Titisee, where all the participants stayed for the period of the meeting.

This issue contains nine of the twenty presented papers which were submitted to the *Journal of Chromatography* and chosen by its Editorial Board for publication. They contain such important subjects as the purification of topoisomerase I and II from human leukemic cells, the characterization of chromatographically purified isoforms of angiotensinase A from human kidney, the purification of the C1 repressor of bacteriophage P1 and of P-450 cytochromes from liver microsomes. Scaling-up procedures involving the BioPilot System of Pharmacia are described with the aim of efficiently handling large volumes of recombinant proteins. Fast HPLC is used to purify the large-scale over-production of phosphoenolpyruvate carboxykinase in *Saccharomyces cerevisiae* in order to determine its three-dimensional structure after crystallisation. Vasoactive peptides, obtained by the incubation of human plasma with proteases, are purified by a sophisticated combination of chromatographic procedures and cross-flow filtration; techniques necessary for the rapid removal of the digestive enzyme(s). Immobilized-metal affinity chromatography allows the separation of photosystems I and II from a thermophilic cyanobacterium. Chromatographic studies, involving the effects of light and age, on adenylate kinase from plant tissues are presented.

In addition to these briefly described themes a lot of new information was presented on the use of Pharmacia's SMART™ System for the separation of minute quantities of proteins, as well as on scaling-up procedures aimed at handling large quantities of proteins by sophisticated and automated high-performance chromatographic methods, including Pharmacia's BioPilot System™.

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie, Universität Hamburg, Hamburg (Germany) Rudolf Dernick

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CHROMSYMP. 2570

Application of cross-flow filtration to the purification of biologically active peptides in human plasma after incubation with a protease-rich extract

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ABSTRACT

The aim of this study was to find an experimental procedure to purify biologically active peptides from a complex biological matrix (plasma), which was incubated with a protease-rich extract (submandibular gland extract). Special interest was focused on the practicability of cross-flow filtration for this purpose. Therefore, peptides in the incubation mixture were purified with a combination of high-performance liquid chromatographic steps. Purification of biologically active peptides was monitored by a sensitive bioassay and by laser desorption/ionization mass spectrometry. This permitted not only purity control at each purification step but also identification of one of the peptides with vasoconstrictor properties as angiotensin II. This result demonstrates the practicability of cross-flow filtration for extracting enzymatic reaction products from complex substrate–enzyme mixtures during the incubation.

INTRODUCTION

Croxatto [1] recently suggested that there may be many unidentified, biologically active peptides released from their precursor proteins by limited enzymatic proteolysis. With his idea in mind, a method for the purification of biologically active peptides was devised. Limited enzymatic proteolysis of precursor proteins, to obtain bioactive peptides, demands the rapid removal of the proteolysis products from the incubation mixture on their generation in order to avoid further cleavage into biologically inactive peptides. The conventional solution for this problem, dialysis [2], seems to be unsatisfactory, as the removal of the peptides is based on diffusion and therefore possibly too slow. Another disadvantage is that dialysis is time consuming and the handling is cumbersome. Cross-flow filtration appears to be a better technique for removing the newly generated peptides from the incubation medium, because the filtration rate is larger, the reaction medium is moved continuously, the risk of membrane clogging is low and the temperature from the retentate and the filtrate can be controlled separately. The practicability of the cross-flow filtration had to be proved by purification and identification of the bioactive peptides from the filtrate.

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Rat submandibular gland tissue was chosen, because it contains a broad spectrum of proteases [3]. Human plasma was chosen as a source of precursor hormones, because it is well documented that several prehormones, such as angiotensinogen, are present in plasma [4]. It was not attempted to demonstrate a mechanism which is active *in vivo*, as this would have required substrate and enzymes from the same species. Further, in this work, the submandibular gland proteins releasing vasoactive peptides were not isolated and identified, as a variety of such submandibular gland enzymes have already been described, such as tonin, kallikrein and other enzymes [5–7].

The background of this study was to find and characterize still unknown peptides with vasoactive properties that may be involved in blood pressure regulation like the peptide hormones angiotensin II, endothelin or bradykinin. So far there is no evidence that the known pressure-regulating peptide hormones are involved in the pathogenesis of hypertension [8,9]. This suggests that other, still unknown, peptide hormones may be involved.

EXPERIMENTAL

Materials

Chemicals. High-performance liquid chromatography (HPLC)-grade water, methanol, acetonitrile and HPLC-grade trifluoroacetic acid (TFA) were obtained from J. T. Baker (Deventer, Netherlands). Angiotensin II (AII), norephinephrine (NE) and AII inhibitor ([Sar^1,Val^5,Ala^8]-AII) were obtained from Sigma (Deisenhofen, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

Physiological buffer. The buffer was 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 100 m*M* NaOH, 90 m*M* NaCl, 5 m*M* KCl, 1.8 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$ dissolved in water and titrated to pH 7.4.

Filtration equipment. Tubular dialysis membranes with an exclusion pore diameter of relative molecular mass (M_r) 8000 were obtained from Spectra/Por (Spectrum Medical, Los Angeles, CA, USA). The cross-flow filtration device and the M_r 10 000 membrane (regenerated cellulose) were purchased from Millipore (Bedford, MA, USA).

SDS-PAGE equipment. The Phast system and PhastGel (gradient 8–25) from Pharmacia Biosystems (Freiburg, Germany) were used.

Solid-phase extraction (SPE) equipment. The vacuum manifold column processor was obtained from Burdick & Jackson (Muskegon, MI, USA), glass columns (5 ml), PTFE frits and reversed-phase (RP) C_{18} silica material from J. T. Baker (Phillipsburg, NJ, USA) and a Speed-Vac concentrator from Savant (Farmingdale, NY, USA).

HPLC equipment. The HPLC equipment consisted of an L-6200 gradient pump (Merck) coupled to a Rheodyne injector (Latek, Heidelberg, Germany), a spectrophotometer (Lambda-Max 481; Waters), a two-channel compensation recorder (Pharmacia Biosystems, Freiburg, Germany) and a RediFrac fraction collector (Pharmacia Biosystems, Freiburg, Germany). A semi-preparative reversed-phase column, Protein Plus (25 cm × 2.2 cm I.D.; Zorbax) (DuPont, Dreieich, Germany) was used.

Microbore HPLC equipment. Microbore separations were performed on the SMART system (Pharmacia Biosystems), which integrates a gradient pump, injector, column holder, a spectrophotometer (280 nm), a flow cell for conductivity measurement and a fraction collector. A microbore cation-exchange column (100 × 2 mm I.D., 100-SA; Machery–Nagel, Düren, Germany) was used. The fractions from cation-exchange chromatography were desalted on a reversed-phase microbore HPLC column (RPC C_2/C_{18}, PC 3.2/3; Pharmacia Biosystems). The GROM-SIL 300-ODS-2 microbore reversed-phase column (200 × 2 mm I.D.) was purchased from Grom/Stargroma (Herrenberg, Germany).

HPLC eluents. Eluent A was 0.1% (v/v) TFA in HPLC-grade water, eluent B was 0.1% (v/v) TFA in HPLC-grade acetonitrile and eluent C was 60% (v/v) HPLC-grade acetonitrile in HPLC-grade water. Eluent D was made up of 5 m*M* KH_2PO_4 (pH 3) in 30% (v/v) acetonitrile and eluent E of 5 m*M* KH_2PO_4 (pH 3) in 30% (v/v) acetonitrile and 0.5 *M* KCl. All HPLC eluents were filtered through a 0.2-μm filter (Anotop) (Merck).

Plasma preparation

A plasma pool was prepared by drawing a total of 830 ml of blood from an antecubital vein from sixteen healthy human volunteers (male 9, female 7, age 28 ± 4 years). Blood samples were collected

in chilled polypropylene tubes, containing 1 mg EDTA/ml plasma. This was centrifuged at 2000 *g* for 10 min at 4°C. The resultant plasma (410 ml) was bulked and stirred on ice for 2 min. Aliquots of 25 ml were frozen in dry-ice and then stored under liquid nitrogen.

Comparison experiments: dialysis versus cross-flow filtration

A 10-ml volume of the pooled plasma was divided into two equal parts and treated as follows.

In method I, 5 ml of plasma were dialysed for 24 h at 4°C through tubular dialysis membranes with an exclusion pore diameter of 8000 dalton against a physiological buffer solution.

In method II, the other 5 ml of plasma were diluted to 100 ml with a physiological buffer solution and then filtered in a cross-flow filtration device, using an M_r 10 000 membrane at 4°C until the volume of the filtrate measured 90 ml.

The filtrate from both methods was lyophilized and desalted by gel filtration. The protein concentration was then measured using the Bradford assay [10] and the samples were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out on PhastGel gradient 8–25. Samples treated with SDS were processed in the PhastSystem and the gels were developed with the silver staining procedure of Heukeshoven and Dernick [11].

Incubation experiments

Tissue extraction. An 18.6-g amount of rat submandibular glands obtained from normotensive male Wistar rats aged 3 months were cut into small pieces. The tissue was placed in 40 ml of ice-cold physiological buffer and homogenized in an ice-bath-cooled blade homogenizer ten times at intervals of 30 s. The homogenate was centrifuged and the supernatant was filtered in the cross-flow filtration device using a 0.2-μm membrane. The filtrate (35 ml) was used as an incubation extract. The extraction work was carried out at 4°C.

Incubation of plasma with the submandibular gland extract. Freshly prepared submandibular gland extract was mixed with 390 ml of the pooled plasma. This mixture was filtered in the cross-flow filtration device using an M_r 10 000 membrane. During filtration the plasma–submandibular gland extract mixture (retentate) was incubated at 37°C for 20 h. The retentate was kept at a constant volume with a physiological buffer reservoir (37°C). The filtrate was cooled in an ice–water bath separately.

Concentration of peptides in the filtrate. For concentration of peptides, laboratory-assembled RP-SPE cartridges and a vacuum manifold column processor connected to a water-driven vacuum pump were used. Silanized glass columns were fitted with PTFE frits and filled with 2.5 g of reversed-phase C_{18} silica material. Extraction was carried out with two columns connected in series. The solid-phase extraction was preceded by conditioning the columns with 20 ml of eluent B and subsequently washing with 20 ml of eluent A.

The filtrate from the incubation experiment was acidified with TFA up to a concentration of 0.1% (v/v). This eluent was slowly aspirated (1 ml/min) through the RP-SPE column by vacuum, then the column was washed with 20 ml of eluent A. Elution of the peptides was achieved with 20 ml of eluent C. The eluate was concentrated to dryness in a Speed-Vac concentrator. The filtrate from the control experiments was also processed according to this concentration procedure.

Purification of the peptide-rich RP-SPE fraction by HPLC. The peptide-rich RP-SPE fraction from the incubation experiment (plasma with submandibular gland extract) and the two control experiments (plasma alone, submandibular gland extract alone) were chromatographed first on the semi-preparative RP-HPLC column. The conditions and the procedure are given in the caption of Fig. 4.

The vasoactive fraction from semi-preparative RP-HPLC was purified further on a microbore cation-exchange HPLC column (the conditions and procedure are given in the caption of Fig. 6). Before testing the fractions from the cation-exchange chromatography for vasoactivity, they had to be desalted on a reversed-phase microbore HPLC column. After the sample injection of a fraction into the SMART system, the column was washed for 4 min with eluent A at a flow-rate of 100 μl/min. Elution of the peptides was performed with a steep linear gradient from 0 to 60% eluent B in 2 min and with 60% eluent B for 3 min at a flow-rate of 100 μl/min. The Speed-Vac-dried vasoactive fraction from the cation-exchange separation was chromatographed by microbore RP-HPLC (the conditions and procedure are given in the caption of Fig. 8).

Detection of vasoactivity. Male normotensive Wistar Kyoto rats (WK), aged 4–6 weeks, were used. The isolated perfused kidney was prepared as described previously [12]. Briefly, the abdominal cavity was opened and the infrarenal aorta and left renal artery were isolated and cannulated. Immediately after the cannulation, 500 units of heparin sodium were flushed into the aorta and renal artery. Perfusion was started immediately after the cannulation. The left kidney was excised by blunt dissection, excluding the adrenal gland, and mounted in the perfusion system.

The perfusion was performed with a single-pass system at constant flow-rate of 9 ml/min · g body mass with Tyrode's solution kept at 37°C and equilibrated with 5% CO_2 and 96% O_2, as described previously [13]. The prepared fractions and the vasoactive hormones (concentration of angiotensin II 25 ng/ml; concentration of norepinephrine 300 ng/ml) were injected in bolus injections of 150 μl each to test kidney responsiveness. To block the angiotensin II receptors, the isolated kidney was perfused with Tyrode's solution containing 10 μM of the angiotensin II receptor antagonist saralasin.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) measurements. The vasoactive fractions were examined by MALDI-MS. A reflector-type time-of-flight mass spectrometer, equipped with a nitrogen laser (337 nm, pulse length 4 ns) was used for ion generation and mass analysis. Details of the MALDI-MS have been reported elsewhere [14]. Briefly, Speed-Vac-dried samples were dissolved in 10 μl of water. A 1-μl volume of this eluent was mixed with 10 μl of an aqueous solution of 0.1 M 2,5-dihydroxybenzoic acid (UV absorption maximum at 337 and 355 nm), representing the UV-absorbing matrix. A 1-μl volume of the final eluent was dripped in and dried on a metallic substrate. Desorption of analyte ions was achieved by laser shots of irradiances in the 10^6–10^7 W/cm^2 range focused to spot sizes typically 50–100 μm in diameter. The spectra were registered with a LeCroy 9400 transient recorder and typically accumulated from ten single laser shots. The total time of measurements, including preparation, was 10–15 min. The results were expressed as M_r/electrical charge of the substance (M_r/z in Figs. 5, 7 and 9). Since with this form of mass spectrometry substances with a single charge are produced, M_r/z is identical with M_r [14].

RESULTS

Comparison of dialysis with cross-flow filtration

The efficiency of cross-flow filtration was first compared with that of dialysis. Pooled human plasma was divided into two parts and one part was fractionated by dialysis and the other by cross-flow filtration. Table I shows the protein concentration, estimated by the Bradford assay [10]. This shows that cross-flow filtration is significantly more effective than dialysis. To obtain further insight into the quality of the fractionation, SDS-PAGE was performed and the results are shown in Fig. 1. In the dialysis filtrate even albumin can be demonstrated, whereas in the cross-flow filtration filtrate no protein bands can be shown.

Incubation experiment

The plasma was incubated with submandibular gland extract in the cross-flow filtration device at 37°C for 20 h. During the whole incubation period the mixture was filtered against a 10 000 M_r membrane to remove the proteolysis products continuously. The filtrate (proteolysis products) were cooled at 0°C.

Concentration of peptides

When the incubation was complete, the peptides in the filtrate were concentrated by solid-phase extraction with a reversed-phase sorbent (RP-SPE).

Detection of vasoactivity

The fractions from the HPLC purification steps were tested for vasoactivity in a bioassay. For this purpose an isolated perfused rat kidney was used. Fig. 2 shows a dose–response curve of the vasocon-

TABLE I

PROTEIN CONCENTRATIONS (DETERMINED WITH THE BRADFORD ASSAY) OF DIFFERENT FRACTIONS FROM HUMAN PLASMA (n = 5)

Fraction	Protein concentration (mean ± standard deviation)
Dialysis, retentate	6.9 ± 0.8 g/dl
Dialysis, filtrate	1.0 ± 0.03 mg/dl
Cross-flow filtration, retentate	7.2 ± 0.6 g/dl
Cross-flow filtration, filtrate	0.0 ± 0.0 mg/dl

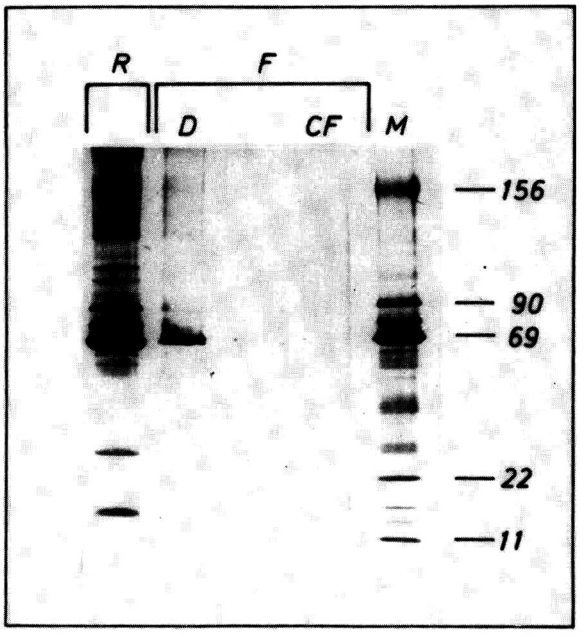

Fig. 1. SDS-PAGE of the plasma fractionation experiments. R = Sample of the retentate; F = samples of the filtrates; D = sample of the filtrate fraction from dialysis; CF = sample of the filtrate fraction from cross-flow filtration; M = marker proteins (desalted human urine proteins); numbers = $M_r \times 10^{-3}$.

strictors angiotensin II and norepinephrine and Fig. 3 an original curve of the time course of the perfusion pressure and the response to vasoactive factors.

Control of the purity of the peptide fractions

MALDI-MS was used to the control the purity of the peptide fractions and to characterize the peptides.

Purification of the vasoactive substance

The peptide-rich SPE-RP fraction was chromatographed first on an RP-HPLC column (Fig. 4). Only in the chromatogram of the incubation experiment (plasma together with submandibular gland extract) was a vasoactive fraction eluted after 34 min. In the fractions from the control experiments (plasma alone, submandibular gland alone) no vasoactivity was registered. The vasoactive fraction from RP-HPLC (Fig. 4) was analysed by MALDI-MS (Fig. 5). Several mass peaks indicate that this fraction is

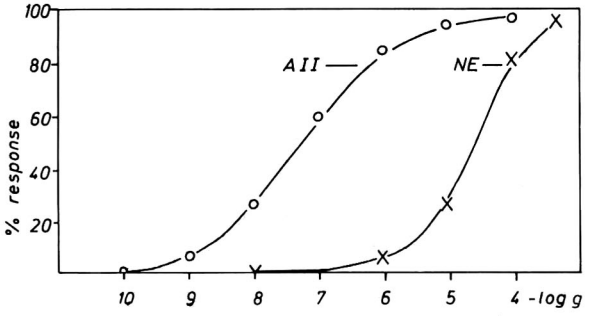

Fig. 2. Dose–response curve of the isolated perfused rat kidney. Abscissa: injected amounts of (○) angiotensin II (AII) and (×) norepinephrine (NE), expressed in −log (grams). Ordinate: change in perfusion pressure (P, mmHg); 100% response of the kidney stimulated with angiotensin II, 90 mmHg; 100% response of the kidney stimulated with norepinephrine, 210 mmHg.

inhomogeneous. One of the peaks has m/z 1047, which is identical with the mass of the protonated ion of angiotensin II. Commercially available angiotensin II, injected to the RP-HPLC column, was eluted at the same retention time as the bioactive fraction.

Next, the vasoactive fraction was purified further with a microbore preparative cation-exchange column (Fig. 6). Again the retention times of the vasoactive fraction and angiotensin II were very similar. The MALDI mass spectrum (Fig. 7) of the vasoactive fraction still shows several mass peaks.

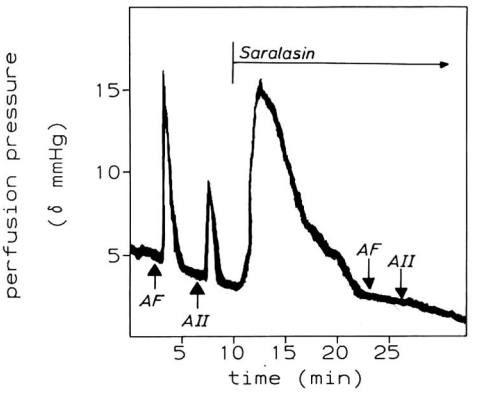

Fig. 3. Typical pattern of changes in perfusion pressure (P, mmHg) in the isolated perfused kidney, caused by 1 ng of angiotensin II (AII) and the bioactive fraction (AF; equivalent to 0.3 ml of plasma) from the chromatographic purification (Fig. 4) before and after adding 10 μM saralasin to the perfusion medium.

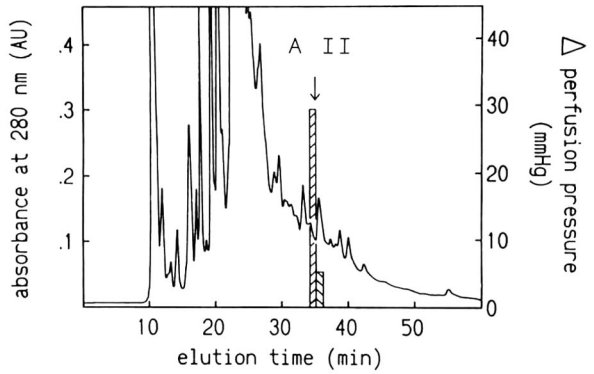

Fig. 4. Typical semi-preparative RP-HPLC of the peptide-rich SPE fraction. AU = Arbitrary units; absorbance range, 0.5. Conditions: column, C_4 reversed-phase Protein Plus (250 × 21.2 mm I.D.); eluent A, 0.1% TFA in water; eluent B, 0.1% TFA in acetonitrile; gradient, 0–5 min 100% eluent A, 5–15 min 0–20% eluent B; 15–55 min 20–40% eluent B; 55–65 min 40–60% eluent B; flow-rate, 6.5 ml/min; sample, peptide-rich SPE fraction (390 ml plasma equivalent), dissolved in 1 ml of eluent A; fraction size, 6.5 ml. The hatched area indicates the vasopressor activity, equivalent to 1 ml of plasma. AII indicates the retention time of angiotensin II.

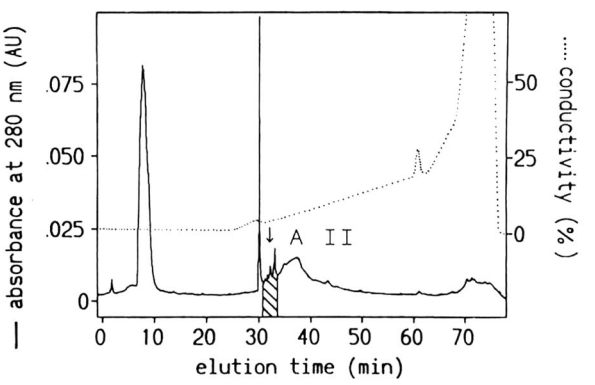

Fig. 6. Typical micropreparative cation-exchange chromatography of the bioactive fraction from the semi-preparative chromatography (Fig. 4). Absorbance range, 0.1. Conditions: column, 100-SA (100 × 2 mm I.D.); eluent D, 5 m*M* KH_2PO_4 (pH 3) in water–30% acetonitrile; eluent E, eluent D + 0.5 *M* KCl; gradient, 0–7.5 min 100% eluent D, 7.5–47.5 min 0–20% eluent E; 47.5–62.5 min 20–35% eluent E, 62.5–64.5 min 35–80% eluent E, 64.5–67.5 min 80% eluent E; flow-rate, 100 μl/min; sample, bioactive fraction from the semi-preparative chromatography (Fig. 4; 350 ml plasma equivalent) dissolved in 1 ml of eluent D; fraction size, 600 μl. The vasoactive fraction, equivalent to 1 ml of plasma, caused an elevation of perfusion pressure of 11 mmHg (hatched area) in the bioassay. AII indicates the retention time of angiotensin II.

One of the mass peaks is identical with the mass of the protonated ion of angiotensin II.

The vasoactive fraction was re-chromatographed on a preparative microbore HPLC column, using a less steep gradient (Fig. 8). The fraction was separated into several UV-absorbing peaks (Fig. 8). Vasoactivity was eluted with the peak at 31 min. The vasoactive fraction and angiotensin II were eluted with the same retention time.

Pharmacological proof of the identity of the purified vasoactive peptide with angiotensin II

To confirm that the vasoactivity is due to angiotensin II, the bioactive fraction from semi-preparative RP-HPLC (Fig. 4) was tested in the isolated,

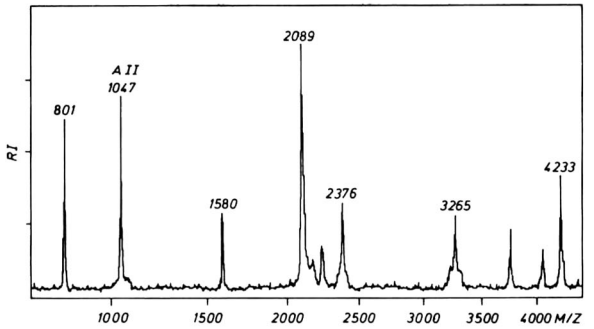

Fig. 5. MALDI mass spectrum of the bioactive fraction obtained after RP-HPLC (Fig. 4). AII indicates a signal of a protonated ion, which is identical with the mass of the protonated ion of angiotensin II.

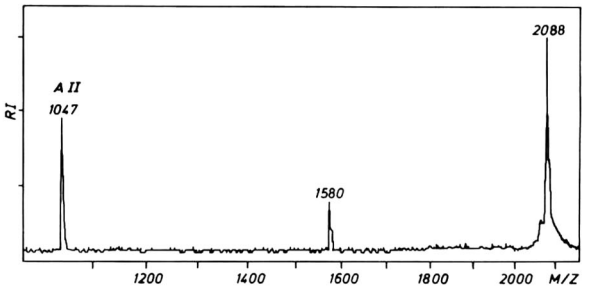

Fig. 7. MALDI mass spectrum of the desalted bioactive fraction from cation-exchange chromatography (Fig. 6). AII indicates a signal of a protonated ion which is identical with the mass of the protonated ion of angiotensin II.

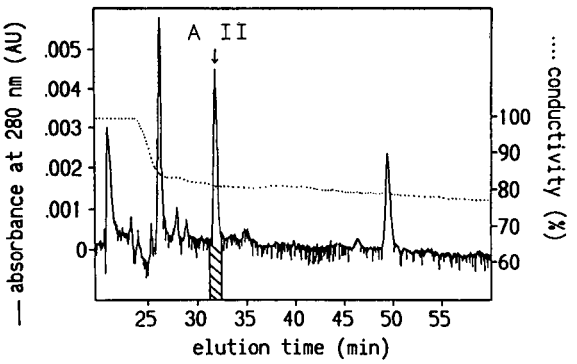

Fig. 8. Micropreparative RP-HPLC of the bioactive fraction of the cation-exchange chromatography (Fig. 6). Absorbance range, 0.1. Conditions: column, C_{18} GROM-SIL 300-ODS-2 (100 × 2 mm I.D.); eluent A, 0.1% TFA in water; eluent B, 0.1% TFA in acetonitrile; gradient, 0–14 min 100% eluent A, 14–20 min 0–22% eluent B, 20–70 min 22–27% eluent B; flow-rate, 100 μl/min; sample, bioactive fraction from the semi-preparative chromatography (Fig. 6; 300 ml plasma equivalent) dissolved in 1 ml of eluent A; fraction size, 200 μl. The vasoactive fraction, equivalent to 1 ml of plasma, caused an elevation of perfusion pressure of 6 mmHg (hatched area) in the bioassay. AII indicates the retention time of angiotensin II.

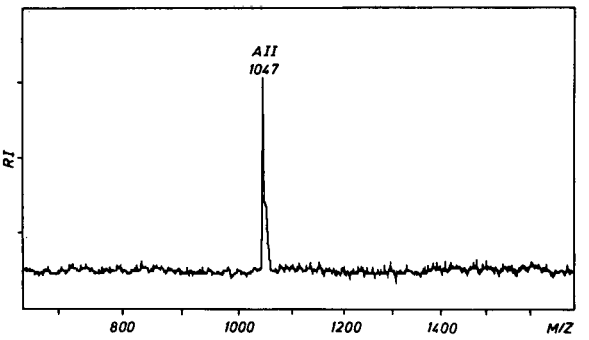

Fig. 9. MALDI mass spectrum of the bioactive fraction obtained after RP-HPLC (Fig. 8). AII indicates a signal of a protonated ion which is identical with the mass of the protonated ion of angiotensin II.

perfused rat kidney before and after incubating the kidney with the angiotensin receptor antagonist saralasin. The action of the fraction was completely blocked by saralasin (Fig. 3).

Determination of the recovery of angiotensin II liberated by the proteolysis

Table II shows that the amount of angiotensin II produced under the experimental conditions described above is about 1000 times higher than the amount of free angiotensin II in plasma.

DISCUSSION

Cross-flow filtration is not very commonly used as an alternative to dialysis or pressure ultrafiltration. In the literature on protein purification [2,17–20] cross-flow filtration has received little interest compared with dialysis, pressure ultrafiltration and ultrafiltration by centrifugation. One of the reasons may be that it was originally designed for biotechnological filtration and therefore it is felt to be too crude to handle small-scale amounts. Our results show that cross-flow filtration adjusted to laboratory-scale volumes has several advantages. First, the major benefit of cross-flow filtration is that even crude liquids can be filtered, such as plasma with submandibular gland extract, because the construction of the cross-flow filtration prevents membrane clogging [21]. Second, cross-flow filtration compared with dialysis shows better results in retaining molecules larger than the labelled retention limit

TABLE II

AMOUNTS OF ANGIOTENSIN II IN PLASMA, ANGIOTENSINOGEN AND THE BIOACTIVE FRACTION OF THE SEMI-PREPARATIVE RP-HPLC (FIG. 4)

Angiotensin II	Concentration
Free angiotensin II in plasma [15]	4–26 pg/ml
Total angiotensin II released from angiotensinogen [16]	10–55 ng/ml
Angiotensin II released by incubation of plasma with submandibular gland extract[a]	25 ± 5 ng/ml

[a] Mean ± S.D. (n = 5) calculated from the dose–response curve (Fig. 2).

(Fig. 1). Third, as one or more pretreatment steps may become unnecessary, the recovery rate should be higher. Fourth, cross-flow filtration is ideal prior to chromatography, because the filtrate can be easily chromatographed and the column following the filtration step is protected, because the filtration removes particles. Fifth, cross-flow filtration is not only useful for the separation of peptides, or other biologically molecules, from the reaction mixture but also allows the reaction to be controlled. In contrast to the conventional dialysis technique or pressure ultrafiltration, the retentate and filtrate can be monitored (*i.e.*, pH) and manipulated continuously and independently of each other during the reaction. For example, as was shown above, the retentate and filtrate can be kept at different temperatures.

Following the cross-flow filtration step the peptides had to be concentrated from a large volume of buffer (1 l). For this RP-SPE, which had been shown by many investigators to be a fast and reproducible method with high recovery rates [17,22–24], was chosen and modified to fit our needs. RP-SPE was not performed on ready-to-use cartridges but the RP material was packed into silanized glass tubes with PTFE frits. This procedure has the advantage that several grams of sorbent can be packed in one column. Also, the elution of interfering materials from the frits and polymer column material is significantly reduced [25]. This is important in bioassays and in structural investigations of the bioactive fractions.

Next, the biologically active peptides, usually present in trace amounts, had to be isolated from a complex substance mixture. Thus, specific and sensitive detection methods and an optimum purification procedure were required.

The requirement for specific detection was accomplished with the bioassay. The detection limit of this bioassay is in the range 0.1–1 ng of angiotensin II (total amount injected into the kidney perfusion system, Fig. 2). The control of the purity of the fractions and the identification of the vasoactive peptide were possible with the help of the sensitive method of MALDI-MS [14] (Figs. 5, 7 and 9).

With regard to an optimum purification procedure, the benefits of the first two sample preparation steps, cross-flow filtration and RP-SPE, are mentioned above. After this, the peptide-rich fraction was further purified with three chromatographic steps, preparative RP-HPLC (Fig. 4), followed by microbore cation-exchange HPLC (Fig. 6) and finally by microbore RP-HPLC (Fig. 8), giving a homogeneous biologically active peptide fraction. The homogeneity of the purest fraction was confirmed by MALDI-MS, shown in Fig. 9. The particular benefit of the chromatographic steps is the use of micropreparative HPLC, which is optimized to handle low-μg–ng amounts.

The active peptide was clearly identified as angiotensin II. The identity was proved by MALDI-MS (Fig. 9), by comparison of the retention times of the active fraction and angiotensin II, which appeared to be very similar (Figs. 4, 6 and 8), and by specific pharmacological inhibition (Fig. 3). The action of the vasoactive fraction was blocked by the angiotensin II receptor antagonist saralasin.

The determination of the recovery of angiotensin II, liberated by proteolysis, demonstrates that after incubation of plasma with an extract, rich in proteases, bioactive peptides can be released. The total recovery of released angiotensin II can be roughly estimated to be more than 50% of total releasable angiotensin II.

The biochemical aspects of the results are due to the fact that the submandibular gland contains tonin [6] and several tonin-like serine proteases [7], which may be responsible for the liberation of angiotensin II. Tonin is known to cleave angiotensin II directly from angiotensinogen [5]. Further, it should be emphasized that the isolation and determination of angiotensin II was not the primary aim of the study, although the analyses finally revealed angiotensin II as the reaction product.

CONCLUSION

A method based on cross-flow filtration, which is suitable for the isolation of bioactive peptides released by proteases, has been developed. The method is attractive for isolating peptides released by proteases from any biological material. It should be possible to extend the use of the benefits of the cross-flow filtration not only to the study of proteolysis and the reaction products but also to other applications dealing with reactions of biological systems.

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CHROMSYMP. 2579

Adenylate kinase from plant tissues

Influence of ribonuclease on binding properties on Mono Q

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ABSTRACT

Adenylate kinases modulate the three adenine nucleotide pools and were found to be localized as isoenzymes in different tissues and organelles in animals and plants. For investigations of adenylate kinase isoenzymes from plant tissues different plant extracts were examined by anion-exchange chromatography. During investigations with the strong anion exchanger Mono Q, adenylate kinase activity eluted in the void volume. This void volume activity did not always occur, but depended on the age of the plants and light treatment. The nature of the factors affecting void volume activity could only be partially resolved. It could be shown that RNase treatment at the beginning of extraction led to the disappearance of void volume activity, whereas an untreated extract still showed this activity.

INTRODUCTION

Adenylate kinases (ATP:AMP phosphotransferase, E.C. 2.7.4.3) are globular, low-molecular-mass proteins with the following catalytic activity:

$$Mg \cdot ATP + AMP \rightleftharpoons Mg \cdot ADP + ADP$$

Their small size and their ubiquitous presence in all living systems made them a tool in clinical chemistry for recognizing diseases (*e.g.*, [1–3]). When Fildes and Harris (1966) [4] first showed the occurrence of specific patterns of adenylate kinase isoenzymes in human erythrocytes, investigations in this field advanced rapidly.

Because of their catalytic capability and their distribution among different tissues and organelles, the enzymes seem to play an important role in regulating energy metabolism [1,5–10]. In 1968, Atkinson [11] and Bomsel and Pradet [12] presented their hypothesis of energy charge control of metabolism by adenine nucleotides. Bomsel and Pradet showed that adenylate kinase regulates the equilibrium between adenine nucleotide pools and that the energy charge does not change even under extreme conditions. Adenylate kinase might be of great importance in regulating coupling in oxidative phosphorylation [13].

In C_3-plants, adenylate kinase levels are low in comparison with C_4-plants [14–16]. An increase in adenylate kinase activity on greening in C_4-plants and higher contents in these plants are taken as an indication of its importance in regenerating the photosynthetic primary CO_2 acceptor phosphoenolpyruvate [14–19].

In higher plants, five different isoenzymes are localized in different organelles. Most activity is localized in chloroplasts and only a small portion is cytoplasmatic or found in the nucleus and mitochondria [7,8,20,21]. Two enzymes could be isolated from chloroplasts. The major activity in these orga-

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nelles results from a soluble form [22] while a small portion is bound to the envelope [23]. These two isoforms might be involved in the control of adenylate pools and adenylate translocation in plastids.

In developing seedlings of *Chenopodium rubrum*, the adenylate kinase capacity shows circadian rhythmicity and increases under phytochrome control [5,6,24]. In addition, isoenzyme patterns found after ion-exchange chromatography on DEAE-cellulose could be modulated by changes in light and temperature treatments or glucose feeding. Imposition of flower-inducing conditions led to a significant increase in mitochondrial adenylate kinase capacity, whereas the chloroplastic enzyme capacity decreased. Glucose feeding experiments led to characteristic changes in isoenzyme patterns, indicating a glucose concentration-dependent modulation of cytoplasmatic, mitochondrial and chloroplastic forms. Therefore, adenylate kinase might be involved in fine control of energy transduction [7,8].

Whereas amino acid sequences, gene loci and conformations of the enzymes from different animal and human tissues, bacteria and yeast are known (*e.g.*, [25–27]), information about the enzymes from plants is scarce.

The aim of these investigations was to purify adenylate kinase from leaves of *Chenopodium rubrum* and to separate isoenzymes from different organelles with a new and efficient chromatographic procedure [fast protein liquid chromatographic (FPLC) system].

EXPERIMENTAL

Growing conditions

Seedlings of *Chenopodium rubrum* L. (ecotype 184) were grown in moist vermiculite in small pots in a transparent container closed with a transparent lid and transferred to a growth chamber with continuous white light (270 μmol m^{-2} s^{-1}) and changing temperature conditions (12 h/12 h: 305.5 K/283 K) for 4 days and thereafter to constant conditions (297 K, white light, 70% relative humidity). After transfer to constant conditions, the plants were supplied with 40% Hoagland's nutrient solution [28]. For synchronization, plants were transferred after 20 days to a box with constant temperature (297 K) and alternating light conditions (12h/12h : dark/white light). After 4 days of synchronization the plants were kept in complete darkness for 2 days or under continuous white light for 3 days. Leaves were harvested from the second and third internodes.

Chemicals

AMP, ATP, NADH, PEP, LDH, PK and pancreatic RNase (DNase-free) were obtained from Boehringer (Mannheim, Germany), ethanolamine from Serva (Heidelberg, Germany) and 2-amino-2-methyl-1-propanol from Sigma (Deisenhofen, Germany). All other chemicals were obtained from Roth (Karlsruhe, Germany). Ready-to-use Coomassie Protein Assay Reagent was purchased from Pierce (Oud-Beijerland, Netherlands).

Instrumentation

A modular FPLC system was used, consisting of a liquid chromatographic controller (LCC-500), two pumps (P-500), four motor-driven valves (MV-8) and one motor-driven injection valve (MV-7). Absorbance at 280 nm was monitored with a UV spectrophotometer and a 10-μl flow cell. Further instruments were a mixing chamber (24 V), a fraction collector (FRAC-100), a peristaltic pump (P-1) and a superloop (50 ml) for application of large sample volumes. Small volumes were injected with a syringe by sample loops of different sizes. All instruments were obtained from Pharmacia Biosystems (Freiburg, Germany).

Chromatography

For subsequent anion-exchange chromatography a prepacked Mono Q HR 5/5 column (1 ml) was used. For prepurification a column with DEAE-Sepharose CL-6B (8 ml) was prepared. For separation the following two buffer systems were used: (I) A = 20 m*M* ethanolamine–HCl (pH 9.5)–14 m*M* 2-mercaptoethanol, B = 20 m*M* ethanolamine–HCl (pH 9.5)–1 *M* NaCl–14 m*M* 2-mercaptoethanol; and (II) A = 40 m*M* 2-amino-2-methyl-1-propanol–HCl (pH 10.25)–14 m*M* 2-mercaptoethanol, B = 40 m*M* 2-amino-2-methyl-1-propanol–HCl (pH 10.25)–1 *M* NaCl–14 m*M* 2-mercaptoethanol. All buffers for FPLC were degassed prior to use. Membranes for degassing and filtration (Millex GV, Millex GVWP) were purchased from Millipore (Eschborn, Germany). Mono Q HR 5/5, DEAE Sepharose CL-6B, Sephadex and FPLC are registered

trade-marks of Pharmacia Biosystems (Freiburg, Germany).

Extraction procedure

All preparations took place in a cold room or on ice. The centrifuge was cooled to 275 K. The extraction medium contained 250 m*M* Tris–HCl (pH 7.4), 8 m*M* $MgSO_4$, 5 m*M* EDTA and 14 m*M* 2-mercaptoethanol. Adenylate kinase was prepared according to the following procedure: 10 g of plant material were harvested and frozen with liquid nitrogen. The frozen material was transferred into PTFE cells each with a tungsten carbide ball at liquid nitrogen temperature. The material was shaken in a microdismembrator (Braun, Melsungen, Germany) for 60 s. The frozen powder was transferred into a beaker with extraction medium (1 g fresh weight per 5 ml of medium) and stirred until the solution became homogeneous. This crude extract was centrifuged for 20 min at 39 000 *g*. Solid ammonium sulphate was added to the supernatant to reach a final concentration of 75% (w/v). After 45 min of stirring the suspension was centrifuged again at 39 000 *g* for 10 min. The resulting pellet was resuspended in 10 ml of 20 m*M* ethanolamine–HCl (pH 9.5)–14 m*M* mercaptoethanol, stirred for 45 min and again centrifuged at 39 000 *g* for 10 min. The remaining ammonium sulphate was removed from the supernatant by Sephadex G-25 columns. These columns (16-ml bed volume) were especially equilibrated with the appropriate elution buffer. Between 4 and 5 ml of supernatant were applied to one of these columns and the filtrate from this step was applied to another column. The extract obtained by this procedure was filtered through a membrane of 0.22 μm pore size and thereafter applied (8–9 ml) to a column with DEAE Sepharose CL-6B for prepurification. After application of the extract to the column proteins were eluted with 30 ml of 20 m*M* ethanolamine–HCl (pH 9.5)–1 *M* NaCl–14 m*M* 2-mercaptoethanol. All combined fractions from this chromatographic step resulted in a clear protein solution without chlorophylls and phenols. Solid ammonium sulphate (75%, w/v) was added and all steps for concentrating and desalting were repeated as described above using 10 ml of the appropriate elution buffer for resuspension of precipitated pellets. The extract was filtered (0.22-μm pore size) and applied to Mono Q.

RNase treatment

In RNase-treatment the first supernatant was divided into two aliquots. To one aliquot 2 μg/ml of pancreatic RNase (DNase-free) were added while the other half was not treated. The extracts were left in the cold overnight in extraction buffer at pH 7.4. The next step was the precipitation of extract with ammonium sulphate and all other steps were as described under *Extraction procedure* using 10 ml of 40 m*M* 2-amino-2-methyl-1-propanol–HCl (pH 10.25)–14 m*M* 2-mercaptoethanol as resuspension buffer for the second precipitation step.

Adenylate kinase assay

Adenylate kinase activity was determined in the backward reaction by the formation of Mg · ADP and ADP. The reaction sequence is described elsewhere [3]. Under the chosen conditions, activity is monitored by measuring the decrease in NADH at 366 nm.

A 1-ml volume of assay mixture contained 75 m*M* Tris (pH 7.4 with HCl), 3 m*M* $MgSO_4$, 7.5 m*M* KCl, 1.8 m*M* ATP, 1.6 m*M* PEP, 2 m*M* AMP, 0.35 m*M* NADH, 260 nkat LDH and 50 nkat PK. Reactions were initiated by various amounts of sample and run at 25°C. Because of enzymes starting the reaction by bypassing adenylate kinase, all activities were tested in a double-beam spectrophotometer having a reference without AMP. Volume-dependent enzyme activities (A_v) were calculated from changes in absorbance per second.

Results for adenylate kinase activity profiles are representative for at least three independent experiments.

Determination of protein

Protein was determined according to Bradford [29] using ready-to-use Coomassie Protein Assay Reagent.

RESULTS

Anion-exchange chromatography

Prepurification on DEAE-Sepharose CL-6B with subsequent precipitation leads to clear protein extracts without chlorophylls and phenols. When leaves were harvested 2 days after transfer to continuous darkness, application of prepurified extracts to Mono Q led to elution of most activity in the void volume (Fig. 1).

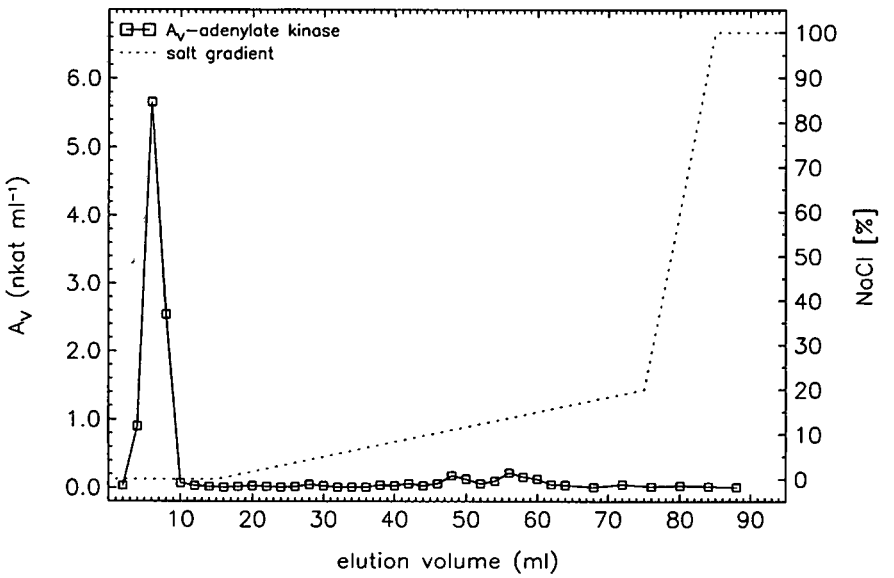

Fig. 1. Adenylate kinase activity profile on Mono Q. Leaves were harvested 2 days after transfer to continuous darkness. Buffer: A = 20 m*M* ethanolamine–HCl (pH 9.5)–14 m*M* 2-mercaptoethanol; B = 1 *M* NaCl in A. Flow-rate, 1 ml min^{-1}; protein applied, 3.7 mg; activity applied, 22 nkat; recovery, 22 nkat (100%).

For Mono Q HR 5/5 a charge of 20 mg of protein is considered to be the upper limit for the capacity of the column. To avoid overloading of the column only small amounts of protein (3–4 mg) were applied.

Collection of void volume activity and reapplication to Mono Q led to binding of *ca.* 90–100% of total activity (Fig. 2) when the column was previously washed with 1 *M* NaCl. Five activity peaks could be detected after elution with a salt gradient.

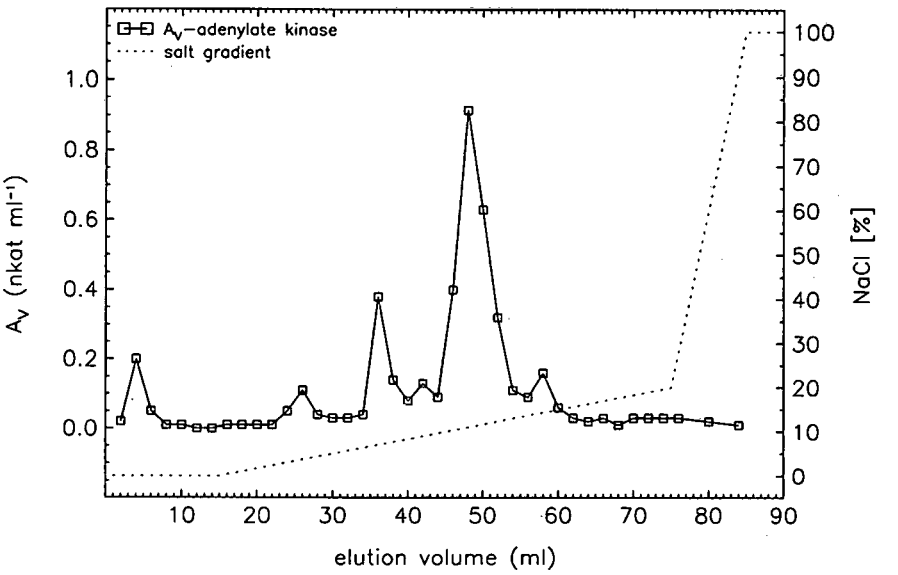

Fig. 2. Rechromatography of void volume activity. Buffer: A = 20 m*M* ethanolamine–HCl (pH 9.5)–14 m*M* 2-mercaptoethanol; B = 1 *M* NaCl in A. Flow-rate, 1 ml min^{-1}; activity applied, 10 nkat; recovery 9 nkat (90%).

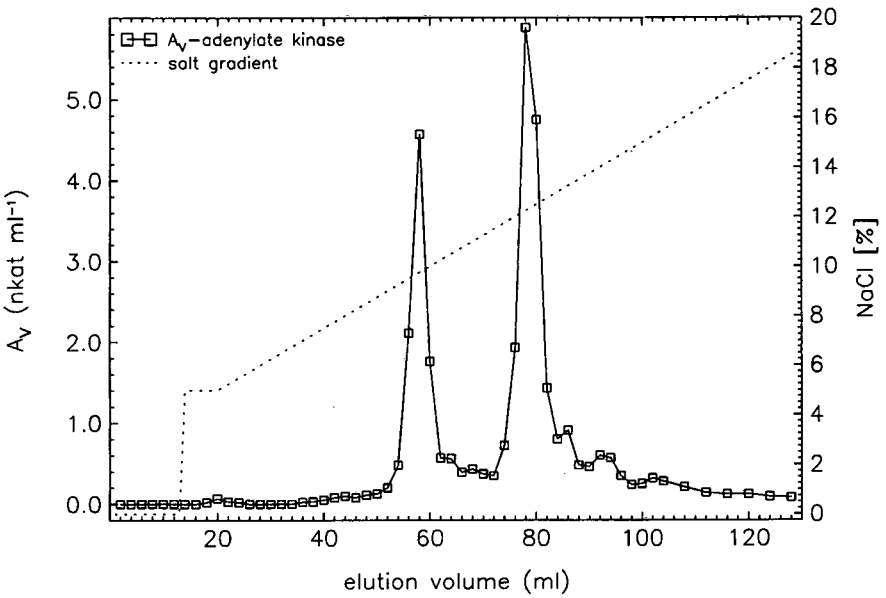

Fig. 3. Anion-exchange chromatography on Mono Q. Plants were harvested after continuous illumination for 3 days. The amount of protein applied was at capacity limit of Mono Q. Buffer: A = 20 m*M* ethanolamine–HCl (pH 9.5)–14 m*M* 2-mercaptoethanol; B = 1 *M* NaCl in A. Flow-rate, 1 ml min^{-1}; protein applied, 19.8 mg; activity applied, 72 nkat; recovery, 66 nkat (92%).

When leaves were harvested from plants that were kept in continuous white light for 3 days after synchronization, application of the prepurified extract to Mono Q led to no void volume acitivity. To probe the capacity of Mono Q for adenylate kinase, 72 nkat were applied to Mono Q. This activity corresponded to nearly 20 mg of protein, being the limit for Mono Q HR 5/5. Application of this activity and amount of protein resulted in complete binding and five activity peaks could be detected during elution with salt (Fig. 3). This result showed that overloading of Mono Q could be excluded as a reason for the appearance of void volume acitivity.

Anion-exchange chromatography: influence of RNase on binding of adenylate kinase to Mono Q

To elucidate the reason for the occurrence of void volume activity, various enzymes were added to crude extracts in order to separate adenylate kinases from lipids, DNA or sugars. Lipase, DNase, *endo*-β-N-acetylglucosaminidase F and peptide-N-glycohydrolase F were not able to remove void volume activity (data not shown). In contrast, the addition of pancreatic RNase (DNase-free) to half of the crude extract and resuspension of precipitated prepurified extract in 40 m*M* 2-amino-2-methyl-1-propanol–HCl–14 m*M* 2-mercaptoethanol led to binding of all adenylate kinase activity to Mono Q (Fig. 4, I) whereas the untreated half showed void volume activity of about 50% (Fig. 4, II). After RNase treatment, four clear activity peaks could be detected (Fig. 4, I) whereas no clear peaks of activity at the appropriate elution volumes appeared without RNase (Fig. 4, II). RNase not only prevents void volume activity but also seems to sharpen activity peaks.

DISCUSSION

Adenylate kinases have been purified from different sources (*e.g.*, [1,9,15,25,30]) and their distribution as isoenzymes in different organelles and tissues has been shown (*e.g.*, [1,4,7,8,10,31,32]). During our investigations on the distribution of adenylate kinases in plant tissues from different sources with the strong anion exchanger Mono Q, it was found that most adenylate kinase activity eluted in the void volume. The occurrence of this void volume activity depended on the age of the plants and light treatment.

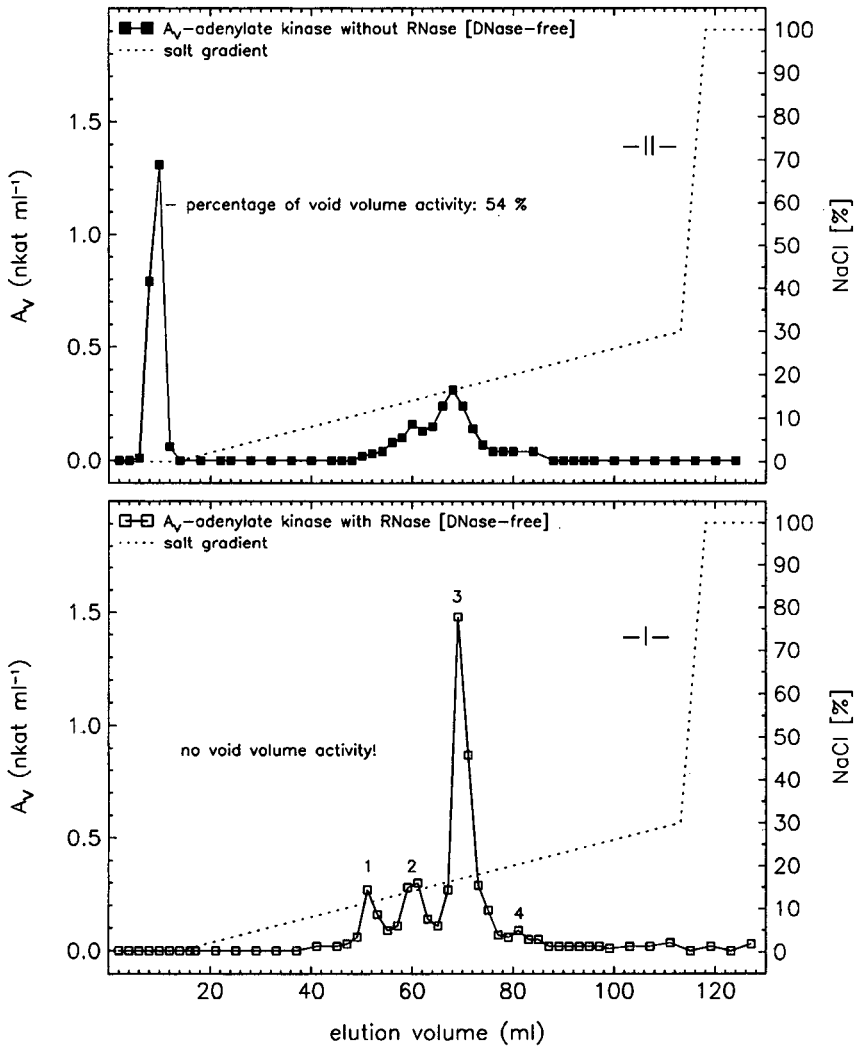

Fig. 4. Anion-exchange chromatograms of adenylate kinase activity on Mono Q. (I) With RNase (DNase-free). Protein applied, 3.2 mg; activity applied, 10 nkat; recovery, 10 nkat (100%). (II) Without RNase (DNase-free). Protein applied, 3.4 mg; activity applied, 8.4 nkat; recovery, 8 nkat (95%). Buffer: A = 40 m*M* 2-amino-2-methyl-1-propanol–HCl (pH 10.25)–14 m*M* 2-mercaptoethanol; B = 1 *M* NaCl in A. Flow-rate, 1 ml min^{-1}.

De Looze and Wagner [33–35] were able to show that chloroplast glyceraldehyde-3-phosphate dehydrogenase isoenzyme patterns could be modulated by the addition of pyridine nucleotides at the time of extraction. Moreover, aggregated ($\geqslant 10^6$) and disaggregated ($165 \cdot 10^3$) molecular mass forms were obtained in the presence of NAD^+ and $NADP^+$, respectively. The aggregation phenomenon could be prevented by RNase treatment. Thus, the occurrence of adenylate kinase activity in the void volume might also have its reason in aggregation of isoenzymes mediated by small molecules. Rechromatography of void volume activity showed that it is composed of all adenylate kinase activities. Gel filtration of different materials showed adenylate kinase activity eluting in a range between 25 and 35 kilodalton, thus excluding covalent aggregation of adenylate kinases (data not shown).

Another explanation for void volume activity could be that the factor responsible for void volume activity might block binding sites on Mono Q, thus leading to void volume activity. If this is the case, it is not a specific phenomenon for adenylate kinase but might concern other proteins also. This can be clarified by examination of other enzymes.

Nevertheless, the influence of RNase treatment on the binding of adenylate kinase activity to Mono Q may suggest that ribonucleoprotein interactions could be involved in the occurrence of void volume activity. As no leader sequences have been characterized for adenylate kinases, the question arises of how proteins are directed to different organelles. Therefore, post-translational modifications might be responsible for protein targeting. According to the signal hypothesis [36] for translocation of proteins across membranes, ribonucleoprotein interactions might play a role in light-regulated targeting [37].

The question of why the observed phenomenon occurred only with Mono Q and not with other chromatographic material might be explained by the pore size of these materials. Mono beads have a pore size of about 5 nm, so that larger or modified molecules are not able to enter the pores. The nature of the factors affecting void volume activity and dependence on light treatment is the aim of further investigations.

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CHROMSYMP. 2577

Immobilized metal affinity chromatography for the separation of photosystems I and II from the thermophilic cyanobacterium *Synechococcus elongatus*

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ABSTRACT

Immobilized metal affinity chromatography (IMAC) of solubilized, photosystem II (PS II) enriched particles from the thermophilic cyanobacterium *Synechococcus elongatus* was studied. A chelating Sepharose Fast Flow column was charged with various metal ions (Mn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Co^{2+}, Ca^{2+}, Sr^{2+}, Zn^{2+} and Cu^{2+}) and their affinity to photosystem I (PS I) and PS II was examined. Among all the metal ions tested, only copper was able to bind the two protein complexes. For elution of the column, a pH gradient, a pH step gradient and gradients of imidazole, amino acids, organic acids and various other eluents were tested; only the pH step gradient, which selectively eluted PS II at a pH between 6 and 5, was useful for the separation of PS I and PS II. All other gradients proved to be inappropriate for the separation of these two photosystems. Mechanisms of protein elution by these compounds are discussed. Alternatively, a separation of PS I and PS II at pH 7.5 could be achieved when an IMAC column was used on which the free coordination positions of the bound copper ions were occupied by imidazole. When solubilized photosystems were loaded on to this column, PS I replaced imidazole and remained bound on the column, whereas PS II was highly enriched in the effluent.

INTRODUCTION

Cyanobacteria contain, similarly to higher plants, two photochemical reaction centres [1]. They are localized in the thylakoid membranes and enable the cyanobacteria to evolve oxygen by splitting water and to reduce $NADP^+$. Water is split in the so-called photosystem II (PS II) and $NADP^+$ is the terminal electron acceptor of photosystem I (PS I). Both photosystems are multi-protein complexes that consist of various amounts of proteins and pigments depending on their state of purification.

In thylakoid membranes (TM), PS II of cyanobacteria consists of at least six subunits: the reaction centre protein D1 with an apparent molecular mass (M_r) of 31 000, the reaction centre protein D2 with an apparent M_r of 32 000, the M_r 47 000 chlorophyll (Chl) protein (CP 47), the M_r 43 000 chlorophyll protein (CP 43), the M_r 33 000 water splitting protein and cytochrome *b* 559 with M_r *ca.* 13 000. These proteins are encoded by the psbA, psbD, psbB, psbC, nuclear psb1, psbE and psbF, respectively [2].

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The primary charge separation takes place in the reaction centre proteins D1 and D2, which contain the primary electron donor P 680, two pheophytin molecules, two molecules of chlorophyll, two quinones and one non-haeme iron [2].

Photosystem I consists of two reaction centre proteins with M_r *ca.* 83 000 [3], containing the primary electron donor P 700, the primary acceptor A_0 (chlorophyll *a*), the electron acceptor A_1 (phylloquinone) and the iron–sulphur centre F_x. These proteins are encoded by the genes psaA and psaB. The reaction centre proteins also contain about 60 molecules of chlorophyll *a* and ten carotinoid molecules [4]. In addition to these two polypeptides, PS I consists of at least two other polypeptides: an M_r 10 000 protein (psaC gene product), containing the iron–sulphur centres F_a and F_b, and an M_r 15 000 polypeptide. Both are localized on the stromal side [5].

In the last few years, many different purification procedures for PS I and PS II complexes from thylakoid membranes of various organisms have been developed [6–8]. Most of these preparations include a selective extraction of the photosynthetic complexes from the membranes by the use of detergents. For the solubilization of the membranes the nature of the detergent (ionic or non-ionic), the ratio of detergent to chlorophyll (Chl) and other parameters such as pH and concentration of salts are of importance. Usually these preparations yield complexes which are pure for most purposes. Sometimes, however, it is necessary to eliminate even small amounts of impurities, especially when kinetic studies are done or the crystallization of the protein is attempted.

In 1984, Schatz and Witt [9] introduced a method for the selective extraction of PS II from the thermophilic cyanobacterium *Synechococcus* sp. by the use of the zwitterionic detergent SB 12. This purification was further improved by Rögner *et al.* [10] using density gradient centrifugation for the elimination of remaining impurities.

We applied a similar method to the thermophilic cyanobacterium *Synechococcus elongatus* and found a small amount of PS I still present even after density gradient centrifugation. A further purification by either anion-exchange chromatography (Q-Sepharose) or gel filtration (Superose 6 B) (data not shown) did not result in a satisfactory separation of PS I and PS II. We only succeeded in eliminating this contamination by immobilized metal affinity chromatography (IMAC) on a Chelating Sepharose column using Cu^{2+} as the immobilized metal ion.

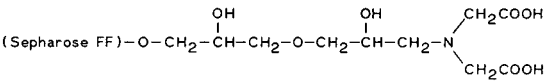

Fig. 1. Partial structure of Chelating Sepharose Fast Flow stationary phase. Assuming an octahedral coordination of the metal ion, iminodiacetic acid can only saturate three coordination positions, while the remaining three positions are free for interaction with proteins [12].

Chelating Sepharose (see Fig. 1) consists of iminodiacetic acid groups on spacers, coupled to a highly cross-linked agarose matrix by stable ether linkages [11].

Immobilized metal affinity chromatography [13] offers a new approach for the purification of PS I and PS II because the binding of metal ions is a specific property of a protein and differences in the binding of these metal ions may be used for the separation of these proteins. In addition, it is possible to alter parametes such as adsorption and desorption conditions and salt concentrations, which may determine the purity and stability of the sample.

EXPERIMENTAL

Prepartion of PS II particles

The thermophilic cyanobacterium *Synechococcus elongatus* strain KOVROV 1972/8 was grown in an inorganic medium according to Castenholz [14], supplemented with 10 m*M* $NaHCO_3$, at 57°C in a chemostat culture aerated with 2% (v/v) CO_2. The irradiation regime was chosen so as to favour a high ratio of PS II to PS I. For this purpose the cyanobacteria were grown in the tubular culture unit at a dilution rate of $D = 0.1\ h^{-1}$. The cells were irradiated at 50 W m^{-2} for several hours. Then a dark period of 12 h was inserted and the flow was stopped during that time. After this period, the cells were irradiated at 200 W m^{-2} for 6 h at the same dilution rate. This resulted in an increase in the PS II/PS I ratio from 1:2 at an irradiation of 50 W m^{-2} to *ca.* 2.5:1 when the cells were grown under the conditions mentioned above.

For the preparation of the thylakoid membranes (TM), the method of Schatz and Witt [9] was slightly modified. The cells were harvested at a density of 10^8 cm^{-3} by centrifugation, washed with buffer A [30 m*M* K_2HPO_4–5 m*M* $MgCl_2$–1 m*M* ε-aminocaproic acid (pH 7.5)] and treated with 0.3% lysozyme in buffer B [30 m*M* K_2HPO_4–500 m*M* mannitol–5 m*M* $MgCl_2$–35 m*M* NaCl–1 m*M* $CaCl_2$–1 m*M* ε-aminocaproic acid (pH 6.5)] at 47°C for 1 h in the dark with shaking. Lysozyme was removed by a centrifugation step at 6000 *g*. The spheroplasts in the pellet were disrupted by osmotic shock with buffer A and washed twice with the same buffer to remove phycobilisomes (PBS). Thylakoid membranes were collected by centrifugation at 24 000 *g* for 30 min at 2°C. They were resuspended in buffer B (pH 6.5), stirred for 15 min and centrifuged at 6000 *g* to remove whole cells. The supernatant was stored in the presence of 20% glycerol in liquid nitrogen. The activity of TM was determined to be 180 μmol O_2 (mg Chl)$^{-1}$ h^{-1} at 40°C with benzoquinone (BQ) as an electron acceptor.

For the preparation of the PS II particles, the TM were diluted with buffer B (pH 6.5) to a concentration of 1 mg Chl ml^{-1} and stirred with the detergent sulphobetaine 12 (SB 12; N-dodecyl-N,N-dimethyl-3-amino-1-propanesulphonate) at a ratio of detergent to chlorophyll of 3.3:1 (w/w) at room temperature for 30 min in the dark. Then the sample was centrifuged at 160 000 *g* for 2 h at 2°C. PS II particles were solubilized in the superatant, while the sedimented membrane fragments were enriched in PS I. This supernatant (further designated as PS II particles) was stored in the presence of 20% glycerol in liquid nitrogen. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that this PS II preparation still contained a large amount of low-molecular-mass proteins, free PBS and a small amount of PS I.

Purification of PS II particles by sucrose density gradient centrifugation according to Rögner et al. [10]

PS II particles prepared as described above were layered on to a six-step sucrose density gradient (20–45%) and centrifuged at 160 000 *g* for 16 h at 2°C. We obtained six bands which differed in absorption spectra, photochemical activity (Hill reaction) and content of protein. The highest oxygen evolution of 230 μmol O_2 (mg Chl)$^{-1}$ h^{-1}, measured polarographically using a Clark-type electrode in the presence of 0.5 m*M* *p*-benzoquinone and 1 m*M* hexacyanoferrate(III) was observed in fraction 5 (designated as purified PS II particles), which corresponds to a sucrose concentration of 35%. The absorption maximum of this fraction in the red region was at 673 nm, and SDS-PAGE

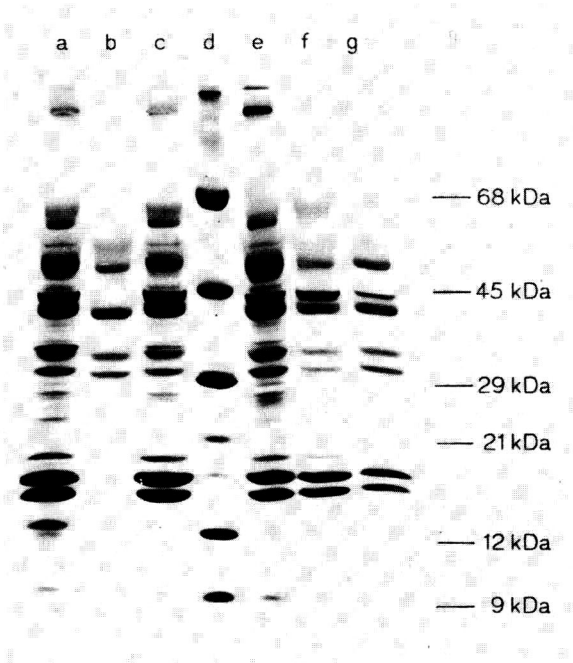

Fig. 2. SDS-PAGE for the purification of PS I and PS II from purified PS II particles (see Experimental). The bands at about M_r 110 000 and at M_r 80 000 belong to the PS I complex, the bands at M_r 47 000, 43 000, 33 000, 31 000 and 9000 belong to the PS II complex. Phycobilisomes have an apparent M_r of 15 000–18 000. No other bands will be considered here. Lanes a–c show the purification of the two photosystems with the pH step gradient. Lane a shows the purified PS II particles (see Experimental), lane b shows the fraction that was obtained by washing the column with buffer of pH 5, lane c shows the proteins which remained bound after the pH 5 washing and could be eluted with a buffer at pH 4, lane d contains marker proteins at M_r 68 000, 45 000, 29 000, 21 000, 12 000 and 9000 and lanes e–g show the separation of PS I and PS II by means of an imidazole-pre-charged column. In lane e the fraction is shown which bound to the first imidazole-pre-charged column (fraction G_1). It is obvious that both PS I and PS II were bound. Lane f shows the fraction which was bound to the second imidazole-pre-charged column (fraction G_2) and lane g shows the effluent of the second imidazole-pre-charged column (fraction E_2). It is clearly visible that the content of PS I in the effluent is drastically decreased in comparison with the purified PS II particles.

showed, in addition to the proteins of the PS II complex, the presence of PBS proteins and some PS I (see Fig. 2, lane a).

Immobilized metal affinity chromatography

For IMAC, Chelating Sepharose Fast Flow was packed in an HR 10/10 column (Pharmacia–LKB, Uppsala, Sweden). The bed height was 8 cm and the diameter was 10 mm. Then the Chelating Sepharose was loaded with a solution of a metal salt in distilled water at a concentration of 1 mg/ml and washed with three column volumes of distilled water [13] and three column volumes of buffer C [25 m*M* 2-(N-morpholino)ethanesulphonic acid (MES) (pH 7.5)–500 m*M* NaCl–0.02% β-D-dodecyl maltoside (DM)] at a flow-rate of 0.5 ml min^{-1}.

Then 8 ml of a solution of PS II particles or purified PS II particles (prepared as described above) at a concentration of 0.5 mg ml^{-1} in buffer B were applied to the column at 4°C and a flow-rate of 0.5 ml min^{-1}. We found that both PS I and PS II were bound to the column under these conditions.

For elution of the protein complexes, either a pH gradient, a pH step gradient or a gradient of eluents (imidazole, amino acids, organic acids or various other substances) were used.

The pH gradient was run by applying to the column a linear binary gradient (five column volumes) consisting of buffer C and a buffer of pH 4 containing 50 m*M* sodium acetate, 500 m*M* NaCl and 0.02% DM at a flow-rate of 0.7 ml min^{-1}. The pH gradient was controlled by measuring the pH of the fractions with a pH electrode (Ingold Messtechnik, Steinbach/Ts., Germany).

The pH step gradient was run by applying to the column buffers containing 50 m*M* sodium acetate, 500 m*M* NaCl and 0.02% DM, which were adjusted to pH 6, 5 and 4, respectively. The flow-rate was 0.7 ml min^{-1}. To perform the gradient of eluents, a linear gradient consisting of buffer C and this buffer containing the respective eluent was applied to the column. The gradient was run for five column volumes at a flow-rate of 0.7 ml min^{-1}. First gradients were run with a maximum concentration of eluent of 15 m*M*. If the protein did not elute at that concentration, this gradient was followed up by another gradient containing the concentration of eluent given in Tables II–IV.

Samples were concentrated with an ultrafiltration cell (Amicon, Witten, Germany) equipped with a Diaflo YM-100 membrane and run at a nitrogen pressure of 1.0 bar.

The spectra of the samples were measured with a Lambda 17 UV–VIS spectrophotometer (Perkin Elmer, Überlingen, Germany).

SDS-PAGE was performed according to Laemmli [15] using gels with a continuous polyacrylamide gradient of 12–24%, containing urea at a concentration of 7 *M*. The sample buffer consisted of 0.13 *M* tris(hydroxymethyl)aminomethane (Tris) (pH 9)–5.0% (w/v) SDS–2.5% (w/v) dithiothreitol–4 *M* urea. The electrophoresis was run at 7 mA constant current for 18 h at 18°C.

All chemicals were of analytical-reagent grade from Merck (Darmstadt, Germany).

RESULTS

Metals

Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Sr^{2+}, Ca^{2+}, Cu^{2+} ions were bound to the Chelating Sepharose. The column was equilibrated with buffer C and then PS II particles were applied. It was found that PS I and PS II only bound to copper whereas the other metal ions showed no binding capacity for these two protein complexes.

Buffers

Only buffers that have no strong interaction with metal ions can be used. The best buffers in this respect are 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), piperazine-N,N′-bis(ethanesulphonic acid) (PIPES) and MES whereas, for example, N-tris(hydroxymethyl)methylglycine (Tricine) elutes copper from the column at concentrations below 30 m*M*. Tris does not elute copper from the column at concentrations comparable to that of Tricine but it interacts in some way with copper and so reduces or modifies significantly the binding of proteins to the column. Buffers containing Tris should only be used for proteins that have a high affinity for metal ions.

TABLE I

ELUTION DATA FOR IMIDAZOLE AND IMIDAZOLE ANALOGUES

The top row gives the concentration of eluent that is necessary for protein elution and the bottom row the maximum concentration of eluent tested. Mg–ATP probably does not elute because of steric effects.

	Eluent			
	Imidazole	4-Methylimidazole	Adenine	Mg–ATP
Concentration for protein elution (m*M*)	<5	<5	<5	>15
Concentration for Cu^{2+} elution (m*M*)	>15	>15	>10	>15

Elution

pH gradient

When a pH gradient (pH 7.5–4) was applied to the column (see Experimental) we obtained an elution diagram (not shown) that showed two distinct but overlapping bands. PS II was eluted in fractions with pH between 6 and 5, whereas PS I was enriched in the fractions below pH 5.

pH step gradient

A satisfactory separation of PS I and PS II could only be obtained when a step gradient was applied to the column. When the column was washed with acetate buffer (pH 5), pure PS II eluted (see Fig. 2, lane b). PS I, PBS proteins and a residue of PS II remained bound on the column (see Fig. 2, lane c). These proteins could be eluted when the column was washed with acetate buffer of pH 4. Although this method resulted in a good separation of PS II from PS I, we observed partial inactivation of PS II photochemical activities (since light-induced kinetic measurements at 820 nm did not show the characteristic microsecond donation of an electron from the secondary donor Z (Tyr-161 of the reaction centre protein D1) to the primary donor P 680 [16] (data not shown). For this reason we tested eluents that eluted the protein complexes at higher pH.

Imidazole and imidazole analogues

Imidazole and imidazole analogues as eluents. Imidazole is commonly used for protein elution in IMAC. The elution properties of imidazole and some imidazole analogues are given in Table I.

We found that imidazole eluted both PS I and PS II at any concentration below 5 m*M*. We obtained an elution diagram (see Fig. 3) that showed that PS I and PS II were eluted closely together. The separation could not be improved by applying a less steep gradient. Therefore, imidazole was not useful

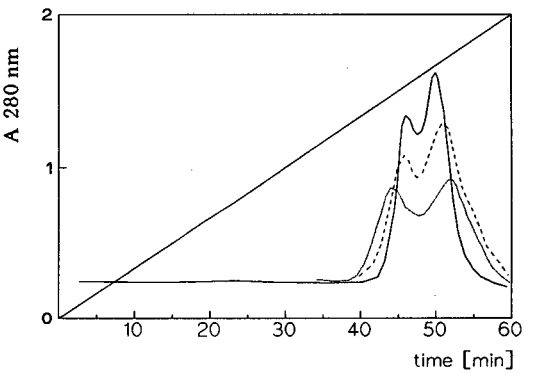

Fig. 3. Schematic elution diagrams of the elution of purified PS II particles (see Experimental) with gradients of imidazole (solid line) and amino acids (dashed line, group A; dotted line, group B). In order to be able to compare the elution profiles of the different eluents, the eluents and their concentrations are not given. The elution data are presented in Table I for the elution with imidazole and in Table III for the elution with amino acids. The amino acids listed in group A elute the protein complexes at concentrations below 15 m*M* and those amino acids listed in group B elute the protein complexes at concentrations below 60 m*M*. These elution profiles demonstrate that the two peaks cannot be separated, so that both peaks contain PS I and PS II. PS II is enriched in the peak eluting earlier, whereas PS I is enriched in the peak eluting later in the gradient. It is obvious that the resolution is best in the gradients with the amino acids eluting at higher concentrations.

for the separation of these two protein complexes. Similar results were obtained with its analogues 4-methylimidazole and adenine, while the strongly reduced elution capability of Mg–ATP is probably due to steric effects.

Pre-charging the column with imidazole. Alternatively, we did not use imidazole as an eluent, but pre-charged the column with imidazole. In this experiment we washed the Cu^{2+}-loaded column with 15 m*M* imidazole in buffer C until the colour of the column changed from light to dark blue, indicating that all free binding sites of copper were occupied with imidazole. The column was then washed with three column volumes of buffer C in order to remove surplus imidazole. After sample application we washed the column again with three column volumes of buffer C. In contrast to the experiments with the non-pre-charged column, only a minor part of the protein complexes was bound to the column, while the major part passed through (further mentioned as fraction E_1). Together with fraction E_1 there also eluted some imidazole, which was released from the column when protein was bound. The bound material was eluted in a step gradient (three column volumes, 0.7 ml min^{-1}) with 15 m*M* imidazole in buffer C (further mentioned as fraction G_1). Then the column was equilibrated with three column volumes of buffer C.

SDS-PAGE (see Fig. 2, lanes e–g) showed that fraction G_1 consisted mainly of PS I and a small amount of PS II whereas fraction E_1 contained mainly PS II complexes. For further purification, fraction E_1 was concentrated in an Amicon cell equipped with a YM 100 membrane. The concentrated sample was diluted tenfold with buffer C and concentrated once again in order to remove imidazole. This is necessary because imidazole at higher concentrations prevents the protein complexes from binding to the column. (The more PS I is bound, the more imidazole is released. As a consequence, imidazole concentrates along the column until a concentration is reached where it acts as an eluent. Then the remainder of the protein that did not yet bind to the column is prevented from binding.) The concentrated fraction E_1 was then applied once more to the same column. This time almost all the PS I that did not bind to the column in the first run and some PS II were bound.

The bound material was eluted with 15 m*M* imidazole as described above (further mentioned as fraction G_2). Again most of the PS II did not bind and was found in the effluent (further mentioned as fraction E_2). The UV–VIS spectrum of the purified PS II particles (see Experimental) is given in Fig. 4a and the spectra of fractions G_1 and E_2 are shown in Fig. 4b.

This method removes PS I satisfactorily but it cannot remove PBS proteins from the sample (see Fig. 2, lane g).

In Fig. 2 (lanes e–g) an SDS gel is shown where the successive extraction of PS I from purified PS II particles is monitored. It can be seen that in the first run almost all PS I is bound. Later PS I and PS II are bound. This is probably due to the changing PS I/PS II ratio in the sample during the procedure. With regard to the results obtained by Porath and Olin [12], we assume that there is a competitive

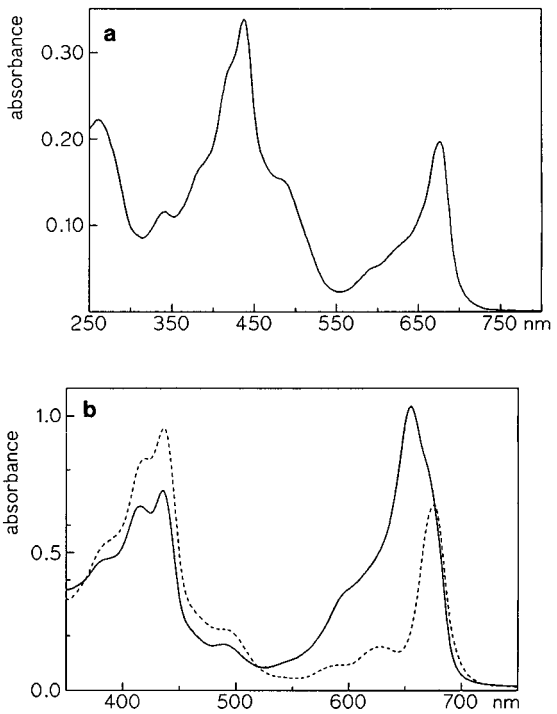

Fig. 4. Spectra from different states of purification. (a) Spectrum of the purified PS II particles derived from the sucrose density gradient centrifugation (see also Fig. 2, lane a). (b) Spectrum of the fraction that was bound to the first imidazole-pre-charged column, fraction G_1 (dashed line; see also Fig. 2, lane e), and that of the effluent, fraction E_2 (solid line; see also Fig. 2, lane g). The strong absorption of the effluent at about 654 nm is due to the high concentration of allophycocyanine in the sample.

TABLE II

ELUTION DATA FOR VARIOUS ORGANIC ACIDS THAT HAVE DIFFERENT CHELATING PROPERTIES

The concentrations listed are the maximum concentrations of eluent checked for the purpose of elution. The top row gives the concentration of eluent that is necessary for protein elution. The values in the bottom row indicate that no copper is eluted at the concentration of eluents tested.

	Eluent			
	Ascorbate	Acetate	Malonate	Oxalate
Concentration for protein elution (m*M*)	>50	>500	$\leqslant 60$	<30
Concentration for Cu^{2+} elution (m*M*)	>50	>500	>60	>30

binding of PS I and PS II to the copper where PS I shows a greater affinity for copper than PS II.

Finally all PS I could be removed from the sample.

Eluents

We also tested other compounds as possible selective eluents of the protein complexes (carboxylic acids, amino acids and others), but no separation of PS I and PS II comparable to the results just described could be obtained. We shall nevertheless briefly describe the results because they provide valuable data about the mechanism of protein elution in IMAC.

Carboxylic acids. Carboxylic acids present an ideal system for the investigation of the elution mechanism of proteins from the metal chelate column because they are available in all chain lengths. In addition, many oligofunctional carboxylic acids are available. Their elution characteristics are shown in Table II.

These data indicate that acetate, which is a monodentate ligand, did not elute protein at concentrations below 500 m*M*, whereas the bidentate ligands malonate and oxalate eluted protein at much lower concentrations. The elution diagrams of these carboxylic acids are comparable to those of the amino acids group B (see Fig. 3, and also the next sub-section. All these acids did not elute copper at the concentrations tested.

Amino acids. With amino acids as eluents, again no good separation of PS I and PS II could be obtained, similarly to the carboxylic acids. According to the data in Table III the amino acids can be divided into two groups: (A) amino acids that elute the protein complexes below a concentration of 15

TABLE III

ELUTION DATA FOR PROTEIN AND COPPER OF SOME AMINO ACIDS

The top row gives the concentrations of the individual amino acids that were necessary for protein elution. The bottom row gives the maximum concentration for the use of these amino acids because above these concentrations Cu^{2+} is released. N-Acetylhistidine is an exception; in contrast to all the other amino acids tested, no copper was eluted at the end of the gradient.

	Amino acids group A						Amino acids group B			
	Arg	Lys	Asn	Pro	Trp	Cys	NAc-His	His	Met	Leu
Concentration for protein elution (m*M*)	<15	<15	<15	<15	<15	–	<50	<50	$\leqslant 50$	<60
Concentration for Cu^{2+} elution (m*M*)	$\geqslant 15$	$\geqslant 15$	$\geqslant 15$	$\geqslant 15$	>10	>0	>100	$\geqslant 50$	$\geqslant 50$	$\geqslant 100$

TABLE IV

ELUTION DATA OF SUBSTANCES ASSUMED TO HAVE A HIGH AFFINITY TO COPPER AND THEREFORE BE GOOD ELUENTS

The concentrations given are the maximum concentrations that were checked for the purpose of elution. With thiourea and cystamine, copper was released from the column at the end of the gradient. In all other instances no copper was eluted.

	Eluent								
	Urea	Thiourea	Thiamine	Thiophene-2-carbonic acid	Cystamine	IPTG	Piperazine	Sodium azide	Biotin
Concentration for protein elution (m*M*)	> 2000	> 500	> 500	> 100	< 100	> 50	> 50	> 20	> 15
Concentration for Cu^{2+} elution (m*M*)	> 2000	⩾ 500	> 500	> 100	⩾ 100	> 50	> 50	> 20	> 15

m*M* (arginine, lysine, asparagine, proline and tryptophan) and (B) those which elute proteins below 60 m*M* (N-acetylhistidine, histidine, methionine and leucine). The elution profiles are shown in Fig. 3. Cysteine is an exception; it does not elute protein, but elutes copper immediately from the column.

The elution patterns of both classes of amino acids (see Fig. 3) are very similar. In all instances two distinct but overlapping bands were observed. This indicates that the elution mechanism should be similar. The only difference is that the elution peaks of the amino acids eluting at higher concentrations are much broader than those of the amino acids eluting at lower concentrations. Therefore, a slightly better separation of the two protein complexes can be obtained when amino acids are used for elution which elute at higher concentrations. Again it was not possible to improve the separation even with flatter gradients.

A comparison of the elution properties of imidazole and histidine (both containing an imidazole ring) indicates the different elution mechanisms for the two compounds. Whereas imidazole did not release copper from the column, copper was released by histidine at concentrations that were slightly above the concentrations at which protein was eluted.

Eluents containing nitrogen or/and sulphur atoms. We also tested various other compounds that we thought might be good competitors with the protein for the coordination positions of the metal ions. As copper has a great affinity to sulphur and nitrogen [17], substances were tried that contain either one or both of these atoms. The results are given in Table IV. Again a separation of PS I and PS II could not be obtained.

From the results, it is striking that with the exception of cystamine (which contains a sulphur–sulphur bond), none of the eluents eluted the protein at the concentrations tested. The results obtained with cystamine as an eluent are not very good, however. Thus it is not possible to separate PS I and PS II and it is a disadvantage that copper is eluted from the column at or even below a concentration of 100 m*M* cystamine in the elution buffer.

DISCUSSION

Separation of PS I and PS II

This work has shown that IMAC is a useful method for the separation of PS I and PS II. This can be achieved either by elution of the protein complexes at low pH or by using their different binding properties to the imidazole-pre-charged column.

The elution mechanism at low pH is probably based on the protonation of the amino acid residues responsible for the binding of the proteins to the metal ions. The protonation finally results in a release of protein from the column. Elution with buffer at low pH results in extensive purification of the PS II complex and can be used as a one-step procedure omitting the density gradient centrifugation.

Pre-charging the column with imidazole differs from conventional elution procedures because here the protein competes with the eluent for the coordination positions of the metal ions and not the reverse. When imidazole was used as an eluent on a non-pre-charged column, an excess of imidazole was necessary to elute the protein complexes and therefore only a mixture of PS I and PS II could be eluted. With an imidazole-pre-charged column however, imidazole only saturates the coordination positions of the metal ions and therefore no surplus imidazole is present. This technique allows PS I and PS II to be separate on the basis of their different abilities to replace imidazole from the metal ion binding sites, which is a consequence of their different affinities to Cu^{2+}.

Elution with various eluents

Selective elution of PS I and PS II was not achieved with various substances as eluents. The probable elution mechanisms of each group of eluents are discussed.

Imidazole and imidazole analogues

Imidazole and adenine cannot form a ring complex with copper and therefore they should act as a monodentate ligand, which coordinates the metal ion with its basic nitrogen. The other nitrogen is not available as an electron donor because its free electron pair is part of the aromatic system of the imi-

dazole ring (we assume that the delocalization of the electrons in the aromatic system is disturbed in the complexation state, so that the two nitrogen atoms can be distinguished). Kinetic studies also show that imidazole behaves like NH_3 in complex formation reactions [18]. The fact that Mg–ATP does not elute protein at concentrations comparable to that of adenine is probably due to steric hindrance [19,20].

Carboxylic acids

The results obtained with carboxylic acids indicate that those substances which form five-membered rings in the complexation state are most effective in eluting protein, whereas those which form larger or smaller rings are less useful.

Amino acids

The difference in the elution of protein between the two groups of amino acids may be explained by considering their mechanism of elution. In general, two ways of interaction of amino acids with metal ions are possible.

One is that they act as bidentate ligands which coordinate the free positions of the bound copper with the carboxylate group and the α-amino group [21]. In this instance the amino acids form five-membered ring complexes and the different elution concentrations among the various amino acids tested could be explained primarily by either steric effects or hydrogen bonding. For amino acids the elution capacity is not primarily due to the basicity of the nitrogen [20].

The second possibility is the interaction of the amino acid residues with the metal ion. In this instance one should observe various elution behaviours for the different amino acids. This was only true for cysteine, which complexed the copper ion immediately, probably with its sulphydryl group. As we did not observe significant differences in the elution properties of the other amino acids tested, our results confirm the data obtained by Makinen *et al.* [22] and thus show that amino acid residues are not primarily involved in the complexation of metal ions and therefore do not contribute much to the elution process.

Eluents containing nitrogen or/and sulphur atoms

Regarding the other eluents tested, it is surprising that piperazine does not elute protein at concentrations below 50 m*M*, though ethylenediamine is one of the best chelating agents for copper [23]. This ought to be due exclusively to steric effects.

Thiophene-2-carboxylic acid should also be able to form a five-membered ring complex and therefore elute protein from the column, but as discussed before, the sulphur atom is part of the aromatic system and so the lone electron pairs of the sulphur atom are not available for coordination of the copper ion.

Thiamine (vitamin B_1) was also tested as an eluent because of its substituted thiazole ring. We expected this structure to be an analogue of an N-substituted imidazole ring, which would help to clarify the role of the basic nitrogen in the elution mechanism. The fact that no elution of protein was observed at concentrations below 500 m*M* again supports the model that the free electron pair of the basic nitrogen is involved in the elution mechanism. Additionally, steric effects have to be taken into account when explaining the elution properties of this large system.

Among the compounds containing one sulphur atom which is not part of an aromatic system, only those with electron-rich sulphur atoms such as in sulphydryl groups (see *Amino acids*) are appropriate for copper complexation. The disadvantage is that these substances elute the copper. Less electron-rich compounds such as thiourea are not able to elute protein at comparatively low concentrations. Even at high concentrations thiourea did not elute protein, but eluted copper from the column. Isopropyl thiogalactoside (IPTG) may additionally be sterically hindered. Biotin was tested because the sulphur atom is part of a cyclic non-aromatic system so that the angles of the free electron pairs of the sulphur atom are different to those in linear systems. However, no elution of protein could be observed up to a concentration of 15 m*M*.

Sodium azide was used to investigate the elution-properties of a nitrogen ligand. As the azide anion is linear it can only act as a monodentate ligand. No elution of protein was observed up to a concentration of 20 m*M*.

Urea, which can provide two amino groups for the complexation of metal ions, can only form a four-membered ring with copper and is therefore a poor competitive ligand. This is the reason why the

column can be washed with urea at a concentration of at least 2 *M* without elution of either protein or copper. This may be advantagous for certain purification problems [13].

Although we were not successful in separating PS I and PS II, the results obtained with the various eluents tried showed that there are differences in their elution properties. It became clear that the elution properties of the eluents cannot be exactly predicted, either from the chemical nature or from the structure of the eluent, which gives room for further efforts to find new eluents.

Imidazole and adenine proved to be the "strongest" eluents for IMAC, although they are monodentate ligands. They eluted the protein early in the gradient, but did not elute copper from the column. Among the "weaker" eluents, those forming five-membered ring complexes with the metal ions elute protein much better than those which form larger cyclic systems [22]. In this respect it would be interesting to try β-amino acids. As they can form only six-membered ring complexes they should elute protein less effectively than α-amino acids, and this could result in a better separation of the proteins. Additionally, it would be worth trying N-substituted systems as they do not elute copper at concentrations where unsubstituted systems already do, but show the same elution patterns as unsubstituted systems.

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CHROMSYMP. 2574

Scale-up of recombinant protein purification by hydrophobic interaction chromatography

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ABSTRACT

The scale-up of hydrophobic interaction chromatography is described. Human recombinant superoxide dismutase was used as a model. The scale-up was performed by keeping the height to diameter (H/D) ratio of the column constant. The success of scale-up was evaluated by reversed-phase high-performance liquid chromatography of the eluted material. The wrong H/D ratio causes decreased resolution.

INTRODUCTION

There are several strategies for scaling up preparative and process chromatography. Gareil *et al.* [1] reported increasing the throughput by overloading the column. Both volume and concentration overload are applicable. Changing the column geometry is another approach, which is often used in pilot-scale and industrial chromatography [2]. The guidelines for the scale-up of chromatography are relatively simple. The bed height, linear flow-rate, sample concentration, ratio of gradient volume and total column volume are held constant and only the column diameter is increased. The sample load and the volumetric flow-rate must be increased by the factor $(D_1/D_0)^2$, where D_0 is the original diameter and D_1 is the diameter of the larger column. However, some chromatographic rules must be considered to ensure that the entire process is scaled up efficiently. The sample distribution system at the column inlet and the bottom of the column and the plumbing of the entire system are important features. Care must be taken to ensure that laminar flow is achieved throughout the system to avoid additional band spreading by turbulent flow. Another approach involves a complete change of the chromatographic system. The fluid stream is changed from axial flow to radial flow [3].

In the early days of preparative chromatography for protein purification, scale-up was achieved by enlarging the column with a constant height-to-diameter ratio [4]. Many complicated equations have been advocated for this type of scale-up of process chromatography. However, for gel filtration a simple means of obtaining the same elution pattern with increasing scale of operations was found to depend on maintaining dynamic similarity. Dynamic similarity is obtained by arranging both large and small columns to be the same with respect to H/D and $HV\rho/\mu$, where H = column height, D = column diameter, V = velocity (flow-rate/cross-sectional area of the column), ρ = liquid density and μ = viscosity.

In this paper scale-up of hydrophobic interaction chromatography (HIC) is described following the concept of constant H/D ratio, but without maintaining dynamic similarity. Human recombinant superoxide dismutase was used as a model. The prepurified protein was subjected to HIC on Phenyl Sepharose Fast Flow, using a linear descending salt gradient. The purification was monitored by re-

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versed-phase high-performance liquid chromatography (RP-HPLC).

EXPERIMENTAL

Starting material

Escherichia coli broth [JM 105, expressing recombinant human copper–zinc superoxide dismutase (SOD)] according to Bayer *et al.* [5] was used. In the laboratory-scale experiments 10-l fermenters were used, whereas in the pilot-scale work the material was produced in a 100-l fermenter in the fully automated pilot plant of the Institute of Applied Microbiology. No difference was observed in the composition of the harvested culture broth between the laboratory- and pilot-scale experiments.

Homogenization

The chilled *E. coli* cells were homogenized using a two-valve high-pressure homogenizer (SHL 05) from Brahn and Lübbe (Hamburg, Germany). The homogenization valve was diamond coated. The pressure drop at the first valve was from 1000 to 150 bar and at the second valve from 150 bar to atmospheric pressure. The flux was 50 l/h. The culture broth was delivered to the homogenizer under a 4-bar pressure. Cell breakage was between 95% and 99%. Less than 0.001% of viable cells could be detected after homogenization.

Flocculation

Flocculation was carried out as described previously [6] with charged pellicular flocculents (BPA 1050; Rohm and Haas, Philadelphia, PA, USA). Flocculent was added to the *E. coli* homogenate. The final flocculent concentration was 3000 ppm. After flocculation, the clarified supernatant was filtered through 0.8- and 0.2-μm filters connected in series.

Chromatographic media for hydrophobic interaction chromatography

Phenyl Sepharose Fast Flow High Sub from Pharmacia–LKB (Uppsala, Sweden) was used for HIC. The degree of substitution is *ca.* 40 μmol of phenyl groups per millilitre of swollen gel. The mean particle size is 90 μm and the bead-size range is 45–165 μm. The gel is incompressible over a wide pressure (flow-rate) range. These data were obtained from the manufacturer.

Chromatographic columns

Two different types of columns, a 0.7-l column (P 90 X 250) from Amicon (Stonehouse, UK) and a 20-l column (BPG 200) from Pharmacia–LKB were used. The columns were connected to either a Biopilot or a Bioprocess system from Pharmacia–LKB. Instead of the original 0.8 mm I.D. tubing of the Biopilot system, 2.0 mm I.D. tubing was used. The Bioprocess system was equipped with 1/4-in. tubing.

Packing of columns

Packing of columns was carried out in a manner slightly different to the manufacturer's recommendation. About 250 ml (P 90 X 250 column) and 1 l (BPG 200 column) of 20% ethanol were poured into the column, followed by the gel slurry. The gel was allowed to settle, then the adaptor was fitted to the column and 20% ethanol was percolated through the column at a flow-rate of 250 cm/h. The adaptor was lowered stepwise and fixed in a position 1–2 mm above the packed gel. The packed column was stored in 20% ethanol at room temperature.

Chromatographic conditions

The column was operated in an upward direction. As equilibration buffer, 25 m*M* Tris buffer (pH 7.5) (titrated with 25% HCl) treated with ammonium sulphate to 60% saturation was used. The starting material was a prepurified protein solution (*E. coli* homogenate was flocculated), which was treated with solid ammonium sulphate to 60% saturation. The filtered solution was applied to the column. The unbound material was washed out with equilibration buffer. A linear descending ammonium sulphate gradient was used for elution. The gradient was formed by mixing 60% saturated ammonium sulphate solution which was diluted with 25 m*M* Tris buffer (pH 7.5) to a conductivity of 200 mS/cm at 20°C and 25 m*M* Tris buffer titrated with ammonium sulphate to a conductivity of 120 mS/cm at 20°C. The gradient volume corresponded to ten total column volumes (v_t). The column was regenerated with 25 m*M* Tris buffer (pH 7.5), cleaned with 0.1 *M* NaOH and stored in 20% ethanol. Loading and washing were carried out at a flow-rate of 200 cm/h and elution at a flow-rate of 40 cm/h.

Metal chelate chromatography (MCC)

Final purification was performed by copper-chelate chromatography, using Chelating Sepharose Fast Flow, as described by Bayer *et al.* [5]. Briefly, the column was activated with 50 m*M* $CuSO_4$. After activation, unbound copper was washed out with 20 m*M* potassium phosphate buffer (pH 6.4). After loading, unbound proteins were washed out with equilibration buffer. SOD was eluted with 100 m*M* citric acid buffer, made up by titrating trisodium citrate to pH 5.0 with 25% HCl. After elution, the column was stripped with one column volume each of 25 m*M* EDTA and 1 *M* acetic acid. The column was operated at a flow-rate of 40 cm/h. Activation and stripping were performed at a flow-rate of 90 cm/h.

Superoxide dismutase

SOD was determined by enzyme-linked immunosorbent assay (ELISA) according to the method of Porstmann *et al.* [7] and by the activity assay of Steindl *et al.* [8]. .

Protein and purity

Protein was determined according to Bradford [9]. The method was modified for microtitre plates and carried out according to the supplier's instructions (Bio-Rad Labs., Richmond, CA, USA).

Purity was monitored by RP-HPLC on a 5-μm, 300-Å C_{18}, column (125 mm × 4.6 mm I.D.). Eluent A was 0.1% aqueous trifluoroacetic acid (TFA); Eluent B was 0.1% TFA in acetonitrile. The UV absorbance was monitored at 214 nm.

RESULTS

Binding capacity and elution conditions

After prepurification of the starting material, the protein solution was filtered through 0.8- and 0.2-μm filters. The maximum loading capacity was determined with a 75-ml column. An excess of protein solution was pumped through the column and the breakthrough of SOD was determined. The maximum load capacity without any loss of the material in the breakthrough was 30 mg of SOD per millilitre of gel (Table I). Elution was optimized, but complete elution with sufficient purity at a defined single step could not be obtained (Table II). The problem was circumvented by applying a linear gradient for elution. Under these conditions (10 v_t gradient volume), SOD was always eluted in a conductivity window of 170 and 150 mS/cm with a yield higher than 80% (Fig. 1A and B).

Resolution

Resolution was improved by varying the height of the laboratory-scale column. At maximum load capacity the minimum H/D ratio for obtaining sufficient resolution is 5. This is a prerequisite for the next step of obtaining 99.9% pure SOD in the final purification step by metal chelate chromatography. This unconventional approach was used because the aim of the purification process was to obtain pure protein, and in order to construct an optimum continuous process from several individual steps, it is necessary for each step to fit in with the previous one.

TABLE I

PROTEIN LOAD OF PHENYL SEPHAROSE FAST FLOW

Column size (ml)	Protein load (g)	Protein per ml of gel (mg)	Specific load (v_t)	Volume (ml)	C_0[b] (ng/ml)
75[a]	2.25	30	36	2700	0.83
700	17.71	25.3	30	21 000	0.84
20 000	263	13.15	13.25	265 000	0.99

[a] The 75-ml column was used only for determining the dynamic capacity.

[b] C_0 is the protein concentration of the material loaded on to the column.

TABLE II

SUMMARY OF RECOVERY OF SOD BY STEPWISE ELUTION FROM PHENYL SEPHAROSE FAST FLOW

Experiment No.	Conductivity of elution buffer at 20°C (mS/cm)	Yield of SOD per elution step (%)
1	180	40
	160	30
2	160	5
	130	59
3	160	5
	110	70

In Fig. 2 the effects of sub-optimum and optimum H/D ratios on the purification are compared. RP-HPLC of the eluted peaks shows more hydrophobic proteins in relation to SOD under sub-optimum conditions. The resolving power of the next step was too low to remove the bulk of the hydrophobic proteins. Under optimum conditions (H/D = 5), the final purification (MCC) yields a pure protein. The two peaks in RP-HPLC, the small one in front of the large one, are characteristic of human copper–zinc superoxide dismutase, depending on the buffer system used. This is because SOD shows some microheterogeneity [10]. Under optimum conditions (H/D = 5) the eluate from HIC contained

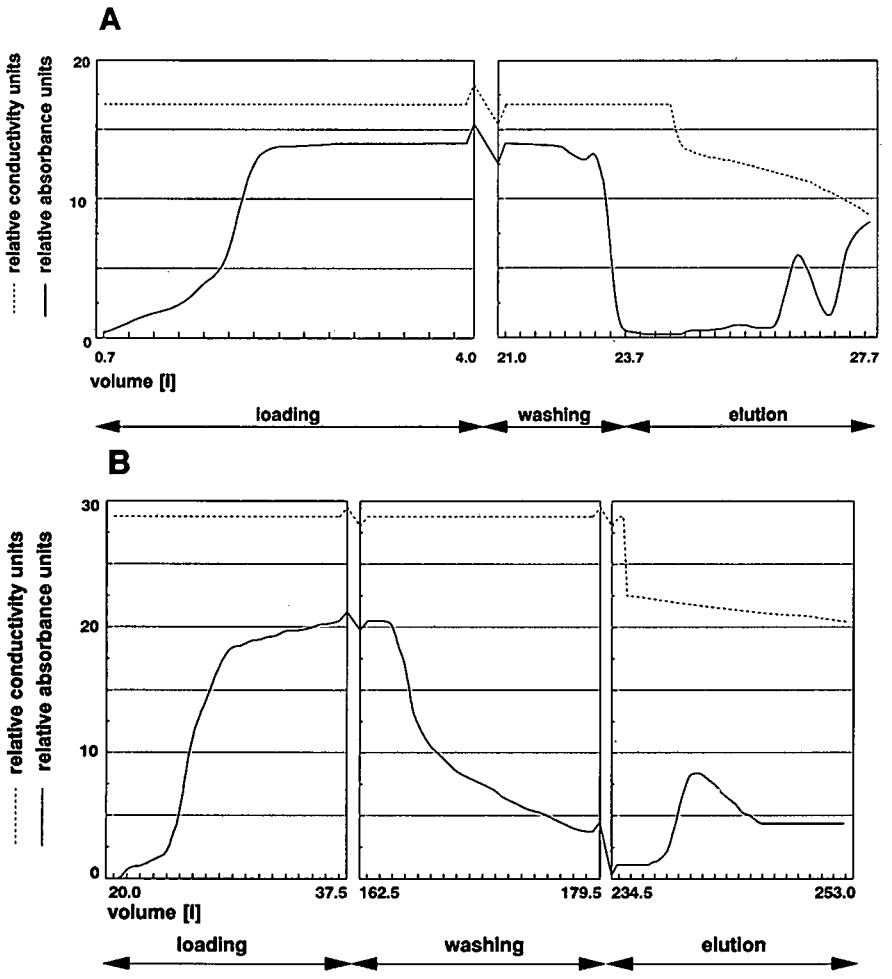

Fig. 1. HIC of SOD with a descending linear salt gradient. The gradient was prepared from (A) an ammonium sulphate buffer of 200 mS/cm at 20°C and (B) an ammonium sulphate buffer of 120 mS/cm. (A) Laboratory-scale experiment; (B) pilot-scale experiment. In both instances, the gradient volume corresponded to 10 v_t.

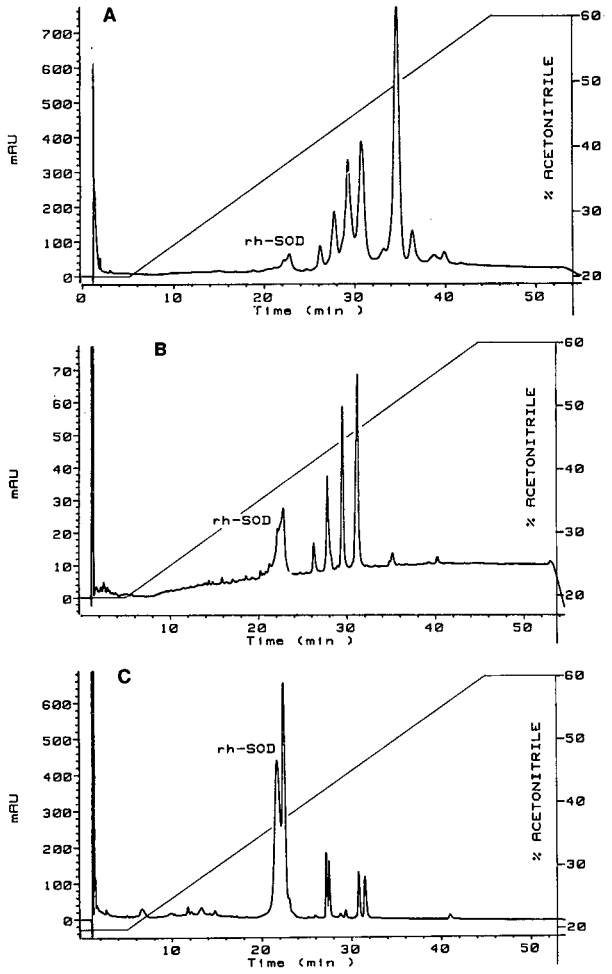

Fig. 2. RP-HPLC of the eluted fraction from Phenyl Sepharose Fast Flow with different H/D ratios. H/D = (A) 1.1; (B) 2.1; (C) 5.7.

SOD in excess. A small fraction of hydrophobic proteins can still be detected. The resolving power of MCC is strong enough to remove these substances (Fig. 3A and B).

Scale-up of HIC

The 0.7-l column was enlarged by a factor of 28. A 20-l Phenyl Sepharose Fast Flow column was used. A protein solution which corresponds to 100 l of *E. coli* broth could be processed in a single run. In Fig. 4 the RP-HPLC traces for the 0.7- and the 20-l column eluates are shown. For both column sizes the ratios of SOD and hydrophobic proteins

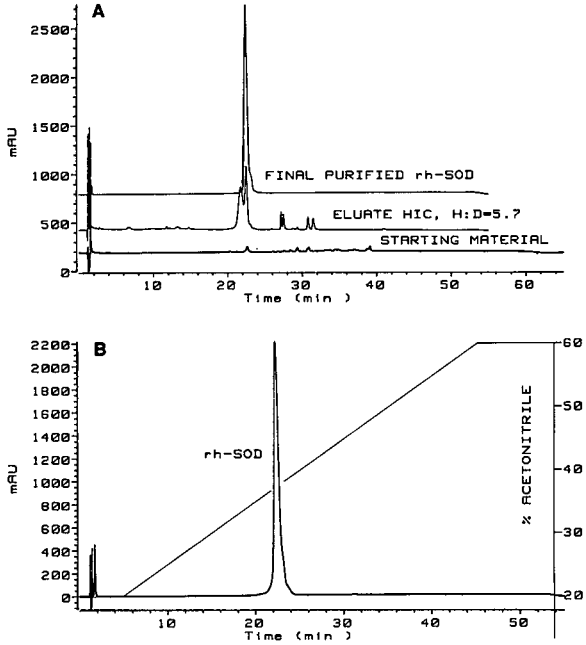

Fig. 3. RP-HPLC of final purified SOD (pretreated *E. coli* broth, HIC and MCC) with (A) a sub-optimum H/D column at the HIC step and (B) an optimum H/D column at the HIC step.

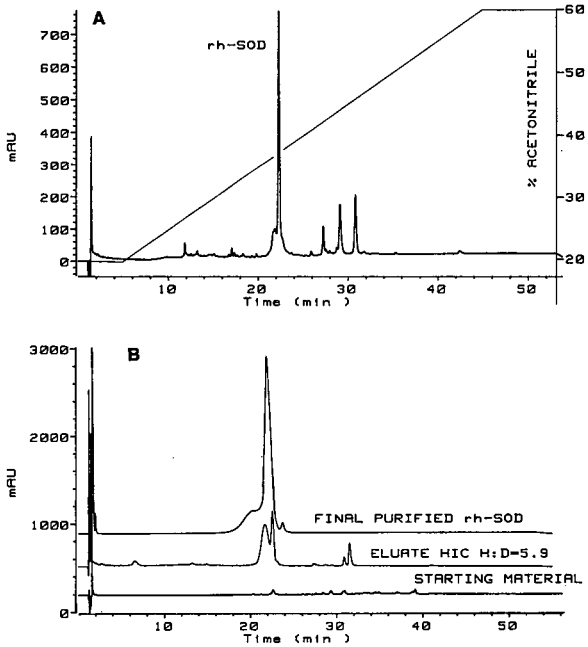

Fig. 4. RP-HPLC of pretreated *E. coli* homogenate eluate from HIC and final purified material of MCC. (A) A 0.7-l column was used in the laboratory-scale experiment and (B) a 20-l column in the pilot-scale experiment.

were in the same range. The final purification yielded homogeneous material. In Table II the protein load and volumetric load of the scales studied are summarized.

DISCUSSION

The objective of this work was to investigate the scale-up of a purification process. The process consisted of flocculation, HIC and MCC. The critical step in the scale-up was the HIC.

As shown in Fig. 4, the chromatographic purification of HIC could be scaled up successfully. Experiments were performed to find an optimum system (Table II and Figs. 2 and 3).

To reduce the column size and mobile phase volume for large-scale work, maximally loaded columns are desirable. The dynamic capacity is not completely used in order to save material. Sample application is stopped when product (SOD) can be detected in the flow-through. After optimization of the load capacity, the elution conditions were optimized.

According to theory, elution should be carried out at the minimum mobile phase modifier (MPM) concentration where elution is accomplished. The MPM concept, described by Velayudhan and Horváth [11], generalizes the mobile phase composition (acetonitrile, salts, etc.) that promotes elution. At higher MPM concentrations more tightly bound proteins are also desorbed. This material contaminates the product. Optimum elution is obtained only with a small window of MPM concentration. We could not find an MPM concentration at which SOD was quantitatively eluted in a single step in appropriate purity. To avoid losses of product by incomplete desorption, a descending linear gradient [12] was used for the subsequent experiments in stead of a descending step gradient [13]. To make the elution conditions as reproducible as possible, the ammonium sulphate concentration was controlled by conductivity at a given temperature (20°C). The Tris buffer was titrated with ammonium sulphate to give a defined conductivity. This approach led to very reproducible elution. Small variations in temperature, load, etc., led only to a shift in the peak position, but complete elution was still obtained. The large buffer volumes necessary to produce a linear gradient compared with stepwise elution are regarded as a disadvantage in process chromatography. In our opinion, this disadvantage is compensated by complete elution.

An incomplete distribution over the gel surface at the column inlet may also contribute to losses in resolution. The effects of both perturbation propagated by centres of disturbances and incomplete distribution of fluid may be one explanation why scale-up at constant H/D is necessary. HIC in general is more sensitive than ion-exchange chromatography or affinity chromatography to external changes. Several workers have described a partial unfolding during adsorption [14]. Unfolding increases with increasing residence time of the protein in a column [15]. By the scale-up concept of keeping H/D constant, unfolding of proteins could be likely, because the residence time of a protein in the column also increases with increasing size. Therefore, band spreading should occur. This event causes decreases in resolution. Nevertheless, we did not observe significant decreases in resolution in the large-scale experiments (Fig. 4). Hjertén *et al.* [16] could not find an influence of residence time on resolution. They pointed out that "the residence time of proteins on the column has no observable influence on the appearance of the chromatograms". Fausnaugh and Regnier [17] assumed that the alteration of the retention behaviour of proteins may be caused in some instances by the salt composition, which is responsible for the alteration of protein structure. SOD is regarded as one of the most stable proteins described so far. This unusual high stability may explain why the resolution does not decrease when the residence time is increased.

In our particular case, separation of SOD from hydrophobic proteins is of great importance for obtaining a pure product. We assume that these proteins are heat-shock proteins from *E. coli*. Some representatives of the *ca.* 40 heat-shock proteins have accessible histidines on the surface. They are able to interact with the chelated copper in the column. If these proteins are present in excess, they cannot be separated from SOD by subsequent MCC.

Scale-up of process chromatography cannot be performed apart from the other purification steps. Scale-up of HIC by keeping H/D constant led to the same product purity as observed in the laboratory-scale experiments.

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CHROMSYMP. 2572

Purification of the C1 repressor of bacteriophage P1 by fast protein liquid chromatography

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ABSTRACT

A fast protein liquid chromatographic method is described for the purification of the C1 repressor of bacteriophage P1 and its truncated form C1*. By using one crude extract, both repressor proteins were purified in parallel to homogeneity and were shown to interact specifically with P1 operator DNA *in vitro*. The method involves an affinity chromatographic step on heparin-Sepharose, followed by a combination of ion-exchange chromatography on Q Sepharose and S Sepharose. The availability of a homogeneous preparation of the phage repressor is a prerequisite for studies on its structure–function relationship.

INTRODUCTION

Proteins that regulate gene expression generally bind to specific DNA sequences. These regulatory proteins can control gene expression negatively or positively. Many of these regulatory proteins were reported to be repressor proteins, which act by binding to specific operator DNA sequences. Usually, an operator is overlapping with a promoter, and therefore an operator-bound repressor is preventing the RNA polymerase from binding to this promoter.

We are interested in the molecular mechanisms regulating gene expression in bacteriophage P1. As temperate phage, P1 is able either to (i) lyse an infected bacterial cell after forming new phage particles and thus release many progeny phages or (ii) lysogenize its host by maintaining the injected phage chromosome as a prophage in the cell. At various stages in its life cycle the phage has to switch on or off different sets of genes. A key element in this circuit of phage P1 gene control is the C1 repressor as a negative regulator of phage gene expression. As long as this repressor is active, the phage is maintained as a stable extrachromosomal low-copy plasmid in the cell. C1 turns off the lytic phage genes by binding to several specific operators, which are scattered over the P1 chromosome. Analysis of the C1 protein sequence did not reveal any homology to the classical helix-turn-helix motif [1] or to any other of the known DNA-binding structures. A review on phage P1 was published by Yarmolinsky and Sternberg [2].

By using conventional chromatographic methods for the purification of the C1 repressor and of mutant C1 repressor proteins [3,4], the repressor activity was resolved into two overlapping peaks. Two polypeptides, called C1 and C1*, were found to be active as P1 operator-binding proteins *in vitro*. The polypeptide C1* (268 amino acids; relative molecular mass, M_r = 30 900) has been shown to be a truncated form of C1 (283 amino acids, M_r = 32 500 [3,5]. The C1 repressor protein is positively charged (net charge of +4 at pH 7.0, pI = 9.1) and exists as a monomer in solution [6].

On the basis of the previously published purification procedure [3], this paper describes the application of fast protein liquid chromatography (FPLC) to the isolation of the P1 C1 and the C1* repressor proteins. By using one crude cellular extract, both

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repressor proteins were purified in parallel. During the purification, the repressor proteins were monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and by a DNA mobility shift assay that measured selective binding to P1 operator DNA.

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade and were purchased from Merck (Darmstadt, Germany), Boehringer (Mannheim, Germany) or Sigma (Deisenhofen, Germany). Heparin-Sepharose CL-6B gel and the prepacked fast protein liquid chromatographic columns Q Sepharose High Performance 16/100, S Sepharose High Performance 16/100, Mono S HR 5/5 and Fast Desalting Column HR 10/10 were obtained from Pharmacia (Freiburg, Germany). The DNA (1000 base pair ladder) and protein molecular mass markers were obtained from Bethesda Research Labs. (Eggenstein, Germany) and Bio-Rad Labs. (Munich, Germany).

Preparation of the crude extract

The method used for the preparation of extracts and the isolation of C1 and C1* repressor proteins is based on previously described procedures [3,4]. For the overproduction of the C1 repressor the recombinant plasmid pMV2w [4] was used. Here, the *c*1 repressor gene is under the control of the *tac* promoter, which is inducible by isopropyl β-D-thiogalactoside (IPTG). Unless noted otherwise, all steps were performed on ice and dialysis was performed against the appropriate buffer.

The *Escherichia coli* strain HB101 harbouring plasmid pMV2w was grown at 37°C in 1.2 l TY medium [1.0% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 0.5% (w/v) NaCl]. To induce repressor synhesis, IPTG was added to a final concentration of 2 m*M* at a cell density of about $2 \cdot 10^8$–$3 \cdot 10^8$ cells/ml. Incubation was continued at 37°C for 2 h and finally the cells were harvested by centrifugation [6000 *g* (Sorvall GS3 rotor), 10 min, 4°C]. The resulting pellets (13.7 g of wet cell paste) were resuspended in 2.5 m*M* EDTA (pH 8.0), 200 m*M* NaCl and 20 m*M* spermidine (5 ml/g of wet cell paste). The addition of one volume of lysis buffer containing 25 m*M* Tris–HCl (pH 8.0), 120 m*M* NaCl, 3.5% (w/v) sucrose, 0.2% (w/v) Brij 58, 1.25 m*M* EDTA (pH 8.0), 10 m*M* spermidine and 0.5 mg/ml of lysozyme led to bacterial lysis. After incubation for 30 min at 0°C the samples were warmed to 30°C and stirred at the same temperature for 5 min to complete lysis. The suspension was immediately cooled again, adjusted to 1 *M* NaCl and centrifuged [105 000 *g* (Beckman 45Ti rotor), 60 min, 4°C]. The supernatant (105 ml) was diluted 1:1 with buffer A [20 m*M* Tris–HCl (pH 7.6), 0.1 m*M* EDTA (pH 7.5), 50 m*M* NaCl, 1 m*M* dithiothreitol (DTT) and 10% glycerol]. An ammonium sulphate precipitation (60% saturation = 390 mg/ml) was followed by centrifugation [105 000 *g* (Beckman 45Ti rotor), 60 min, 4°C]. The pellets were dissolved in buffer A (11 ml) and subsequently dialysed against buffer A, resulting in a final volume of 13.5 ml with a protein concentration of 21.6 mg/ml (fraction I).

Fast protein liquid chromatography

An automated FPLC system (Pharmacia) was used, consisting of a controller (LCC-500 Plus), two high-precision pumps (P-500), solvent mixer, prefilter, a UV monitor (UV-M), one MV-7 and four MV-8 multi-position valves, a recorder (REC-482) and a fraction collector (FRAC 200). For the FPLC application of samples of up to 2 ml, loops of various sizes were used. A 50-ml Superloop was used for sample volumes larger than 2 ml.

Heparin-Sepharose CL-6B gel was laboratory-packed in an XK 16/20 glass column with an attached RK 16/26 packing reservoir [7]. The column was packed, washed and equilibrated under FPLC low-pressure conditions (operating pressure $\leqslant 0.5$ MPa). The calculated column volume was 22.0 ml. The prepacked FPLC columns were handled as recommended by Pharmacia. High Performance (HP) 16/100 columns were equilibrated and developed under FPLC low-pressure conditions (operating pressure range 0–0.5 MPa). During the purification procedure the temperature of the columns and of the collected fractions was kept constant at 2°C by using a MultiTemp II refrigerated thermostatic circulator unit (Pharmacia) as an external cooling system. All buffers were degassed and filtered through 0.22-μm membrane filters (Sartorius, Göttingen, Germany) prior to use. The protein absorbance was monitored at 280 nm.

C1 repressor assay

The *in vitro* interaction of C1 repressor with P1 Op86 operator DNA in buffer E [20 m*M* Tris–HCl (pH 7.6), 0.1 m*M* EDTA, 1 m*M* DTT, 50 m*M* NaCl, 10% glycerol, 100 μg/ml bovine serum albumin (BSA)] was studied by a DNA mobility shift assay [8,9] as modified by Velleman *et al.* [5].

Protein analysis

SDS-PAGE of proteins was performed on 15% slab gels as described [10]. Proteins were stained with Coomassie Brillant Blue R250 (Sigma). Protein molecular mass markers were myosin (200 000), phosphorylase *b* (97 400), BSA (68 000), ovalbumin (43 000), carbonic anhydrase (29 000), soybean trypsin inhibitor (21 500) and β-lactoglobulin (18 400). The protein concentrations were determined by the method of Miller [11] using BSA as a protein standard.

RESULTS AND DISCUSSION

Heparin-Sepharose chromatography

Affinity chromatography is commonly used as one of the final steps in protein purification, but it has been shown that chromatography on heparin-Sepharose is a very effective first step in the purification of C1 repressor [3]. Heparin is a linear glycosamine–glycan composed of mostly sulphated 1,4-linked glycosamine and glucuronic acid residues, covalently coupled to Sepharose CL-6B.

A crude cellular extract was prepared from an *Escherichia coli* strain which overexpresses the *c*1 repressor structural gene from the *tac* promoter. Proteins of the crude extract were concentrated by salting-out with ammonium sulphate at a concentration of 60% saturation (fraction I) and finally loaded on to a laboratory-packed heparin-Sepharose column (50-ml Superloop, flow-rate 0.5 ml/min), equilibrated with buffer A. Unbound proteins were washed from the column, at a flow-rate not exceeding that used for loading, until the absorbance of the flow-through dropped to the absorbance of buffer A. Under these conditions, all repressor material is bound to the matrix. The repressor protein was eluted with a flow-rate of 2.5 ml/min using an NaCl gradient in buffer A (Fig. 1). The SDS-PAGE analysis of protein extracts from uninduced and induced cells, fraction I, the flow-through and fractions 23–30 eluted from heparin-Sepharose is shown in Fig. 2. The preparation of fraction I (Fig. 2, lane 3) is a very critical step in the purification of the C1 repressor (see purified C1 re-

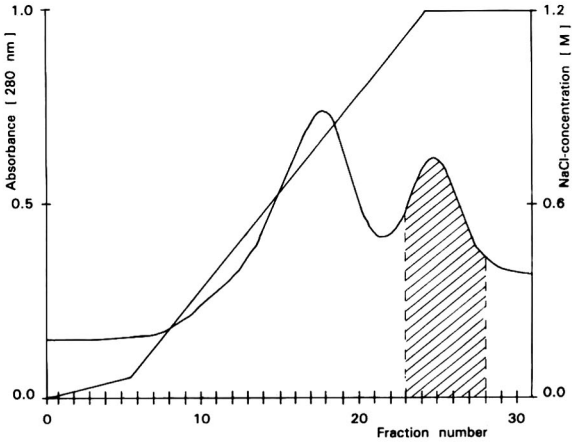

Fig. 1. Elution profile of C1 repressor on heparin-Sepharose. Fraction I (13.5 ml, 21.6 mg/ml) was applied to a heparin-Sepharose column (22 ml), pre-equilibrated and washed with buffer A. Bound proteins were eluted using a gradient: 0% buffer B (buffer A containing 1.2 *M* NaCl) in 5 ml, 0–5% buffer B in 40 ml, 5–100% buffer B in 150 ml, 100% buffer B in 30 ml. Fractions of 8 ml were collected at a flow-rate of 2.5 ml/min. The fractions containing the repressor protein (hatched area) were pooled and dialysed against buffer C (fraction II).

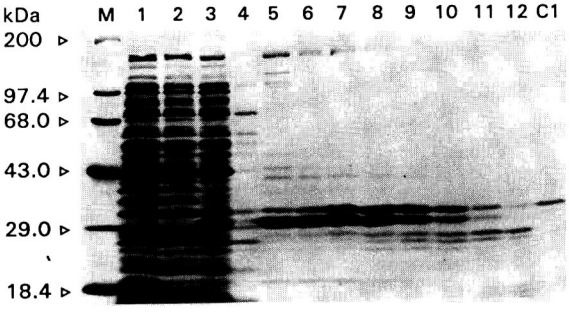

Fig. 2. SDS-PAGE of protein fractions eluted from heparin-Sepharose on a 15% gel. Lanes: M = protein molecular mass markers [in kilodaltons (kDa) as indicated] in descending order (8 μg each): myosin, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin; 1 and 2 = crude cellular extracts (15 μl each) of uninduced (lane 1) and induced cells (lane 2); 3 = fraction I (5 μl); 4 = flow-through heparin-Sepharose (70 μl); 5–12 = fractions 23–30 eluted from heparin-Sepharose (20 μl each); C1 = purified C1 repressor protein (1 μg).

pressor as marker in the last lane of Fig. 2 on the right). The truncation of C1 (upper band at *ca.* 32 500) to C1* (lower band at *ca.* 31 000) is drastically increased in this step, possibly owing to the activity of proteases in fraction I (compare lane 2 with lane 3). Therefore, the portion of C1* in the pooled fractions 24–28 eluted from heparin-Sepharose (Fig. 2, lane 5–10) is very high. The pooled fractions were dialysed against buffer C [20 m*M* Tris–acetate (pH 6.5), 0.1 m*M* EDTA (pH 7.5), 1 m*M* DTT, 15% glycerol] and applied to ion-exchange chromatography (fraction II).

Ion-exchange chromatography

For further purification of heparin-Sepharose fractions containing a mixture of C1 and C1* repressor and other proteins, we chose a combination of anion- and cation-exchange chromatography. Under the experimental conditions, C1 and C1* repressor were bound to the cation-exchange column.

Collected fractions from the affinity chromatography on heparin-Sepharose were passed through a Q Sepharose HP column (50-ml Superloop, flow-rate 0.5 ml/min), equilibrated with buffer C. The flow-through of this anion-exchange column was loaded directly on to an S Sepharose HP column coupled in series. The columns were washed free from unbound protein using buffer C and were subsequently decoupled. The bound proteins were eluted from the cation-exchange column with a flow-rate of 1.5 ml/min using an NaCl gradient in buffer C (Fig. 3). The overlapping peaks of the C1* and the C1 repressor proteins resulting from the heparin-Sepharose step were separated on this cation-exchange column. Two well defined peaks were obtained, the first peak containing only C1* (Fig. 4, lanes 2–4, corresponding to fractions 18–20 in Fig. 3) and the second peak mainly containing C1 repressor (Fig. 4, lanes 5 and 6, corresponding to fractions 21 and 22 in Fig. 3, hatched area).

For further purification and concentration of C1, fraction 22 (Fig. 4, lane 6) was desalted on a Fast Desalting HR 10/10 column by using buffer C at a flow-rate of 3 ml/min and subsequently rechromatographed on an analytical cation-exchange column (Mono S). The C1 repressor was eluted from the column with an NaCl gradient in buffer C as shown in Fig. 5. In contrast to the C1 repressor, which was eluted from the Mono S column at ≥ 0.6 *M* NaCl (hatched area in Fig. 5), the C1* repressor was elut-

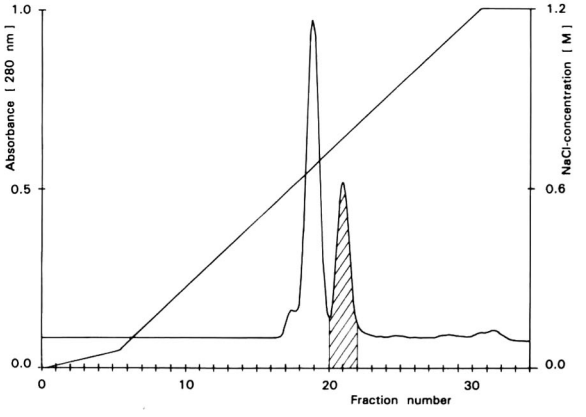

Fig. 3. Elution profile of C1 and C1* repressor proteins on S Sepharose. Fraction II (42 ml, 3.3 mg/ml) was applied to a Q Sepharose column (20 ml) coupled in series with an S Sepharose column (20 ml), each pre-equilibrated and washed with buffer C. The C1 and C1* proteins were only bound to S Sepharose and eluted from this column with a gradient: 0% buffer D (buffer C containing 1.2 *M* NaCl) in 5 ml, 0–5% buffer D in 40 ml, 5–100% buffer D in 200 ml, 100% buffer D in 30 ml. Fractions of 8 ml were collected at a flow-rate of 1.5 ml/min. The fractions containing the C1 repressor protein (fraction 21 and 22, hatched area) or the C1* repressor (fractions 18–20) were separately desalted on a Fast Desalting column and concentrated on Mono S.

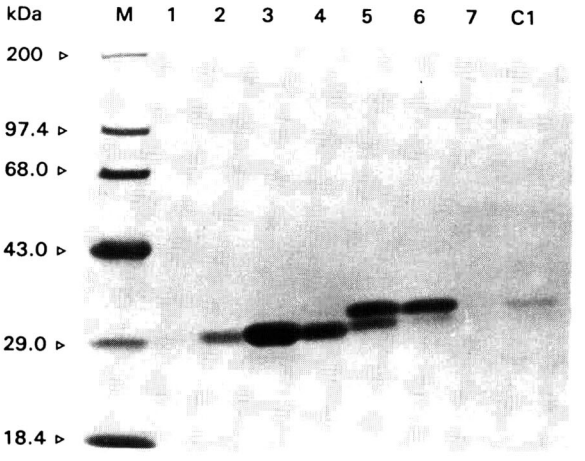

Fig. 4. SDS-PAGE of protein fractions eluted from S Sepharose on a 15% gel. M = protein molecular mass markers as described in the legend of Fig. 2; lanes 1–7 = fractions 17–23 from S Sepharose (20 μl each); C1 = purified C1 repressor protein (1 μg).

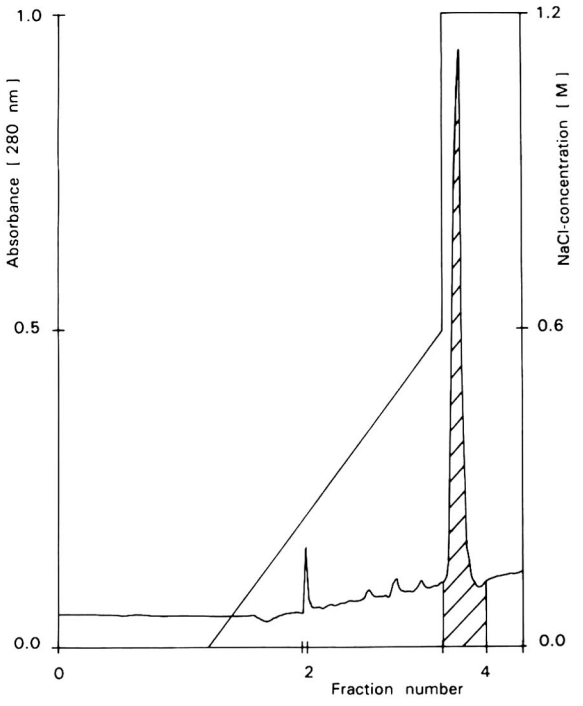

Fig. 5. Elution profile of C1 repressor on Mono S. The desalted fraction 22 obtained from S Sepharose chromatography (3 ml, 0.53 mg/ml) was loaded on to an analytical Mono S column (1 ml), pre-equilibrated and washed with buffer C. Elution was performed using a gradient: 0% buffer D in 10 ml, 0–50% buffer D in 15 ml and 100% buffer D in 5 ml. The flow-rate was 1 ml/min and the operating pressure range 0–3.5 MPa. Only peaks with a peak threshold of 3% full-scale were collected and the maximum fraction size was set to 8 ml. The hatched area indicates the fraction containing only the C1 repressor.

ed at $\geqslant 0.45$ *M* NaCl (data not shown), corresponding to a very small peak in Fig. 5, which is due to contamination of the applied C1 repressor fraction 22 with C1*.

The Mono S column is the most effective step in separating the C1 from the C1* repressor. Desalting of the repressor-containing fraction and the final exchange of buffer were done by using a Fast Desalting HR 10/10 column, equilibrated with buffer F [20 m*M* Tris–HCl (pH 7.6), 0.1 m*M* EDTA, 1 m*M* DTT, 50 m*M* NaCl, 50% glycerol). The same purification and concentration procedure was applied to fraction 21 and the pooled fractions 18–20 obtained from S Sepharose chromatography (data not shown). The C1 and C1* fractions resulting from rechromatography of fraction 21 (Fig. 4, lane 5) on Mono S were pooled with the corresponding C1 and C1* fractions.

The results of the final purification step of C1 and C1* repressor are shown in Fig. 6. SDS-PAGE revealed highly purified C1 and C1* repressor proteins. In addition, the identity and purity of the repressor preparations were tested by the very sensitive Western blot analysis (data not shown). Aliquots of the purified proteins were kept at −70°C and were stable for at least 1 year.

C1 repressor assay

This assay is based on the decreased mobility of protein–DNA complexes during electrophoresis compared with the mobility of free DNA [8,9]. C1 repressor has been shown to bind specifically to P1 Op86 operator DNA *in vitro* as revealed by DNAse I footprinting and by a DNA mobility shift [5].

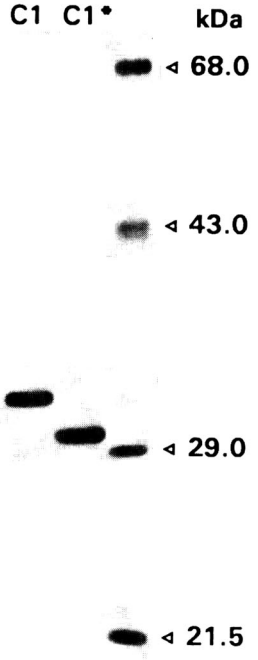

Fig. 6. SDS-PAGE of purified C1 and C1* repressor proteins eluted from Mono S on a 15% gel. The peak fractions from Mono S were desalted and the buffer was changed prior to electrophoresis. C1 and C1* = purified repressor proteins (2.5 μg each in buffer F); M = protein molecular mass markers (in kilodaltons as indicated) in descending order (3 μg each): bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor.

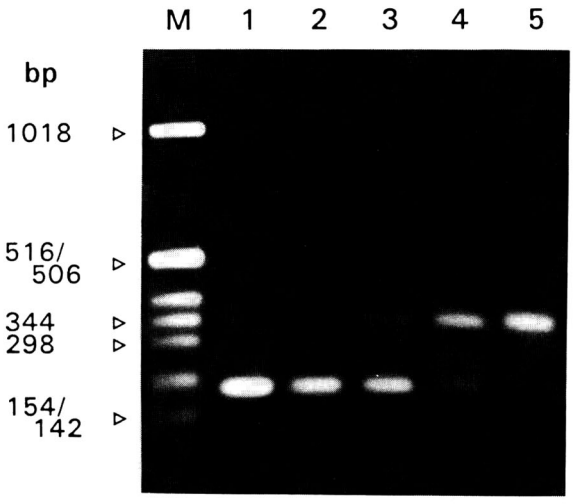

Fig. 7. C1 repressor assay. The purified C1 repressor protein was incubated with Op86 operator DNA for 15 min at 37°C in buffer E (total volume 25 μl) and subsequently subjected to analysis on a 1.5% agarose gel. Lanes: 1 = 198–base pair DNA fragment with P1 operator Op86 (1.2 pmol); 2–5 = increasing molar ratios of C1 repressor to constant amounts of operator DNA (1.2 pmol each lane); 2 = 0.6 pmol C1 (0.5:1); 3 = 1.2 pmol C1 (1:1); 4 = 2.4 pmol C1 (2:1); 5 = 4.8 pmol C1 (4:1); M = DNA molecular mass marker [in base pairs (bp) as indicated].

The P1 Op86 operator DNA was incubated with increasing amounts of FPLC-purified C1 repressor (Fig. 7). On binding of the purified C1 repressor, the operator DNA is gradually shifted to an upper position with decreased electrophoretic mobility (Fig. 7, lanes 2–5). At a molar ratio of four C1 repressor molecules per C1 repressor binding site (Fig. 7, lane 5) all operator DNA is shifted. Therefore, we conclude that the purified C1 repressor is active *in vitro*. When the experiment was repeated with C1* repressor, the DNA-binding activity was reduced at least twofold compared with the C1 repressor (data not shown).

CONCLUSIONS

This chromatographic procedure has been found to be well suited for the purification of C1 and C1* repressor proteins in parallel. Especially for the crystallization of the C1 repressor highly purified proteins are an essential requirement. Also, a reliable determination of biophysical data, such as binding or equilibrium constants, depends very much on homogeneous protein preparations. The use of the described procedure is not restricted to the isolation of the P1 C1 repressor but can also be used for the purification of other repressor proteins. We have already successfully purified the Bof co-repressor from bacteriophage P1 by a very similar procedure [12].

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Fast high-performance liquid chromatographic purification of *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase

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ABSTRACT

A procedure was established for the rapid isolation of *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase (PEPCK) from an overproducing strain. Overexpression was achieved by the transformation of yeast cells with the multicopy plasmid YEp352 harbouring the PEPCK structural gene. The enzyme was purified to homogeneity using first anion-exchange chromatography on Q-Sepharose followed by hydrophobic interaction chromatography on phenyl-Sepharose and gel filtration on Sephacryl S200. The purified phosphoenolpyruvate carboxykinase was further characterized with respect to the molecular mass, displaying an apparent molecular mass corresponding to a tetrameric form.

INTRODUCTION

Phosphoenolpyruvate carboxykinase [ATP; oxaloacetate carboxylyase (transphosphorylating), E.C. 4.1.1.49] from yeast catalyses the decarboxylation of oxaloacetate in the presence of ATP and Mn^{2+} ions to give CO_2, ADP and phosphoenolpyruvate [1]. This reaction is an important step in the formation of glucose from three- and four-carbon precursors. Phosphoenolpyruvate carboxykinase (PEPCK) from yeast differs markedly from vertebrate enzymes, because other enzymes are specific for GTP (or ITP) as a donor of the phosphate group [1]. In addition, the yeast enzyme exists as a tetramer composed of identical subunits [2,3] whereas the PEPCKs from other sources are monomers (relative molecular mass *ca.* 72 000). The primary structures were deduced from cloned DNAs for PEPCK from *Saccharomyces cerevisiae* [4], rat liver [5], chicken kidney [6] and *Drosophila melanogaster* [7]. Comparing the amino acid sequences of the PEPCKs from higher eukaryotes, strong homologies were found [7], and putative GTP-binding regions were suggested to be partially related to a consensus sequence found in different GTP-binding proteins [8]. In the case of the yeast enzyme, no significant similarities to the corresponding en-

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zymes have been obtained [4]. However, a consensus sequence for a putative ATP-binding site [6] can be found in the monomeric PEPCK from yeast at amino acid positions 249–255; further, a conserved sequence interacting with phosphoenolpyruvate [6] may be located at residues 356–382.

Although no detailed investigations of the reaction mechanisms of PEPCK are yet available on a molecular level, the presence of a functional arginyl residue and a reactive lysine residue have been determined [9,10]. Fluorescence spectroscopic measurements support the conclusion that a highly reactive sulphydryl group, located in the nucleotide binding site, is important either for the catalytic mechanism or for the maintenance of the active conformation of yeast PEPCK [11].

Comparing all PEPCKs studied so far, it seems possible that monomeric and tetrameric enzymes may have common features in their secondary or tertiary structures. In order to perform crystallization experiments for subsequent X-ray diffraction analysis, we developed an improved large-scale purification procedure for yeast PEPCK. The purification scheme described here involves the construction of a genetically modified yeast strain that facilitates the isolation of milligram amounts of the enzyme.

EXPERIMENTAL

Enzymes and substrates were purchased from Boehringer (Mannheim, Germany). All chemicals used were commercially available and of analytical-reagent grade.

Yeast strain and overexpression

A DNA fragment harbouring the complete *PCK1* gene encoding PEPCK was ligated into the yeast episomal plasmid YEp352 [12] by standard recombinant DNA techniques [13,14]. The resulting plasmid pPEPCK contained a chromosomal *EcoRI/XhoI* 4-kilobase (kb) DNA fragment obtained from yeast strain WAY. 5-4A ligated into the *EcoRI* and *SalI* restricted YEp352. The plasmid was screened by colony hybridization with an oligonucleotide-derived probe obtained from the *PCK1* sequence [4]. The *PCK1* gene was under the control of the native promoter. Transformation marker genes and sequences necessary for replication in *Escherichia coli* and yeast were derived from the plasmid YEp352. The origin of plasmid replication was originally from the 2-μm circle DNA which results in a high plasmid copy number [15]. Plasmid pPEPCK was transformed in *Saccharomyces cerevisiae* strain WAY. 5-4A according to the lithium acetate method of Ito *et al.* [16]. The transformed yeast was grown under selective conditions on synthetic media lacking uracil to prevent plasmid loss in cultures up to a volume of 200 ml. The preculture so obtained was transferred to 1 l of rich medium. This medium contained 3% glycerol and 3% ethanol as carbon sources. Under this condition the glucose repressible *PCK1* gene was derepressed. Incubation was stopped when the wet mass reached 10–15 g/l. After centrifugation the pellet was resuspended in water. The cells were pelleted again by centrifugation and stored at −20°C.

Enzyme assay

PEPCK activity was assayed spectrophotometrically by coupling the formation of oxaloacetate from phosphoenolpyruvate to the malate dehydrogenase reaction [17]. Standard assays were performed at 25°C in a volume of 1 ml, containing 100 m*M* imidazole–HCl (pH 7.0), 50 m*M* potassium hydrogencarbonate, 1.25 m*M* ADP, 1.0 m*M* $MnCl_2$, 2.0 m*M* glutathione, 0.45 m*M* NADH and 1.5 U/ml malate dehydrogenase (Boehringer). The reaction was started by adding of 100 μl phosphoenolpyruvate (25 m*M*).

Protein determination

The protein peaks of all chromatographic separation steps were recorded using an UV detector at 280 nm (Uvicord SD 2158; LKB). Protein concentrations in PEPCK-containing fractions were determined with the Coomassie protein assay reagent (Pierce). Additionally, pooled fractions and purified samples of PEPCK were assayed with biuret reagent (Sigma).

Chromatographic procedures

Protein purification was performed at 4°C in order to avoid losses of enzyme activity. The columns used were connected to a Model 2249 high-performance liquid chromatographic gradient pump (Pharmacia LKB); sample loading was done using a peristaltic pump.

Starting with 10–35 g of yeast cells, the crude extract was prepared as described in a previous paper [18], except that 100 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 7.0) containing 1 m*M* dithiothreitol (DTT) and 0.1 m*M* EDTA was used for cell disruption. Immediately after centrifugation the supernatant was applied (1.5 ml/min) to a Q-Sepharose fast-flow column (6 × 5 cm I.D.) previously equilibrated with 100 m*M* HEPES (pH 7.0) containing 1 m*M* DTT, 0.15 m*M* $MnCl_2$ and 0.1 m*M* EDTA (buffer A). The column was washed with the same buffer until the UV absorbance gave a stable baseline and then eluted with a linear sodium chloride gradient (buffer A containing 1 *M* NaCl, gradient volume 700 ml). Fractions containing more than 5 U/ml of PEPCK activity were pooled and adjusted to 30% saturation by addition of solid ammonium sulphate; no precipitation was visible. The sample was loaded onto an equilibrated (buffer A, 30% or 40% ammonium sulphate saturated) phenyl-Sepharose CL-4B column (23 × 2.6 cm I.D.) at a flow-rate of 1 ml/min. PEPCK was eluted with a 750-ml linear gradient of 30–0% (or 40%–0%) ammonium sulphate saturation in buffer A. Pooled fractions containing PEPCK activity were brought to 60% saturation with solid ammonium sulphate. After gentle stirring for about 30 min at 4°C, the suspension was centrifuged at 20 000 *g* for 30 min at 4°C. The protein pellet was dissolved in about 3 ml of buffer A and then applied to a Sephacryl S-200 gel filtration column (53 × 2.6 cm I.D.) that had been equilibrated with buffer A. Buffer A was also needed for elution of PEPCK.

For analytical anion-exchange chromatography, a Mono Q HR 5/5 column (Pharmacia LKB) was used. Crude extracts were prepared from 1–2 h of freshly thawed yeast cells according to the method of Ciriacy [19]. Before sample loading, the solutions were filtered through 0.4-μm filters (Nalgene).

About 300 μg of purified PEPCK were applied (1 ml/min) to a Superformance 75-5 hydroxyapatite column (Merck) that had been equilibrated with 0.005 *M* sodium phosphate buffer (pH 6.6). After washing with equilibration buffer the column was eluted with a linear gradient of 0.005–0.5 *M* sodium phosphate buffer (pH 6.6) in 25 min.

Purified PEPCK (1 mg/ml; 100 μl per run) was injected onto a Superose 6 HR 10/30 column (Pharmacia LKB) equilibrated with buffer A containing 150 m*M* NaCl. Elution was performed with the same buffer at a flow-rate of 0.5 ml/min and fractions of 0.5 ml were collected. The molecular mass of PEPCK was re-examined using standard proteins (low- and high-molecular-mass gel filtration kit, Pharmacia LKB) for calibration. The void volume was determined with blue dextran.

Immobilized metal ion affinity chromatography (IMAC) was carried out using a Chelating Superose HR 10/2 fast protein liquid chromatography (FPLC) column (Pharmacia LKB) loaded with 250 m*M* $CuSO_4$, $CoCl_2$ or $NiCl_2$ dissolved in water. IMAC columns were washed with degassed water (10 ml) in order to remove the unbound metal ions and equilibrated with 0.02 *M* phosphate buffer (pH 7.5) containing 1 *M* KCl and 0.15 m*M* $MnCl_2$. All iminodiacetate (IDA)–M(II) columns were eluted in a falling pH-gradient protocol by using 0.02 *M* phosphate buffer (pH 4.0) containing 1 *M* KCl and $MnCl_2$ as elution buffer (flow-rate 0.6 ml/min; gradient volume 36 ml).

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

SDS-PAGE was performed on a GE 2/4-LS electrophoresis apparatus (Pharmacia LKB) using the system of Laemmli [20]. Gel electrophoresis under non-denaturating conditions was carried out according to the method of Davis [21] using polyacrylamide gels. Gels were stained with Serva Blue R or with silver nitrate [22].

Crystallization of PEPCK

Before crystallization, samples of purified PEPCK were concentrated by centrifugation in Centricon-30 tubes (Amicon). The crystallization was carried out by the hanging drop vapour diffusion technique [23]. Crystallization experiments were performed at 18°C with 10-μl protein droplets containing 8–16 mg/ml of PEPCK and 1.4 *M* ammonium sulphate (pH 7.0; buffered with 0.2 *M* phosphate) in the presence of 0.001 *M* DTT.

RESULTS

Purification of PEPCK

As the success of protein crystallization is strongly dependent on the total amount of the starting

material, we used the yeast strain WAY. 5-4A harbouring the multicopy plasmid pPEPCK as a source for the isolation of PEPCK. The purification of *Saccharomyces cerevisiae* PEPCK was achieved from this overproducing yeast cells with a yield of about 87%. Crude extracts of the transformed cells showed an enzyme level about ten times higher than wild-type cells. The specific PEPCK activity was about 4.6 U/mg of protein in the crude extract of the recombinant cells.

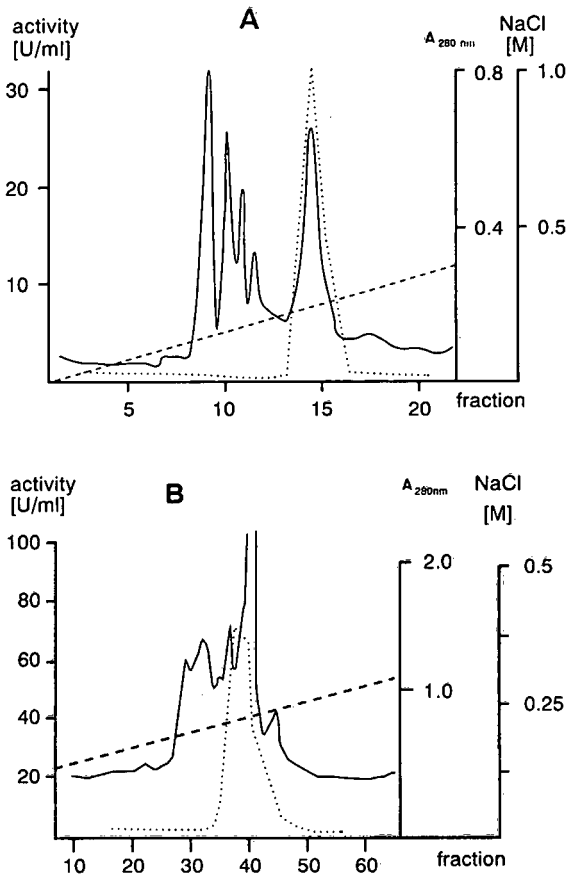

Fig. 1. Scale-up of anion-exchange chromatography of *Saccharomyces cerevisiae* PEPCK form Mono Q to Q-Sepharose. (A) Crude extract (30 U of PEPCK activity) was injected on to a Mono Q HR 5/5 column equilibrated with 100 m*M* HEPES (pH 7.0) containing 1 m*M* DTT, 0.15 m*M* $MnCl_2$ and 0.1 m*M* EDTA (buffer A). Elution was performed using a linear NaCl gradient (0–0.5 *M*) and a gradient volume of 30 ml (flow-rate 1 ml/min). (B) 75 ml of crude extract containing 3670 U of PEPCK were applied to a Q-Sepharose column (bed volume 118 ml) equilibrated with buffer A. Proteins were eluted by using a linear gradient of NaCl (0–1 *M* NaCl in 430 min; flow-rate 1.6 ml/min). Solid lines, protein concentration; dotted lines, enzyme activity; dashed lines, NaCl concentration.

The first step in the purification was anion-exchange chromatography of the supernatant after disrupture of the cells. Fig. 1 illustrates scale-up from a Mono Q HR 5/5 to a K 50/30 column with a 6-cm bed height, packed with Q-Sepharose fast flow. A sharp PEPCK peak was eluted from the Mono Q column at about 250 m*M* NaCl (Fig. 1A). For the preparative run on the Q-Sepharose column, a peak displaying PEPCK activity occurred at about 200 m*M* NaCl (Fig. 1B). This chromatographic step gave an efficient separation of PEPCK from the bulk protein; a recovery reproducibly higher than 100% was observed. The specific activity increased from 4.6 U/mg in the crude extract to 30.9 U/mg in the pooled fractions (Table I).

PEPCK in the combined active fractions was further purified through a phenyl-Sepharose column. All hydrophobic interaction columns resulted in elution profiles comparable to that shown in Fig. 2. The same degree of purification was obtained by using 40% or 30% saturated ammonium sulphate buffer. A relatively large amount of contaminating proteins could be removed during the column washing. A homogeneous peak of PEPCK activity corresponding to a protein peak appeared when the conductivity of the eluting gradient was less than 17 mS (*ca.* 5.5% ammonium sulphate saturation). From the 4600 U that were applied, about 93% could be recovered after hydrophobic interaction chromatography. The specific activity increased slightly from 30.9 U/mg in the pooled Q-Sepharose frac-

TABLE I

PURIFICATION OF YEAST PHOSPHOENOLPYRUVATE CARBOXYKINASE

Data are from a representative purification starting with 11 g of overproducing yeast cells. The yield was calculated as a percentage of the amount of PEPCK present in the crude extract.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	795	3675	4.6	100
Q-Sepharose	150	4640	30.9	126
Phenyl-Sepharose	129	4340	33.6	118
Sephacryl-S 200	80	3218	40.2	87

tions to 33.5 U/mg in the pooled fractions from phenyl-Sepharose. As shown in the inset in Fig. 2, the peak fractions were only poorly contaminated when stained with silver after SDS-PAGE.

The pooled fractions were precipitated and subsequently purified to homogeneity on a Sephacryl S-200 column. Only one nearly symmetric protein peak occurred, which coincided with that of PEPCK activity (Fig. 3). Fractions 17–23 were pooled; the recovery of enzyme activity from Sephacryl S-200 was about 87%. Purified PEPCK showed a specific activity of about 40.4 U/mg.

To illustrate the single purification steps, SDS-PAGE was performed with consecutive samples after anion-exchange, hydrophobic interaction and gel permeation chromatography (Fig. 4A). The results of a representative purification are summarized in Table I. About 80 mg of pure PEPCK were routinely obtained from a purification procedure starting with 11 g of yeast cells. The purified enzyme could be stored at −80°C in buffer A for several months with no detectable decrease in activity.

At room temperature a decrease in of enzyme activity was measured after 3 h. The decay could be considerably reduced when the enzyme was incubated on ice. After 20 h only about 25% of the activity could be detected after storage at room temperature, whereas 80% of the activity was determined after incubation on ice for the same period.

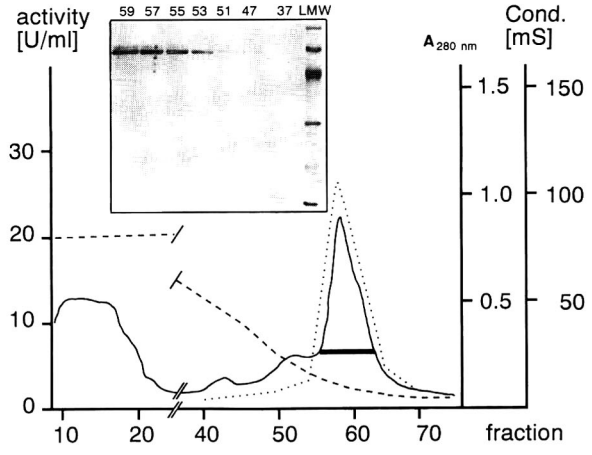

Fig. 2. Hydrophobic interaction chromatography of the pooled fractions from Fig. 1B on phenyl-Sepharose CL-4B. Elution was performed using a linear gradient of decreasing ammonium sulphate saturation (30–0%) in 0.025 *M* piperazine buffer (pH 6.5). PEPCK elution started at about 5.5% saturated ammonium sulphate. Fractions containing more than 5 U/ml (marked with a bar) were pooled. Inset: SDS-PAGE of the peak fractions obtained from phenyl-Sepharose (Coomassie staining). Solid line, protein concentration; dotted line, enzyme activity; dashed line, conductivity. LMW = Low molecular weight standard proteins.

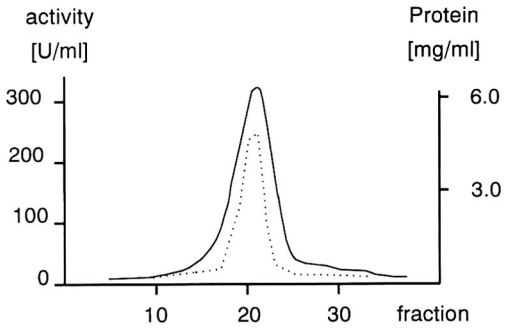

Fig. 3. Gel permeation chromatography of PEPCK on a Sephacryl S-200 column. The active fractions form the phenyl-Sepharose column were precipitated and subjected to gel permeation chromatography on Sephacryl S-200. Solid line, protein concentration; dotted line, enzyme activity.

Criteria of purity

As the quality of an enzyme preparation is an important factor that will influence the crystallization of the protein, the homogeneity of PEPCK was confirmed by different electrophoretic and chromatographic methods. When analysed by SDS-PAGE, the purified PEPCK appeared to be homogeneous, even if as much as 10 μg of enzyme were applied (Fig. 4B; lane 2). A single silver-stained band corresponding to an M_r of about 65 000 was obtained. PEPCK also migrated as a single protein-staining band in a native polyacrylamide gel. After an electrophoresis time of up to 4 h only one protein band was detectable (Fig. 4C). Gel permeation on Superose 6 showed that the PEPCK activity eluted with a single symmetric peak displaying an apparent M_r of about 250 000. The pure enzyme was also injected onto a hydroxyapatite column. The best binding and elution conditions for PEPCK were at pH values in the range 6.6–7.0, where the enzyme eluted in a single symmetric peak. No binding was observed at higher pH values of 7.5 and 8.0. When loading PEPCK onto a column equilibrated with phosphate buffer (pH 6.3), only very poor resolution was achieved.

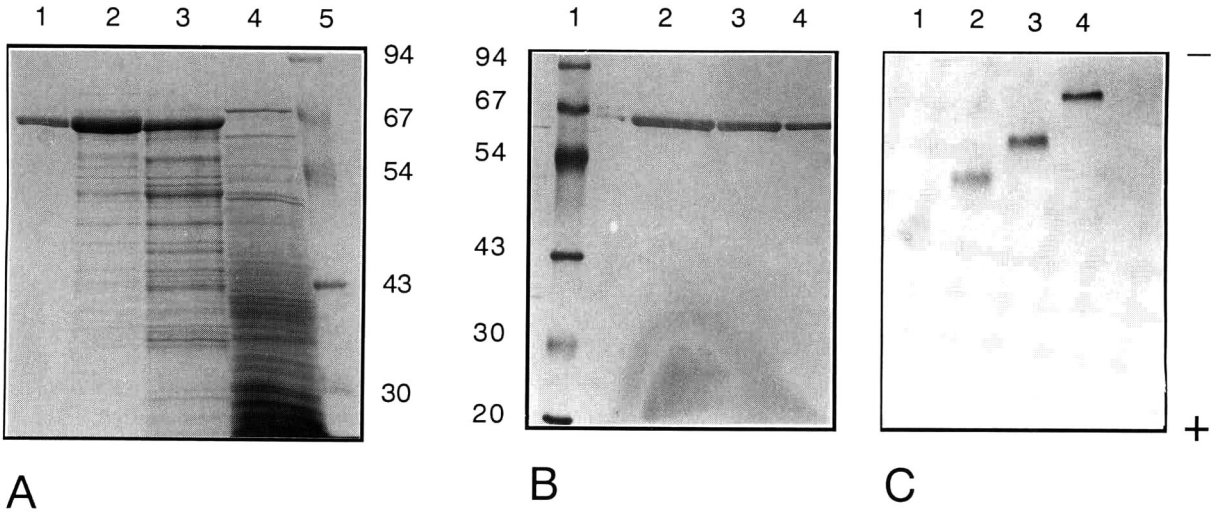

Fig. 4. Electrophoretic analysis of subsequent purification steps of yeast PEPCK and purified PEPCK. (A) SDS-PAGE of pooled fractions from gel permeation (lane 1), hydrophobic interaction (lane 2), anion-exchange chromatography (lane 3) and yeast crude extract (lane 4) (molecular mass markers, $M_r \times 10^3$, lane 5; Coomassie staining). (B) SDS-PAGE of 10 μg (lane 2), 5 μg (lane 3) and 2.5 μg (lane 4) of purified PEPCK (marker proteins, $M_r \times 10^3$, lane 1; silver staining). (C) PAGE of purified PEPCK under non-denaturing conditions electrophoresis time: lane 1, 4 h; lane 2, 3 h; lane 3, 2 h; lane 4, 1 h). The direction of migration is from top to the bottom of the gel.

Crystallization

The protein solution was concentrated by ultrafiltration using Centricon 30 microconcentrators to final concentrations of about 20–30 mg/ml (5000–7000 rpm, 4°C, 1–2 h). Crystals were grown at 18°C in both the presence and absence of ADP at an ammonium sulphate concentration of 1.4 *M*. In spite of the existence of sulphydryl groups in the enzyme [24], the addition of DTT to the protein samples before crystallization had no influence on the size and shape of the PEPCK crystals. Microcrystals were obtained which are too small for X-ray analysis. Further crystallization experiments are in progress.

Affinity of PEPCK for different IDA–M(II) columns

Affinity chromatography on immobilized metal ions was used to investigate the topography of histidine residues on the PEPCK surface [25–27]. The strongest binding was observed when PEPCK was applied to a column loaded with copper. The enzyme could not be eluted from IDA–Cu(II) columns with a decreasing pH gradient. PEPCK displays a weaker affinity for IDA–Ni(II) and IDA–Co(II) columns. Fig. 5 illustrates the chromatography of PEPCK on IDA–Co(II) and IDA–Ni(II) columns. The enzyme was eluted from IDA–Ni(II) columns only after a decrease in the pH to *ca.* 6.3 (Fig. 5A). In chromatography on IDA–Co(II) columns the protein peak occurred at a considerably higher pH value of about 7.0 (Fig. 5B).

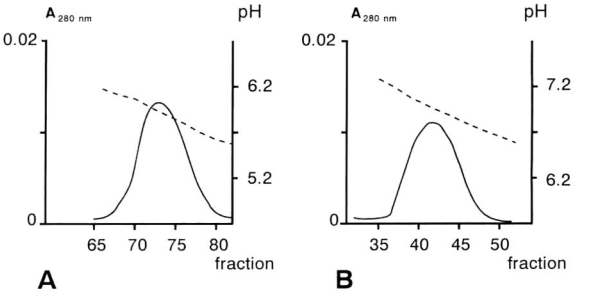

Fig. 5. Chromatography of yeast PEPCK on (A) IDA–Ni(II) and (B) IDA–Co(II) columns. Purified PEPCK was applied to a Chelating Superose column in the equilibration buffer. After washing, the column was developed with a pH gradient using 0.02 *M* phosphate–1 *M* KCl–0.015 *M* $MnCl_2$ (pH 4.0) (0–100% in 60 min; flow-rate 0.6 ml/min). Solid lines, protein concentration; dashed lines, pH gradient.

DISCUSSION

This work involved the overexpression and purification of the *Saccharomyces cerevisiae* PEPCK. The purification was greatly aided by the cloning of the coding region and the overexpression of the inserted gene.

The purification method was adapted to the manipulated yeast strain with the aim of isolating PEPCK in a large amount for subsequent crystallization experiments. The procedure described here indeed resulted in large amounts of highly purified enzyme. The purification of PEPCK using anion-exchange, hydrophobic interaction and gel permeation chromatographies was highly reproducible; results similar to those shown in Table I were obtained from four repeated runs. The conditions required for elution of PEPCK from the analytical Mono Q HR 5/5 column could be applied to fractionation on a large-scale Q-Sepharose column. As shown in Fig. 1, the resolution of the Q-Sepharose column is slightly diminished. Possibly the capacity of the Q-Sepharose column may have been too low, causing the observed decrease in peak resolution. The differences in the separation pattern may be due to the different cell disruption method used for the experiments. However, there is no negative influence on the PEPCK purification efficiency. The peak fractions of both chromatographic steps contained nearly the same specific activity (Mono Q, 25 U/mg; Q-Sepharose, 30 U/mg). Presumably, the removal of adenylate kinase during anion-exchange chromatography caused the apparent increase in PEPCK activity after this purification step [2].

The elution profile of PEPCK on phenyl-Sepharose suggests a relatively hydrophobic character of the enzyme. The average hydropathy of the PEPCK determined according to Kyte and Doolittle [28] was calculated to be -3.1; obviously this value indicates a hydrophobicity that is large enough to be bound to the column material. However, the high ammonium sulphate concentration enhances the binding properties of PEPCK to phenyl-Sepharose. As the purity of the enzyme obtained was the same whether using 40% or 30% ammonium sulphate-saturated buffer, the latter was preferred as the pooled fractions could be adjusted faster.

Gel permeation as the last chromatographic step removed all of the contaminating proteins and the salt that may influence the crystallization experiments. The final specific activity of purified PEPCK after the last step was 40.4 U/mg. This value agrees with the specific activity measured by Encinas *et al.* [11], but is higher than the value reported by Tortora *et al.* [2]. The difference may be due to slightly different assay conditions and different methods of protein determination. The overall recovery of PEPCK was considerably increased (87%) and the yields were at least ten times higher than previously reported [2,29,30].

Although three chromatographic steps were necessary starting from a crude extract, the purification was fast because no time-consuming dialysis was involved. The use of the overexpression yeast strain made it possible to start with a few grams of material. Thus the sample volumes were very small and the columns could be loaded rapidly. Further, the omission of AMP affinity chromatography could lower the cost even in the case of a large-scale purification. We confirmed the results of Tortora *et al.* [2] that the addition of proteinase inhibitors is unnecessary. No significant proteolytic degradation could be detected during the preparations.

The high homogeneity of the purified enzyme was proved by both electrophoretic (native and SDS–PAGE) (Fig. 4) and chromatographic methods using size-exclusion and hydroxyapatite columns. The purified yeast PEPCK exhibited an M_r of *ca.* 65 000 on an SDS-PAGE gel, corresponding to the predicted value calculated by the amino acid sequence (61 500). Although no microheterogeneity of the preparations could be detected, the crystallization of PEPCK resulted in very small crystals not suitable for X-ray analysis.

Chromatography on immobilized metal ions was used in order to investigate the surface topography of histidine residues on the PEPCK molecule. PEPCK showed strong binding affinities for immobilized copper and nickel. PEPCK was also retained on an IDA–Co(II) column. However, the elution from the latter occurred at a considerably higher pH value indicating a weaker binding to cobalt. We interpret the binding of PEPCK to immobilized cobalt as resulting from at least two histidine residues located at the protein surface.

Use of genetic engineering techniques will allow the recovery of large amounts of homogeneous PEPCK, permitting crystallization experiments.

Future work will be aimed at the growth of crystals suitable for X-ray crystallographic studies.

ACKNOWLEDGEMENTS

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CHROMSYMP. 2573

Purification of cytochromes P-450 derived from liver microsomes of untreated and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated marmoset monkeys

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ABSTRACT

The purification of multiple forms of cytochrome P-450 (P450) from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated marmosets using fast protein liquid chromatography (FPLC) is described. The main aim was to achieve a better separation of certain closely related P450 sub-forms from each other than that previously obtained using conventional chromatography. An 8-aminooctyl-Sepharose fraction of cholate-solubilized microsomes was obtained first and, after fast desalting on Sephadex G-25, loaded on to a preparative Mono Q column. Five of the six gradient peaks contained P450 and were each rechromatographed on an analytical Mono Q column. The pass-through peak was fractionated further using a Mono S column. Other HPLC-quality anion- and cation-exchange gels were compared. For removal of excess of non-ionic detergent, five types of hydroxyapatite gels were compared. Seven purified forms of P450 and cytochrome b_5 and P420 were isolated and characterized according to PHAST sodium dodecylsulphate–polyacrylamide gel electrophoretic apparent molecular masses, catalytic, spectral and magnetic properties and also TCDD-binding capacity (molar ratio of [^{14}C]TCDD to P450). There are at least two sub-forms which appear to be TCDD inducible, one showing a substantial ethoxyresorufin-O-deethylase activity and the other having a high TCDD-binding molar ratio. Two other forms appear to be constitutive, as deduced from comparisons with forms purified from untreated animals.

INTRODUCTION

The toxicity of polychlorinated dibenzo-*p*-dioxins (PCDDs, "dioxins") has recently been the subject of much investigation [1]. Of all known PCDDs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to possess the greatest toxic potency, revealing itself in a great diversity of clinical–pathological alterations and over a very wide dosage range [2]. Moreover, toxicity manifestation and dose dependence appear to exhibit some species specificity. TCDD has been found to induce in the rat and other laboratory animals a limited number of cytochrome P-450 forms (P450) equatable to those inducible by the well known class of agents typified by β-naphthoflavone (BNF) or 3-methylcholanthrene (3MC) [3].

The aim of this work was to isolate purified forms of P450 from the marmoset, a non-human primate. Previous initial work in this laboratory involving purification of P450 forms from TCDD-treated marmosets according to established conventional chromatographic procedures for TCDD- and BNF-treated rats [3,4] left open the question as to whether or not with marmosets an adequate separation of certain P450 forms from each other had indeed been achieved. At the same time, the amount of marmoset material available for this purpose was a limiting factor.

In an attempt to provide a better separation and a higher yield of these enzymes, a purification scheme was investigated using fast protein liquid chromatography (FPLC).

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EXPERIMENTAL

Chromatographic gels

All high-resolution (HR) empty glass columns and, unless indicated otherwise, all prepacked columns were purchased from Pharmacia (Uppsala, Sweden).

Animals and animal treatment

Untreated (UT) female marmosets (*Callithrix jacchus*) and TCDD-induced marmosets of both sexes were used. The animals were 9–10 months old, representing the age of a young adult. A single injection of 300–1000 ng of [^{14}C]TCDD per kilogram body mass was given subcutaneously 4 days prior to killing. Each liver weighed between 5 and 10 g.

Sample preparation

All steps were done at 0–4°C. Liver microsomes were prepared and solubilized in sodium cholate as described [5]. 8-Aminooctyl-Sepharose was synthesized following the procedure of Guengerich and Martin [6] and chromatography was carried out as reported previously [7], except that here (after washing with 0.4% cholate) a single elution was effected using Emulgen 911 at a concentration of 0.5% (w/v). The elution pattern was detected by following the absorbance at 405 nm. The collective pool of monooxygenases was concentrated by Amicon PM 30 ultrafiltration and stored at −80°C.

Anion-exchange FPLC

The temperature for this and all further chromatographic steps was 20–25°C, but fractions were collected in a rack filled with ice. Individual portions of the 8-aminooctyl-Sepharose fraction derived from TCDD-induced microsomes (TCDD–P450 8-aminooctyl-Sepharose pool) were desalted on an HR 10/10 column (10 cm × 10 mm I.D. and 10–40-μm particle size) of Sephadex G-25 Superfine or on disposable NAP-25 columns using (as equilibration and elution buffer) Mono Q buffer A, which consisted of 10 m*M* Tris–HCl (pH 7.7), 0.1 m*M* EDTA, 0.5% (w/v) Emulgen 911 and 20% (v/v) glycerol. This sample was loaded on to a Mono Q HR 10/10 column (10-μm particle size) equilibrated with buffer A and, after washing with *ca.* 30 ml, a 115-ml linear gradient of 0–250 m*M* NaCl was applied using 1 *M* NaCl in buffer A (Mono Q buffer B). For this gel, HR 10/10 column size (8 ml) and elution salt anion, this gradient size corresponds to the concentration change of 17.5 m*M*/ml recommended by the manufacturer for HR 5/5 column size (5 cm × 5 mm I.D. = 1.0 ml) [8]. The flow-rate was 0.5–1.0 ml/min in most instances. The pass-through fractions were collected, combined, concentrated and stored as mentioned above until further use.

In place of the Mono Q 10/10 column, a HiLoad 16/10 Q Sepharose HP column (10 cm × 16 mm I.D. and 24–44-μm particle size) was also used. In this instance, selection of the gradient size and flow-rates was based on the scale factors resulting from column volumes and cross-sectional areas, respectively. Peaks eluting from either column during application of the gradient were rechromatographed on a Mono Q HR 5/5 column.

An 8-aminooctyl-Sepharose pool of untreated (UT) P450 was also fractionated on a Mono Q 10/10 column. The HiLoad 16/10 Q Sepharose HP column and rechromatography on Mono Q HR 5/5 were not incorporated here, however.

For the sake of comparison, in a separate experiment, portions of the same 8-aminooctyl-Sepharose pool of UT-P450 were chromatographed on HR 5/5 columns packed in this laboratory [7] with Mono Q, with TSKgel DEAE 5PW (TosoHaas, Stuttgart, Germany) and with LiChrospher 1000 DEAE (Merck, Darmstadt, Germany).

Cation-exchange FPLC

The pass-through fractions from Mono Q 10/10 or HiLoad 16/10 Q Sepharose HP runs which were desalted on Sephadex G-25 or NAP-25 using Mono S buffer A [20 m*M* morpholinoethanesulphonic acid (MES) (pH 6.5), 0.5% Emulgen 911 and 20% glycerol] were loaded individually on to a Mono S HR 5/5 column equilibrated with the same buffer. For this 1-ml column, a gradient volume of 23 ml incorporating 0–500 m*M* NaCl (recommended concentration change of 21.5 m*M*/ml [8]) was effected using 1 *M* NaCl in buffer A (Mono S buffer B).

Mono S chromatography was also carried out using the Mono Q pass-through fraction resulting from an 8-aminooctyl-Sepharose pool of UT-P450. For comparison, portions of the same Mono S load material (UT-P450 Mono Q pass-through fraction) were also chromatographed separately on HR 5/5

columns laboratory-packed with TSKgel SP 5PW and with LiChrospher 1000 SO_3^-.

Hydroxyapatite FPLC

P450 fractions were freed from excess of Emulgen 911 on an HR 10/2 column (2 cm × 10 mm I.D.) packed as described [7] with Hydroxylapatite HPLC Grade (Calbiochem, La Jolla, CA, USA). The column was equilibrated with 10 m*M* potassium phosphate (pH 7.4), 20% glycerol and 0.05% sodium cholate and eluted with the same buffer containing 350 m*M* phosphate as first described by Funae and Imaoka [9]. The flow-rate used for this column of Calbiochem gel was 1.0 ml/min and the temperature was 20–25°C.

HR 10/2 columns were also packed with the hydroxyapatite gels Bio-Gel HTP gel (Bio-Rad, Munich, Germany), IBF HA-Ultrogel (Serva, Heidelberg, Germany), TSKgel HA-1000 (TosoHaas) and Granulated Hydroxyapatite synthesized by us according to Mazin *et al.* [10], and tested in comparison.

In these instances, 1-ml columns were packed at the maximum flow-rates recommended by the manufacturers. The sample load consisted of an 8-aminooctyl-Sepharose pool of phenobarbital-induced Wistar rat P450 gel-filtered on NAP-25 cartridges using the hydroxyapatite equilibration buffer described above but containing in addition 0.5% Emulgen 911. Chromatographic runs, all at a constant temperature of 25°C, were then carried out at various flow-rates between 0.02 and 2.0 ml/min. Each sample load was 6.4 nmol. Using the flow-rate for each column that gave the maximum recovery of residual detergent-free P450 eluent, the capacity was measured by loading each column with 100 nmol, washing and eluting as usual.

Assays

Assays of methoxy- and ethoxy-O-dealkylase activities were carried out as described [5]. The substrate concentrations were 1.0 and 0.5 μM, respectively. NADPH-P450 reductase was purified from Wistar rat liver microsomes using, in a final step, gel filtration on an HR 10/30 Superose 12 column (30 cm × 10 mm I.D.) [7]. This preparation had a specific activity of 65 μmol/min · mg of cytochrome *c* (reduced) when measured in 0.3 *M* potassium phosphate buffer at 30°C [5].

Concentrations of [^{14}C]TCDD were calculated from liquid scintillation countings. The specific activity of this sample was 4.51 Bq/pmol, corresponding to 4.22 fmol of TCDD per dpm. Sodium dodecyl sulphate–polyacrylamide gel electrophoretic (SDS-PAGE) analyses were carried out using 4–15% and 10–15% gradient gels with silver staining in an automated PHAST system (Pharmacia) as described previously [5]. P450 contents, protein assays and assays concerning spectral and magnetic properties were also performed as described [5,11].

RESULTS AND DISCUSSION

Anion exchange using Mono Q

Emulgen 911 was used as a non-ionic detergent based on previous results from us for this type of column [11] and from other workers for HPLC-quality DEAE columns [9]. Use of sodium cholate either alone or together with Emulgen 911 routinely led to decreased resolution and increased back-pressure [11]. Of a variety of elution salts tested, NaCl was still found to be the most suitable [11]. Room temperature was used for this and all subsequent columns [12]; a decrease in column temperature during runs in fact led to poorer resolution.

When an 8-aminooctyl-Sepharose pool of TCDD-induced marmoset microsomes was chromatographed on a Mono Q 10/10 column, a profile was obtained as shown in Fig. 1. This appearance was highly reproducible and consisted of one pass-through (PT) peak, representing consistently 50–55% of the total amount of P450 applied to the column, and six gradient peaks (A–F). The designation of peaks here follows the order in which they were first isolated and characterized. All of these peaks contained P450 except for that peak eluting after 250 m*M* sodium chloride, where cytochrome b_5 was detected (fraction E). Cytochrome P420 was found, if at all, in the pass-through fraction, as also reported by Roos [13] for Mono Q columns. In this work, the amount of P420 did not exceed that originally present in the column load. Total recoveries of P450 from the Mono Q 10/10 column prior to removal of residual detergents were routinely about 70%. We found that the resolution, especially of the smaller peaks, was enhanced when 10 m*M* instead of 20 m*M* Tris buffer was employed.

An increase in resolution was also found when

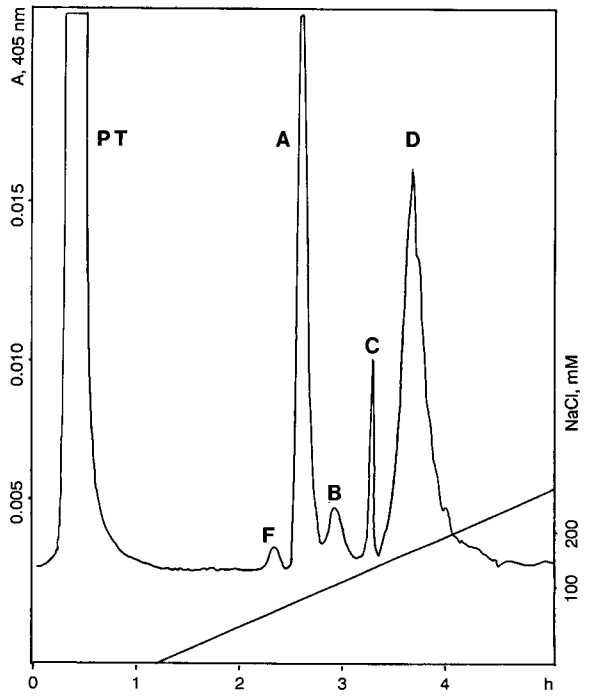

Fig. 1. Anion-exchange chromatography on Mono Q: Column: HR 10/10. Sample load: 17.6 nmol of TCDD-treated marmoset P450, prefractionated on 8-aminooctyl-Sepharose and desalted on Sephadex G-25. Buffer A: 10 m*M* Tris–HCl (pH 7.7), 20% glycerol, 0.1 m*M* EDTA, 0.5% Emulgen 911. Buffer B: 1 *M* NaCl in buffer A. Gradient: 0–250 m*M* NaCl. Flow-rate: 0.5 ml/min. Detection: 405 nm with 0.02 a.u.f.s. The fractions isolated are designated PT (pass-through), A, B, C, D and F. Yield in all fractions: 68%.

lower flow-rates but not when flatter gradients were used. Thus, flow-rates down to 1.0 or 0.5 ml/min were beneficial, but concentration changes of less than 2.2 m*M*/ml were not.

Finally, the best separations of peaks C (from D) and F (from A) were reproducibly obtained on continuous gradients from 0 to 200 or 250 m*M* chloride. Step runs of 0–40–250 m*M* as used previously [7] were disadvantageous for this purpose. Detailed, segmented gradients as exploited by Roos [13], however, may improve the separation of individual peaks.

Mono Q 10/10 versus HiLoad 16/10 Q Sepharose HP

Because in comparison with Mono Q 10/10, HiLoad 16/10 Q Sepharose HP is 2.5 times larger and at the same time significantly less expensive, this column was also considered. The highest flow-rate used for Mono Q 10/10 was 2.5 ml/min, resulting in a back-pressure of 3.5–4.0 MPa. HiLoad 16/10 Q Sepharose HP, in contrast (with its 2.5-fold cross-sectional area), could not be operated at a flow-rate higher than 2.5 ml/min. The back-pressure in this instance was 0.3 MPa.

For fast separations of P450 forms eluting in the pass-through fraction, both columns gave similar results, as judged by SDS-PAGE. However, for the isolation of minor forms, notably those in peaks C and F, or for the isolation of C-free peak D, only Mono Q 10/10 was of value. This type of finding was also reported by Beissmann and Reisener [14], who compared Mono Q and Q Sepharose Fast Flow during the purification of a glycoprotein elicitor. It should be noted that the Fast Flow gel has a particle size range of 45–165 μm and that this gel and the Q Sepharose HP gel are both more hydrophilic than Mono Q.

We observed that, in comparison with Mono Q 10/10 runs, Q Sepharose HP separations were also not as reproducible, especially with regard to the retention and elution of fractions B, C and F.

Comparison of various HPLC-quality anion exchangers

HR 5/5 columns of laboratory-packed Mono Q, TSKgel DEAE 5PW and LiChrospher 1000 DEAE gel were compared for their ability to separate forms of UT-P450. Despite the fact that these gels are all derived from different manufacturers, a first inspection of possible differences due to gel type (strong basic or weak basic anion exchanger) and/or particle size (10 or 5 μm) seemed justified (Table I). One of the aims of this work was to increase the resolution and yield.

Fig. 2 shows the chromatographic profiles of such runs with corresponding SDS-PAGE lanes of individual fractions. The relative sizes of the pass-through fractions in the chromatograms expressed as area under the curve (AUC) for total protein detection (for Mono Q, TSKgel DEAE 5PW and LiChrospher 1000 DEAE of 23, 37 and 39%, respectively) indicate that Mono Q retained the most protein applied. In comparison with both DEAE columns, Mono Q showed in the electrophoretograms of the pass-through fractions much less protein, but with a noticeable restriction more to the

TABLE I

CHARACTERISTICS OF THREE HPLC-QUALITY ANION-EXCHANGE GELS

Gel designation	Gel source	Anion exchanger type	Functional group	Particle size (μm)	Pressure[a] (MPa)
Mono Q	Pharmacia	Strong basic	$-CH_2N^+(CH_3)_3$	10	2.6–3.4
TSKgel DEAE 5PW	TosoHaas	Weak basic	$-(CH_2)_2NH^+(CH_2CH_3)_2$	10	1.2–1.4
LiChrospher 1000 DEAE	Merck	Weak basic	$-(CH_2)_2NH^+(CH_2CH_3)_2$	5	2.6–3.6

[a] Back-pressure at 0.5 ml/min found for a 5 mm I.D. column. For sample load and buffer, see Experimental.

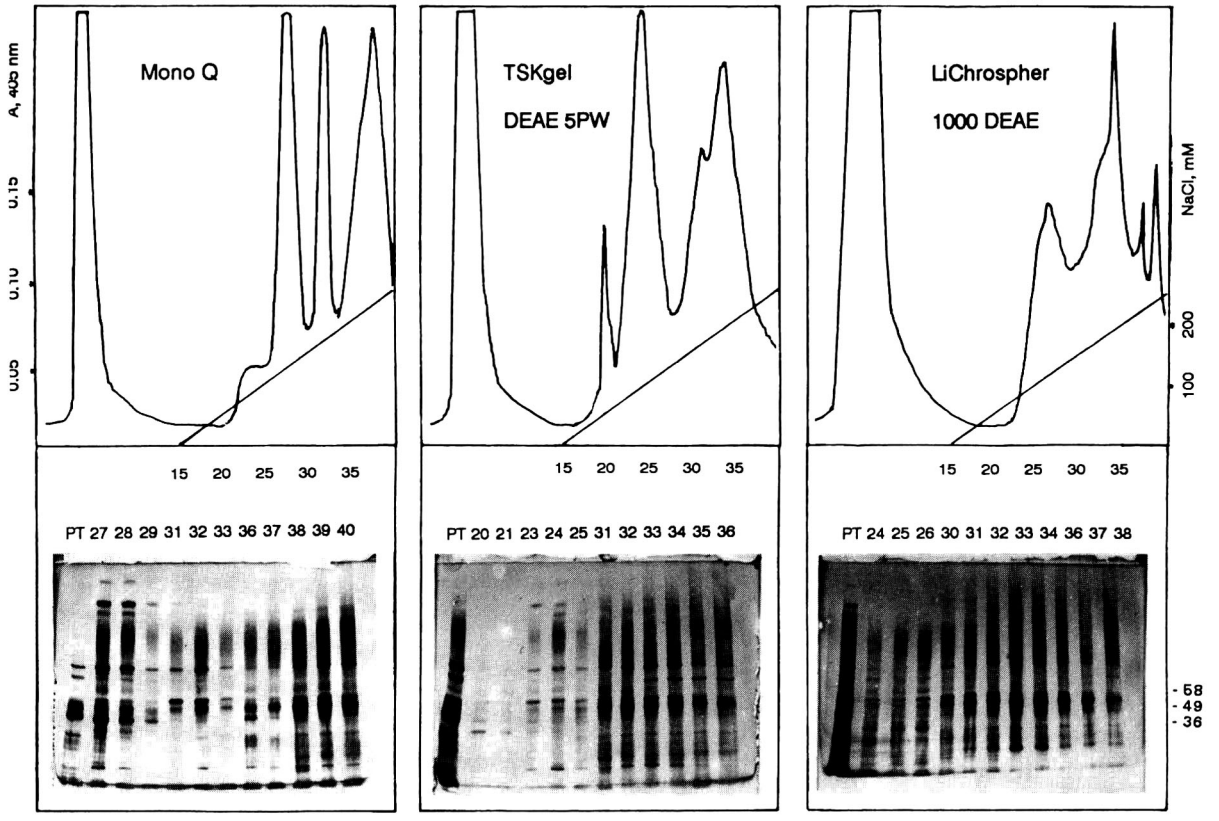

Fig. 2. Comparison of chromatographic profiles and SDS-PAGE patterns of three different HPLC-quality anion exchangers. HR 5/5 columns were laboratory packed with Mono Q, TSKgel DEAE 5PW and LiChrospher 1000 DEAE gels. Sample load: 2.86 nmol of untreated female marmoset P450 prefractionated on 8-aminooctyl-Sepharose and desalted on Sephadex G-25. Buffer A: 10 m*M* Tris (pH 7.7), 20% glycerol, 0.1 m*M* EDTA, 0.5% Emulgen 911. Buffer B: 1 *M* NaCl in buffer A. Gradient: 0–250 m*M* NaCl. Flow-rate: 0.5 ml/min. Yield in all fractions: 65–75%. Detection: 405 nm with 0.25 a.u.f.s. During the chromatographic runs, 0.5-ml fractions were collected (upper row of numbers beneath chromatographic profiles) and the pass-through (PT) fraction and gradient peak fractions were then chosen for electrophoresis (lower row of numbers). Samples of 0.3 μl of those fractions were electrophoresed using 4–15% gradient gels and silver staining in the automated PHAST system [5]. On the right of these are indicated the positions of marker proteins (with $M_r \times 10^{-3}$) catalase (58), fumarase (49) and lactate dehydrogenase (36).

P450 region between relative molecular mass (M_r) *ca.* 50 000 and 52 000 (Fig. 2).

Each gradient region consists basically of three main peaks. Regarding the first main peak here in each run, Mono Q apparently contains the highest concentration of forms in the 49–50-kilodalton range (Fig. 2). The second peaks are all basically similar, whereas among the third peaks Mono Q includes a fraction showing somewhat higher homogeneity (at $M_r \approx 52\,000$).

As might have been expected, the differences between the two DEAE gels themselves appeared to be less striking; the 5-μm LiChrospher 1000 DEAE material possibly yields slightly more homogeneous fractions, especially towards higher salt concentrations. Total recoveries of P450 for all three columns were consistently 65–75%. Judging from all of the above findings, Mono Q was adopted for routine purifications of these enzymes.

Cation exchange using Mono S

Portions of the Mono Q 10/10 pass-through fraction derived from TCDD material were loaded on to a Mono S HR 5/5 column and eluted with a continuous gradient. A typical profile is shown in Fig. 3. The high absorbance of the Mono S pass-through fraction reflects the high concentration of Emulgen 911 present in the Amicon PM 30 retentate of the column load; no P450 was ever detected here. This fraction contained, however, large concentrations of TCDD (57 ± 3% of applied TCDD load, expressed as the average of three separate experiments).

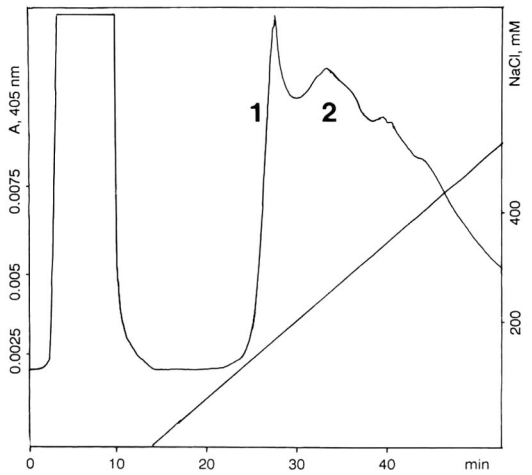

Fig. 3. Cation-exchange chromatography on Mono S. Column: HR 5/5. Sample load: 2.4 nmol of TCDD-treated marmoset P450 derived from the Mono Q pass-through fraction as shown in Fig. 1. Buffer A: 20 m*M* MES (pH 6.5), 20% glycerol, 0.5% Emulgen 911. Buffer B: 1 *M* NaCl in buffer A. Gradient: 0–500 m*M* NaCl. Flow-rate: 0.7 ml/min. Detection: 405 nm with 0.02 a.u.f.s. The fractions isolated are designated 1 and 2. Yield in all fractions: 87%.

The gradient portion revealed an ill-defined pattern consisting of two or more individual, broadly overlapping peaks all containing P450. P420 corresponding to that originally present in the load material eluted at salt concentrations above 300 m*M*. Variations of equilibration buffers and elution salts had only a slight effect on the apparent resolution over the whole gradient area as judged by the absorbance profile. MES as equilibration buffer gave slightly sharper peaks than did sodium or potassium phosphate, and sodium or potassium chloride as elution salts gave sharper peaks than did sodium acetate. The latter salt has been used elsewhere for cation exchange involving sulphopropyl columns [9]. SDS-PAGE in our hands revealed, however, that differences in separation of a lower molecular mass P450 fraction at the gradient start from a high-molecular-mass fraction in the middle using different buffer–elution salt combinations were considerable. MES–NaCl was therefore chosen as the most suitable pair (results not shown).

As we had no knowledge of any possible effects of MES itself on P450, the following checks were made. P450 was not seen spectrophotometrically to be converted into P420 or into any other degradation products, as has indeed been shown to occur elsewhere with other less widely used buffers or elution agents [15]. Substrate-binding spectra likewise were not observed. Finally, no loss of methoxy- or ethoxy-O-resorufin dealkylase activity was seen when TCDD-induced marmoset microsomes were incubated in the presence or absence of MES.

In Fig. 3, the two P450 fractions have been designed 1 and 2. Electrophoreses of such separations showed that within the chromatographic profile, homogeneous fractions in region 2 (as judged by SDS-PAGE) could be obtained. These results are not shown here but are comparable to those in Fig. 4, which depicts a fractionation of UT-marmoset P450. Region 1, however, consisting of a lower-molecular mass fraction, was always contaminated with fraction 2. Use of detailed step gradients here,

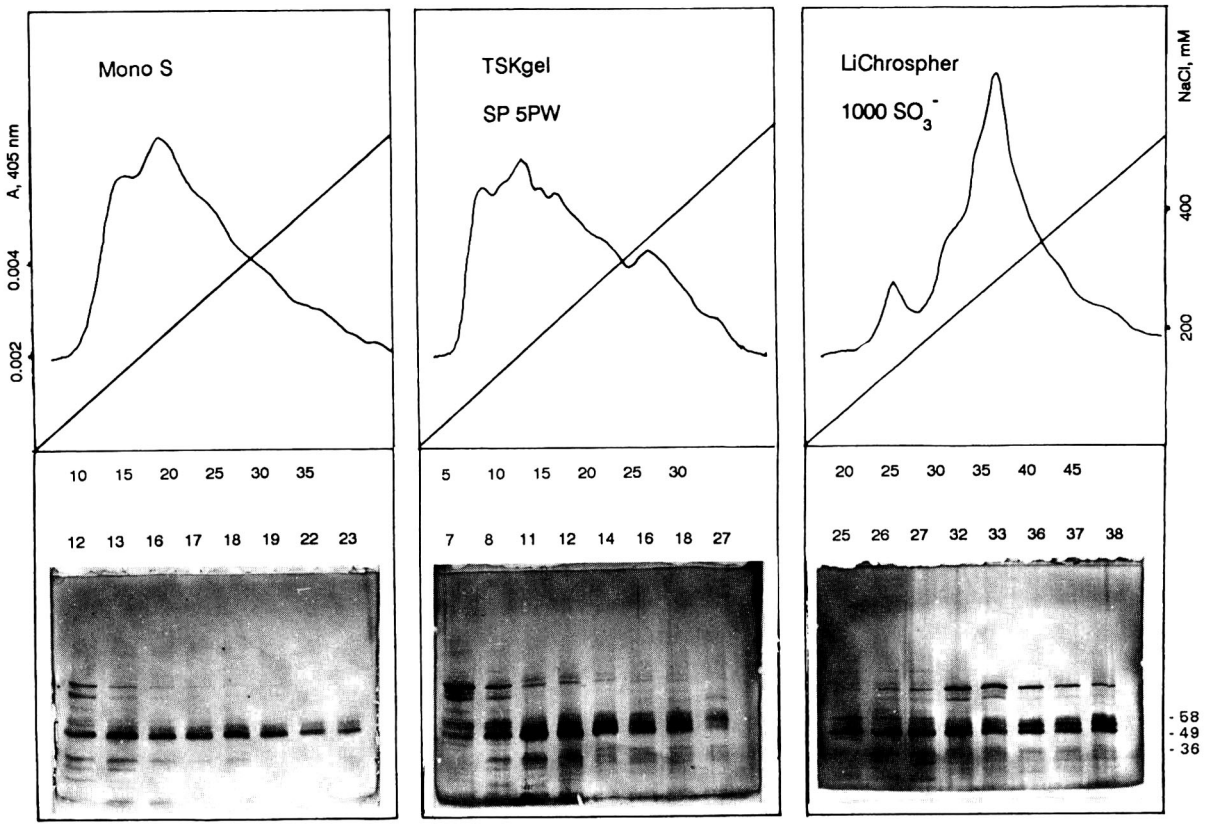

Fig. 4. Comparison of chromatographic profiles and SDS-PAGE patterns of three different HPLC-quality cation exchangers. HR 5/5 columns were laboratory packed with Mono S, TSKgel SP 5PW and LiChrospher 1000 SO_3^- gels. Sample load: 1.32 nmol of untreated female marmoset P450 prefractionated as the pass-through fraction of Mono Q and desalted on Sephadex G-25. Buffer A: 20 m*M* MES (pH 6.5), 20% glycerol, 0.5% Emulgen 911. Buffer B: 1 *M* NaCl in buffer A. Gradient: 0–500 m*M* NaCl. Flow-rate: 0.5 ml/min. Yield in all fractions: 80–90%. Detection: 405 nm with 0.01 a.u.f.s. During the chromatographic runs, 0.5-ml fractions were collected (upper row of numbers beneath chromatographic profiles) and gradient peak fractions were then chosen for electrophoresis (lower row of numbers). Samples of 1.0 μl of those fractions were electrophoresed using 4–15% gradient gels and silver staining in the automated PHAST system [5]. On the right of these are indicated the positions of marker proteins (with $M_r \times 10^{-3}$) catalase (58), fumarase (49) and lactate dehydrogenase (36).

TABLE II

CHARACTERISTICS OF THREE HPLC-QUALITY CATION-EXCHANGE GELS

Gel designation	Gel source	Cation exchanger type	Functional group	Particle size (μm)	Pressure[a] (MPa)
Mono S	Pharmacia	Strong acidic	$-CH_2SO_3^-$	10	1.6–2.1
TSKgel SP 5PW	TosoHaas	Strong acidic	$-(CH_2)_3SO_3^-$	10	1.0–1.1
LiChrospher 1000 SO_3^-	Merck	Strong acidic	$-(CH_2)_3SO_3^-$	5	3.2–4.9

[a] Back-pressure at 0.5 ml/min found for a 5 mm I.D. column. For sample load and buffer, see Experimental.

as employed previously with untreated marmosets [7], did not improve the separation in the sense of increasing the apparent homogeneity, but did, of course, improve the yield of either fraction.

The appearance of more gradient peaks and SDS-PAGE bands here than those found by Funae and Imaoka [9] for Sprague–Dawley rats may possibly indicate for marmosets the presence of a greater multiplicity of P450 forms.

Comparison of various HPLC-quality cation exchangers

As explained above for anion exchangers, three cation exchangers were also examined (Table II and Fig. 4). The profiles consist mainly of two peaks and are all much less well defined than any of those seen for anion exchangers. In spite of such overlapping profiles, SDS-PAGE revealed that the production of some homogeneous P450 fractions was still possible. Here, it is evident that all three columns delivered a P450 fraction with $M_r \approx 50\,000$–$51\,000$.

Surprisingly, Mono S and TSKgel DEAE 5PW are apparently more similar in that a greater degree of P450 homogeneity is seen in the main, middle regions of the chromatograms, whereas for LiChrospher 1000 SO_3^- this holds true for the very first peak. The back-pressures encountered for the 5-μm gel require either lower flow-rates than those used for the other gels (0.5 ml/min for an HR 5/5 column) or HPLC pumps capable of delivering pressures exceeding 5.0 MPa.

Overall, under these conditions, Mono S appeared to provide the electrophoretically purest P450 fractions and, in addition, the largest number of these per run.

Detergent removal and comparison of various hydroxyapatite gels

Prior to analyses of different forms of purified P450, residual amounts of the non-ionic detergent Emulgen 911 were removed on hydroxyapatite. This also effects a lower concentration of TCDD, where decreased stoichiometric ratios of TCDD to P450 were observed (see below). This is also the case with cation-exchange chromatography, where some detergent (but no P450) is eliminated in the pass-through fraction. In the rat, such ratios (for P4501A2 [16]) were mostly found to approach unity and the binding was shown to be very tight [17,18]. The significance of the ratio and the strength of TCDD binding for these primate enzymes is at present not clear, however.

Examination of four commercially available gels and one that we synthesized according to the literature [10] was undertaken because, as pointed out earlier, we had to rely on good yields. In past experience [5,7,11], we have found that however high the yields of P450 subsequent to ion-exchange and hydrophobic interaction chromatography may be, final removal of excess of non-ionic detergent via hydroxyapatite can result in P450 losses of up to 50%. The present experiment was done to ascertain optimum flow-rates (resulting in the highest recoveries of detergent-free P450) and capacities (derived from optimum flow-rates) for each gel (Table III). Recovery in this sense concerns the concentration of spectroscopically intact enzyme determined from difference spectra.

For this purpose, optimum flow-rates and the absolute values of the recoveries themselves were found to differ enormously (Fig. 5). Lower recoveries at lower flow-rates and prolonged contact with the gel are presumably due to more thermal degradation and/or loss of haeme [19]. All back-pressures for all columns at all flow-rates remained constant with the exception of Bio-Gel HTP gel, which repeatedly resulted in strong gel compression and increased back-pressure (>5 MPa), thus allowing only one run per column filling. For these enzymes and at this temperature, Hydroxylapatite HPLC Grade (Calbiochem) gave the highest recoveries of P450 applied. These were apparently but reproducibly found to reach 100%. It may be noted that excellent recoveries were also obtained at flow-rates 5–10 times that recommended by the manufacturer (Fig. 5 and Table III).

Capacities also varied considerably and are given in Table III. The highest value was found for TSKgel HA-100 and represented 1.5–3 times that found for other hydroxyapatite gels.

Properties of purified P450 forms

Anion- and cation-exchange chromatography on Mono Q and Mono S columns resulted in the isolation of seven different fractions of P450 from liver microsomes of TCDD-induced marmosets (Table IV). The total recoveries among these forms were found to vary considerably, this probably

TABLE III

CHARACTERISTICS OF VARIOUS TYPES OF HYDROXYAPATITE USED FOR REMOVAL OF RESIDUAL NON-IONIC DETERGENT FROM P450 FRACTIONS

For sample source and detergent, see Experimental.

Gel designation	Gel source	Particle size (μm)	Recommended linear flow-rate[a] (ml/h · cm²)	Recommended flow-rate[b] (ml/min)	Optimum flow-rate found[c] (ml/min)	Recovery found[d] (%)	Capacity found[e] (nmol/ml)
Bio-Gel HTP	Bio-Rad	10	25–100	0.3–1.3	1.5	53	18
Hydroxyapatite HPLC Grade	Calbiochem	20	15	0.2	0.5–1.0	100	38
HA-Ultrogel	IBF	60–180	5–30	0.07–0.4	0.1	48	20
Granulated hydroxyapatite	Mazin *et al.* [10]	200–250	80	1.0	0.5–1.0	73	29
TSKgel HA-1000	TosoHaas	5	60–120	0.8–1.6	1.5	70	57

[a] By manufacturer for elution purposes.
[b] For a 10 mm I.D. column.
[c] For 6.4 nmol of P450 loaded on to a 1.27 cm × 10 mm I.D. (1-ml) column; flow-rate giving the highest recovery of detergent-free P450 shown in Fig. 5.
[d] Recovery at optimum flow-rate; column and load as above.
[e] Amount of detergent-free P450 in nmoles per ml of gel elutable after 100 nmol were loaded and washed.

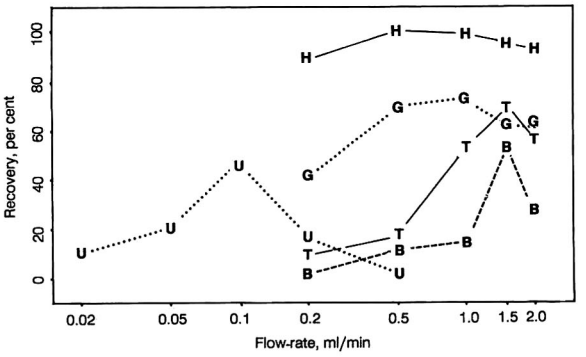

Fig. 5. Removal of excess of non-ionic detergent via hydroxyapatite. Five types of hydroxyapatite gels were laboratory-packed in HR 10/2 columns (1.27 cm × 10 mm I.D. = 1.0 ml), equilibrated, loaded, washed and eluted as described under Experimental. Sample load for each run: 6.4 nmol of an 8-aminooctyl-Sepharose pool of phenobarbital-induced Wistar rat P450 gel filtered on NAP-25 cartridges using the hydroxyapatite equilibration buffer containing in addition 0.5% Emulgen 911. After successive runs, each at flow-rates between 0.02 and 2.0 ml/min, the recovery of detergent-free P450 in percent (based on the load of P450 applied) as a function of flow-rate was determined. Temperature for all runs: 25°C. Symbols: B = Bio-Gel HTP; G = Granulated Hydroxyapatite; H = Hydroxylapatite HPLC Grade; T = TSKgel HA-1000; U = HA-Ultrogel.

reflecting the wide variation in the relative contents of each form originally present. Such diversity has already been found for HPLC-purified P450s isolated from rats treated with similar inducing agents such as 3-methylcholanthrene [9,20].

The degree of purity expressed as specific contents among the P450 forms found in the present work varied between 9.2 and 16.1 nmol per milligram of protein (Table IV). Fig. 6 shows SDS-PAGE results for these forms, where it is evident that at this stage forms A, F and 1 are not homogeneous. As mentioned above, fraction E was not included in this or other assays as it had been identified spectroscopically as cytochrome b_5.

M_r values ranged between 49 000 and 56 000 (Table V). Hence there is no striking difference to the M_r ranges found in different laboratories for a number of different rat P450s [4,9,19].

Marmoset P450s having the lowest apparent values for absorption maxima in CO–dithionite difference spectra (447 and 448 nm) may possibly correspond to one or both of those main forms P450 1A1 and 1A2 inducible in the rat which have the

TABLE IV

P450 FORMS PURIFIED FROM TCDD-TREATED AND UNTREATED MARMOSETS

P450 forms A, B, C, D and F were derived from Mono Q gradient fractions. Fraction E was cytochrome b_5. Forms 1 and 2 were derived from Mono S chromatography of the Mono Q pass-through (PT) fraction. Residual non-ionic detergent was removed from all fractions via hydroxyapatite chromatography. AO = 8-Aminooctyl-Sepharose.

Source	Total protein (mg)	Total content (nmol)	Specific content (nmol/mg)	Recovery (%)
TCDD-treated microsomes	7110	775	0.109	100
AO pool	874	682	0.78	88
Mono Q				
PT	70	333	4.9	43
Form A	2.5	23.2	9.2	3.0
Form B	0.57	8.5	14.8	1.1
Form C	0.34	4.7	13.7	0.6
Form D	2.3	31.0	13.5	4.0
Form F	1.4	17.8	12.4	2.3
Mono S				
Form 1	2.6	40.6	15.8	5.2
Form 2	4.4	71.5	16.1	9.3
Untreated microsomes	828	77.0	0.093	100
AO pool	101	55.4	0.55	72
Mono Q				
PT	6.1	20.0	3.3	26
Form A	0.89	7.7	8.7	10
Form D	0.46	5.1	11.1	6.6
Form F	0.64	2.8	4.4	3.7
Mono S				
Form 2	0.32	4.6	14.4	6.0

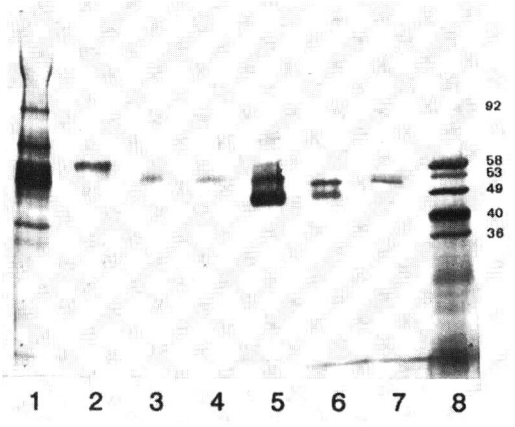

Fig. 6. SDS-PAGE gel of purified fractions of TCDD-induced marmoset P450s. The anode is at the bottom, and the 10–15% gradient gel was stained with silver [5]. Lanes: 1 = form A; 2 = form B; 3 = form C; 4 = form D; 5 = form F; 6 = form 1; 7 = form 2; 8 = marker proteins (with $M_r \times 10^{-3}$) phosphorylase *a* (92), catalase (58), glutamate dehydrogenase (53), fumarase (49), aldolase (40), lactate dehydrogenase (36).

same absorption values. As deduced from the absolute spectra, the contribution to the atypical high-spin state for TCDD marmoset form 2 may possibly relate this form to 1A2 forms found in rats [20] and rabbits [21]. Other forms in the marmoset may, however, correspond more closely to those of other gene families such as P450 3, as has been suggested for a form purified from polychlorinated biphenyl (PCB)-treated crab-eating monkeys [22].

Catalytic activities measured in reconstituted systems containing rat NADPH P450 reductase [5] and the substrates 7-ethoxyresorufin and 7-methoxyresorufin are also shown in Table V. These substrates have been shown to exhibit some specificity towards rat P450 forms 1A1 and 1A2, respectively [23,24]. In this work, it can be seen that forms C and D both have the highest values for these O-dealkylation rates. We therefore gather, for the time being, that one or both of C and D may possibly correspond to 1A1 and/or 1A2.

TCDD binding was monitored by liquid scintillation counting of the fractions, as it is known that a wide variety of inducing agents such as TCDD, pentachlorodibenzofuran (PCDF) and 3MC not only induce P450 but also bind tightly to it [17,18,25]. It was shown there that the highest values obtainable for (detergent-free) rat material were almost unity. Stoichiometric ratios of TCDD to (detergent-free) marmoset P450 are also given in Table V, where it can be seen that the highest value is 0.48 (for form C). Previous work in our laboratory with TCDD-treated rats and marmosets indicated, however, that ratios as high as 6.3:1 could be registered in solutions still containing some excess of non-ionic detergent (results not shown). As yet, we have no explanation for this phenomenon. Over and above the obvious difference in species, it may be conceivable that the dosage of TCDD-like inducing agents utilized plays a role; in this work, for marmosets (and in our previous work on rats), the doses used were 300–1000 ng/kg body mass, whereas for rats treated elsewhere [17,18,20] the doses were 320 μg/kg, 1 mg/kg and 5 mg/kg, respectively. The fact that three different inducing agents are compared (TCDD, pentachlorodibenzofuran and 3-methylcholanthrene) certainly also plays a role. We have found that, in contrast to rats, at doses of 300–1000 ng TCDD per kilogram body mass, an apparent plateau in inductive capacity of EROD activity was reached. Higher doses for marmosets were therefore not used.

Finally, it was considered important to discuss which forms of P450 isolated from TCDD microsomes may not have been TCDD induced but were already present in untreated microsomes. Comparisons of the chromatographic profiles of separations of TCDD-treated and untreated material indicated in a reproducible manner that forms D, F and 2 eluted in the same positions and in basically comparable yields. These three UT forms also had SDS-PAGE molecular masses and difference spectra absorption maxima equivalent to those of TCDD forms. Judging from such biophysical parameters, we conclude that in TCDD-treated marmosets, forms D, F and 2 are probably constitutive. Of all the UT fractions, only UT form D had an appreciable EROD activity (39 pmol of product/min nmol of P450) which is, however, only one tenth of that seen for the TCDD form D (Table V).

We have recently found that monoclonal antibodies raised against purified rat P4501A2 reacted with both TCDD-D and UT-D forms (results to be published elsewhere). Accordingly, this type of P450 form could be classified as constitutive although inducible. A similar phenomenon has been reported

TABLE V

ELECTROPHORETIC, CATALYTIC, SPECTRAL, MAGNETIC AND TCDD-BINDING PROPERTIES OF P-450 FORMS PURIFIED FROM TCDD-TREATED MARMOSETS

For P450 form designations, see Table IV. Residual non-ionic detergent was removed from all fractions via hydroxyapatite chromatography. Abbreviations and explanations: apparent molecular masses were as judged by SDS-PAGE; MROD, EROD = rate of methoxy- or ethoxyresorufin O-dealkylation in pmol of product/min · nmol of P450 from a reconstituted system containing NADPH–P450 reductase; the concentration of reductase was 10 μmol (cytochrome *c* reduced)/min per nmol P450; absorption maximum (in nm) of iron(II) carbonyl complex; L, H = low or high magnetic spin state as deduced from absolute spectrum of oxydized solution; TCDD binding = molar ratio of TCDD-to-P450.

P450 form	Molecular mass	MROD	EROD	Absorption maximum	Spin state	TCDD binding
A[a]	50 000–54 000	15.1	5.3	448–449	L	0.03
B	56 000	2.9	28.5	450	L	0.10
C	53 000	101	125	447	L	0.48
D	52 000	282	431	447	L	0.12
F[a]	48 000	2.3	27.7	452	L	0.09
1[a]	49 000+51 000	<0.2	<0.2	448–451	L	0.003
2	51 500	1.1	37.0	448	L+H	0.096

[a] Non-homogeneous.

for human liver, where a large amount of the microsomal P450 was shown to comprise P450 1A2 [26]. Altogether, adult [27] and prenatal human livers [28] have been shown to contain a great diversity of P450 forms.

Control over induction in humans is, of course, difficult or impossible in most instances, and to this end we consider the marmoset a potential model for assessing the treatment of primates by xenobiotics.

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CHROMSYMP. 2569

Activation of topoisomerase II during partial purification by heparin–Sepharose chromatography

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ABSTRACT

Partial purification of topoisomerase II from small samples (10^7–10^8 cells) of human leukaemic cells was achieved by isolation of cell nuclei, hyper-osmotic extraction of nuclear proteins, sorption of nuclear proteins by heparin–Sepharose and elution with potassium phosphate. Similar results were obtained by gradient and batchwise elution. The calatylic activity of topoisomerase increased *ca.* eightfold after removal of *ca.* 95% of the contaminating nuclear proteins. The conserved enzymatic activity after partial purification indicates that the enzyme was not damaged. The half-life of enzymatic activity is increased by the chromatographic procedure. Owing to its high yield and technical simplicity, this could be a candidate procedure for the study of topoisomerase II in patient-derived blood samples.

INTRODUCTION

Recently, the analysis of DNA topoisomerase (topo) II has come into the focus of oncological interest because many potent anti-cancer drugs formerly regarded as DNA-intercalating or chromosome-fragmenting agents are now known to act as topo II inhibitors [1]. The function of this enzyme system appears to be to disentangle chromosomal DNA during replication and transcription by cleaving both strands of the DNA helix [2]. The enzyme forms a protein bridge across the ends of the divided DNA molecule until continuity is restored, but topo II inhibitors stabilize the DNA–protein complex so that the normally rapid process of strand division, disentangling and rejoining is arrested at mid-stage [3]. Much, if not all, of the cytotoxic acitivity of podophyllotoxins, anthracyclins, mitoxantrone, *m*-amsacrine and a number of other cytostatics is attributable to this specific interaction with topo II [1]. Two forms (α and β) have been recognized in mammalian cells, which are encoded by separate genes and differ biochemically but also with respect to drug sensitivity [4]. Resistance to topo II inhibitors is known to develop in both forms by point mutations in the 5′-triphosphate (ATP)-binding domain of the enzyme [5,6]. In addition, the drug sensitivity can be modulated by post-translational modifications, such as phosphorylation [7,8]. Recently, we observed that both forms of topo II, purified from a human leukaemic cell line, can be further fractionated by anion-exchange chromatography [9] and that the fractions differ considerably with respect to drug sensitivity and optimum reaction [10]. In the treatment of human leukaemia the pre-/peri-therapeutic study of drug sensitivity therefore necessarily includes the preparative fractionation of topo II and the separate analysis of the fractions. For this purpose, we have developed an extraction and partial purification procedure that might be suitable for preparing nuclear topo II from patient-derived samples of leukaemic cells in a routine clinical setting. The emphasis in this study is on the miniaturization and the optimization of a single step of heparin–Sepharose chromatography sufficient for preparing partially purified topo II

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from very small samples of leukaemic cells. The structural and functional characterization of this partially purified enzyme preparation is described.

EXPERIMENTAL

Preparations

The culture of human leukaemic HL-60 cells, isolation of nuclei and extraction of nuclear proteins by 400 m*M* NaCl was carried out as described previously [9]. All buffers for chromatography contained 10% glycerol, 100 m*M* NaCl, 12 m*M* β-mercaptoethanol, 0.5 m*M* phenylmethylsulphonyl fluoride, 1 μg/ml diisopropyl fluorophosphate, 0.1 m*M* benzamidine, 1 mg/ml soybean trypsin inhibitor and 0.5 m*M* EGTA. For heparin–Sepharose chromatography, prefilled 2 × 1 cm I.D. columns (HiTrap-Heparin, 1 ml; Pharmacia, Uppsala, Sweden) were used. Loading, washing and elution were performed at 4°C, using either an FPLC-System (Pharmacia) and a flow-rate of 1 ml/min or a Luer-lock syringe and a flow-rate of 3 drops/s. Usually, 5–7 ml of nuclear extracts were diluted 1:10 with ice-cold 5 m*M* potassium phosphate (pH 7.5) and immediately loaded on to the columns, followed by washing with 6 ml of 5 m*M* potassium phosphate. Bound proteins were either eluted with a 15-ml linear gradient from 5 to 600 m*M* potassium phosphate, as specified later. Eluted proteins were stored at −20°C after addition of concentrated glycerol to a final concentration of 60%. Further purification by gel chromatography was carried out using a 60 × 1.6 cm I.D. Superose 200 column (Pharmacia), which was equilibrated and developed with 50 m*M* Tris (pH 7.5) at a flow-rate of 1.7 ml/min. Fractions of 2 ml were collected and analysed.

Assays

Topo II catalytic activity was detected by ATP-dependent relaxation of 500 ng of pBR322 plasmid DNA as described previously [9,10]. It was determined by serial dilution of the enzyme and expressed as units. One unit was defined according to ref. 11 as the amount of topoisomerase that will give a 90% relaxation of 500 ng of pBR322 plasmid DNA under the described conditions [9]. As a control for topo II-specific activity, phage P4 DNA unknotting or decatenation of kinetoplast DNA was also measured [12,13].

Immunoblot analysis of topoisomerases was carried out as described previously [9]. Protein concentrations were determined according to Peterson [14], and the protein composition was studied by sodium dodecyl sulphate–polyacrylamide (7.5%) gel electrophoresis (SDS-PAGE) [15], followed by silver staining [16].

RESULTS

Topo II from human leukaemic cells strongly binds to heparin-Sepharose at low salt concentrations. After loading the crude extract from *ca.* 10^9 nuclei on to a 1-ml HiTrap-heparin column, only traces of the enzymatic activity were found in the effluent and wash fractions, which contained *ca.* 95% of the nuclear proteins (Fig. 1). Topo II was eluted from the heparin column with 300–400 m*M* potassium phosphate as a single, sharp peak. Interestingly, the total recovery of activity usually significantly exceeded 100% (Table I), indicating that the enzyme is activated during chromatography. A similar result was also obtained when elution was carried out discontinuously, using 200 m*M* potassium phosphate for washing and 400 m*M* potassium phosphate for elution. As Fig. 2b shows (one of

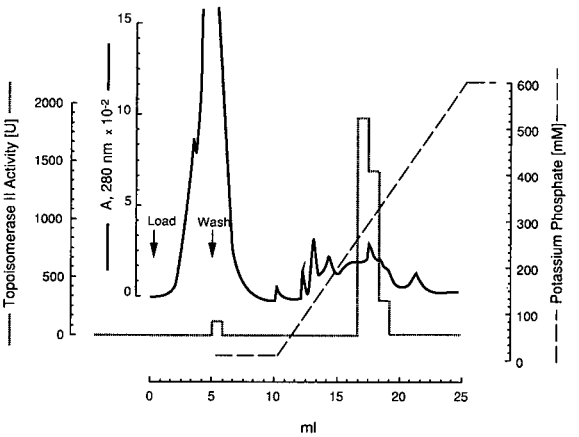

Fig. 1. Chromatography of crude nuclear extracts on heparin–Sepharose. A 5-ml portion of crude nuclear extract was loaded on to a 1-ml HiTrap-heparin column at a flow-rate of 1 ml/min. The column was washed with 5 m*M* potassium phosphate buffer (pH 7.5) and eluted with a 15-ml linear gradient from 5 to 600 m*M* potassium phosphate (pH 7.5). Fractions (1 ml) were assayed for pBR322 plasmid DNA relaxation activity in the presence of 2 m*M* ATP.

TABLE I

PARTIAL PURIFICATION OF TOPO II BY HEPARIN–SEPHAROSE CHROMATOGRAPHY

Mean results of four independent experiments ± standard error.

Fraction	Protein concentration (mg per 10^8 nuclei)	Specific activity of topo II (U/mg)	Recovery of topo II activity (%)
Crude nuclear extract	2.5 ± 1.6	96 ± 33	100
Heparin–Sepharose eluate	0.125 ± 0.078	15 667 ± 1385	816 ± 273

four similar experiments), large proteins (M_r 60 000–20 000) were preferentially recovered in the fraction eluted with 400 mM potassium phosphate.

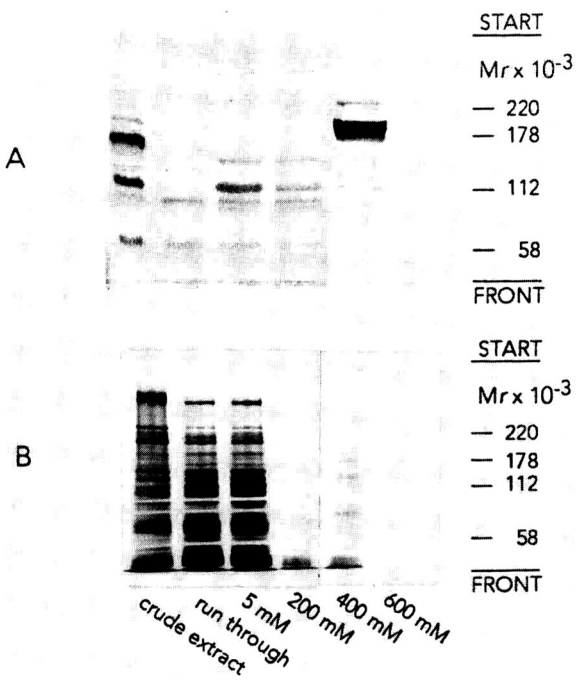

Fig. 2. Enrichment of topoisomerase II by a single-step batch chromatography on heparin–Sepharose. A 50-ml portion of crude nuclear extract, diluted tenfold with 5 mM potassium phosphate buffer (pH 7.5), was loaded on to a 1-ml HiTrap-heparin column with a syringe (3 drops/s). After washing with a 6-ml portion of the above buffer, bound proteins were eluted with 3-ml portions of potassium phosphate (pH 7.5, 200, 400 and 600 mM). Equal proportions of starting material, effluent, wash and eluted fractions were precipitated with 7.5% trichloroacetic acid and subjected to SDS-PAGE 7.5% gels. (A) Immunoblot analysis with rabbit–anti-human topoisomerase II antiserum and (B) silver staining of the precipitated proteins are shown.

By immunoblot analysis of the fractions (Fig. 2a) with a rabbit–anti-human topoisomerase II antiserum, a double band of M_r 170 000–180 000, equivalent to the known α- and β-forms of the enzyme [4], was recognized in crude nuclear extracts and in the fraction eluted by 400 mM potassium phosphate, but not in any of the other fractions. Crude nuclear extracts additionally contained various proteolytic fragments of topo II, which were also recognized by the antibody. These fragments did not bind to the heparin column or exhibited a weaker affinity than the intact enzyme, and they could be removed by washing with 200 mM potassium phosphate.

Titration of topo II pBR322 DNA relaxation activity is shown in Fig. 3 (one of four similar experiments). ATP-dependent DNA relaxation could be observed in crude extracts and in eluate fractions (400 mM potassium phosphate), but not in the effluent or in the wash fractions. The electrophoretic mobility of both relaxed and supercoiled pBR322 DNA was markedly reduced not only in the presence of high concentrations of crude extract or elution fraction but also in the 200 mM wash fractions (Fig. 3). The band-shift phenomenon indicates that some of the DNA-binding proteins present in crude extracts are removed during the chromatography. This might explain why the enzyme is at least eight times more active after removal of *ca.* 95% of the contaminant proteins. By the additive effect of activation and purification, a 160–200-fold increase in the specific activity is obtained in a single chromatographic step (Table I). The half-life of topo II activity in crude extracts was less than 3 days when stored at −20°C (in the presence of 50% glycerol), but it increased to 2 weeks after partial purification (not shown).

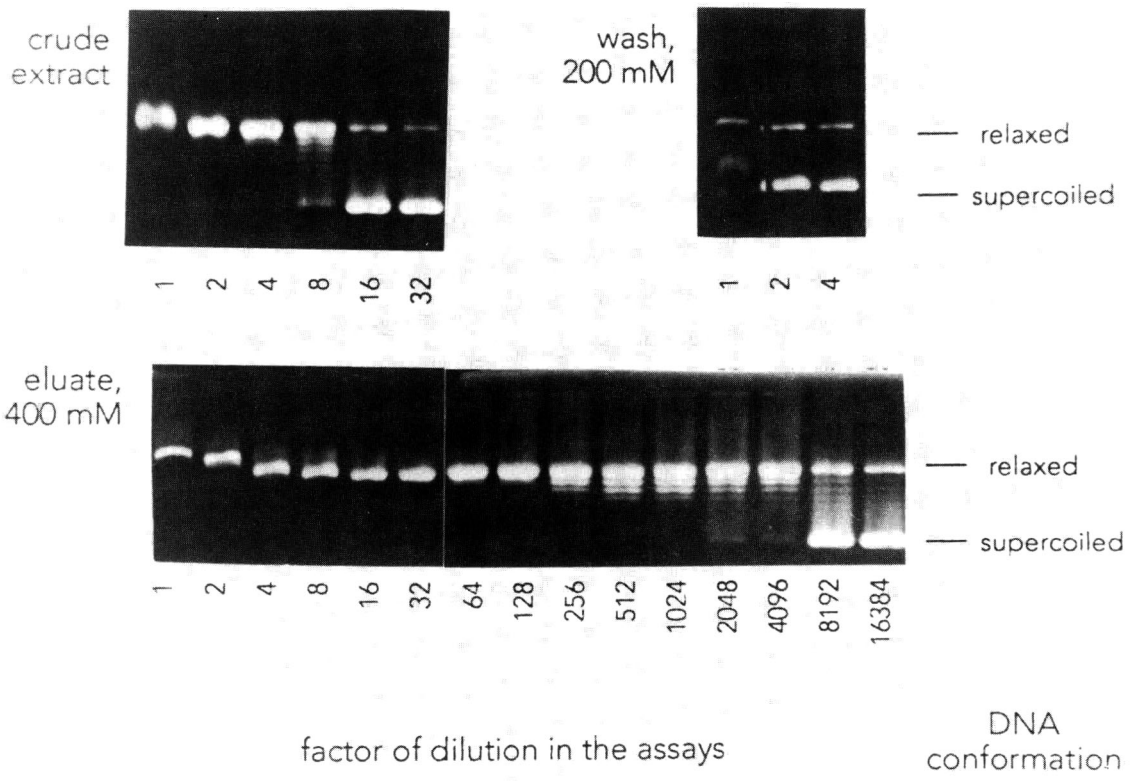

Fig. 3. pBR322 DNA relaxation activity of crude and partial purified topoisomerase II. Batch chromatography on heparin–Sepharose was caried out as described in the caption of Fig. 2. DNA relaxation activity was titrated by serially diluting the fractions prior to assessing the relaxation of 500 ng of pBR322 DBA. DNA conformers were separated by agarose gel electrophoresis and revealed by ethidium bromide staining. The results obtained with the crude extract and with the fractions eluted by 200 and 400 m*M* of potassium phosphate are shown.

DISCUSSION

A variety of purification methods have been described for topo II, all of which start from gram amounts of primary tissue [17,18] or litres of cultured cells [19,20] to obtain milligram amounts of homogeneous enzyme. The yield of these methods (from the cells to the homogeneous enzyme) is usually less than 5%. Whereas these procedures have been suitable for obtaining material for enzymological studies and for raising antibodies, they are of little use when the amount of starting material and/or analysis time are limited, as with patient-derived blood samples.

Studies of the effectiveness of topo II inhibitors in primary leukaemic cells require a certain degree of purification of the target protein for a number of reasons:

(i) As we have reported previously [10] and also shown here, DNA fragments and a variety of DNA-binding proteins present in crude nuclear extracts are potent inhibitors or modulators of topo II activity. The composition and concentration of these interfering substances varies from extract to extract. The determination of topo II activity in crude nuclear extracts is therefore very unreliable and prone to yield artifacts resulting from these variations in the simultaneously extracted nuclear protein matrix.

(ii) Hyperosmotic disintegration of the nucleus releases a high proteolytic potential, which can hardly be inhibited, even by elaborate preparations

of protease inhibitor. We have shown here that topo II quickly becomes degraded in crude nuclear extracts. Partial purification helps to remove proteolytic fragments and to increase the half-life of the enzyme activity.

(iii) We have previously shown [21] that nuclear topo II activity from leukaemic cells is highly dependent on the pH and salt concentration of the reaction medium. Moreover, these cells contain relatively low levels of topo II, so that the enzyme assay is not sensitive enough to allow dilution of the crude extract with the appropriate buffer sufficiently to optimize the assay conditions. Therefore, it is necessary to concentrate the sample prior to the enzyme assay.

The procedure described fulfils the above needs with a single-step heparin–Sepharose batch chromatography. By this procedure the enzyme activity is enhanced eight-fold and the protein concentration is decreased twenty-fold. The increase in specific enzyme activity is virtually 160-fold. Judging from immunoblot analysis, the partially purified enzyme is free from proteolytic degradation products.

This purification procedure is within the capability of a routine clinical application because it is fast (less than 3 h), relatively simple, highly reproducible (prefilled ready-to-use chromatographic columns are commercially available) and does not require complex laboratory equipment. It should be equally suitable for research applications, because it can easily be scaled up by using a larger size of the same brand of prefilled columns. A further purification of topo II to more than 50% homogeneity can be obtained by a single step of gel filtration or anion-exchange chromatography [9].

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CHROMSYMP. 2576

Angiotensinase A (aminopeptidase A): properties of chromatographically purified isoforms from human kidney

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ABSTRACT

Angiotensin-II-cleaving angiotensinase A (aminopeptidase A, E.C. 3.4.11.7, ATA) plays an important role in glomerular haemodynamics, the pathophysiology of essential arterial hypertension and the induction of vascular disorders. In order to study biochemical and immunological properties of ATA, two isoforms (I and II) of the glycoprotein were isolated for the first time from human kidney cortex. Kidney cortex homogenate, digested with bromelain, was fractionated by ammonium sulphate precipitation and subsequent hydrophobic interaction chromatography, using a fast protein liquid chromatographic (FPLC) system. By anion-exchange FPLC (Mono Q column), the isoforms of ATA were eluted in two distinct peaks and were further purified by size-exclusion FPLC and preparative polyacrylamide gel electrophoresis. Biochemical, immunological and immunohistological characterization disclosed differences in the intrarenal localization, glycosylation, Michaelis constant and apparent molecular mass (native and sodium dodecyl sulphate gel electrophoresis) but similar properties in the double-immunodiffusion technique. Polyclonal rabbit antibodies, raised against ATA isoforms I and II, precipitated an analogous antigen in urine from patients with renal tubular damage.

INTRODUCTION

Angiotensinase A [1,2] (ATA, aminopeptidase A [3], E.C. 3.4.11.7) is a membrane protein with a characteristic distribution pattern in human kidney: ATA is predominantly localized in glomerular endothelia and visceral epithelial cells (podocytes) and, to lesser extent, in the brush border (BB) region of the proximal tubule (PT) [4]. This appears to be similar to endopeptidase-24.11 (E.C. 3.4.24.11), which is identical with the common acute lymphoblastic leukaemia antigen (CALLA or CD10) [5]. In contrast to ATA, other kidney marker proteins, such as aminopeptidase M (APM, E.C. 3.4.11.2, identical with CD13 [6]), dipeptidylpeptidase IV (DPP IV, E.C. 3.4.14.5, clustered as CD26 [4,7]) or γ-glutamyltransferase (GGT, E.C. 2.3.2.2) [8] reveal histochemical activity in the brush border region of the proximal tubule only. Human kidney ATA contains N-acetyl-α-D-glucosamine to bind specifically to wheatgerm agglutinin (unpublished results). Previous studies suggested that ATA is involved in the regulation of the intrarenal renin–angiotensin system [9]. The octapeptide angiotensin II (ANG II) is N-terminal-specifically hydrolysed to the heptapeptide angiotensin III (ANG III) [10], and staining of kidney cryosections for ATA activity is inhibited after preincubation with ANG II but not after ANG I and III [11]. Hence, it seems reasonable to assume that the specific cleavage of ANG II to ANG III by ATA modulates the physiological and pathophysiological actions of ANG II reviewed in ref. 12. In this paper we describe, for the first time, the application of fast protein liquid chromatography (FPLC) to the isolation and characterization of isoforms of ATA from human kidney cortex.

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EXPERIMENTAL

Reagents

Bromelain, Fast Blue B salt, L-α-glutamyl-*p*-nitroanilide (-pNA), L-alanine-pNA, L-Ala-4-methoxy-2-naphthylamide (-MNA), neuraminidase (*Vibrio cholerae*) and bovine serum albumin (BSA) were obtained from Serva (Heidelberg, Germany), H-glycylprolyl-pNA and L-α-glutamic acid-MNA from Bachem (Bubendorf, Switzerland), aquacide I (carboxymethylcellulose) from Calbiochem (La Jolla, CA, USA) and ABM-ZK adjuvant from Linaris (Bettingen, Germany). Peroxidase (POD)-conjugated goat anti-rabbit IgG antibodies were purchased from Dako (Hamburg, Germany) and 3-amino-9-ethylcarbazole (AEC) and agarose from Sigma (Deisenhofen, Germany). Carboxymethylindocyanine (Cy3)-conjugated AffiniPure goat anti-rabbit IgG (H + L chain) antibodies for immunofluorescence were obtained from Jackson ImmunoResearch (via Dianova, Hamburg, Germany). Other reagents were of analytical-reagent grade.

Chromatographic equipment

The FPLC system, manufactured by Pharmacia Biosystems (Freiburg, Germany), consisted of an LCC-500 Plus liquid chromatography controller, two P-500 pumps, an MV-7 motor valve, a PSV-100 valve, a P-1 peristaltic pump, a FRAC-100 fraction collector, a UV-M monitor (Hg optics, 280 nm) and an REC-482 dual-chart recorder. The components were mounted in the chromatography rack II together with the following prepacked columns: Mono Q HR 5/5 (0.98 ml), phenyl-Superose HR 5/5 (0.98 ml) and Superose 12 HR 10/30 (23.5 ml). Two XK 26/20 columns were laboratory packed with 67 ml of phenyl-Sepharose CL-4B and 53 ml of Q-Sepharose FastFlow.

Preparation of a crude ATA fraction

Cortex from normal human kidneys, obtained from the Pathology Department, was homogenized using an Ultraturrax RW38 homogenizer (Janke & Kunkel, Staufen i. Br., Germany). The tissue homogenate was digested with bromelain [ratio of renal protein to bromelain = 30:1 (w/w), shaking for 30 min at 37°C] and centrifuged at 13 000 *g* for 20 min at 4°C [13]. The bromelain supernatant then was fractionated with 60% and 80% ammonium sulphate, the 80% pellet was dissolved in a small volume of high-salt buffer [0.05 *M* Tris–HCl (pH 7.2) containing 1.5 *M* $(NH_4)_2SO_4$] and divided into aliquot portions [14].

Hydrophobic interaction FPLC

Samples of 15 ml were loaded on to a 67-ml phenyl-Sepharose CL4B column, integrated into the FPLC system. Eluted proteins were collected in 10-ml fractions during a linear decreasing ammonium sulphate gradient in 0.05 *M* Tris–HCl buffer (pH 7.2) at a flow-rate of 2 ml/min. ATA-active fractions were pooled, rechromatographed, dialysed against 0.02 *M* Tris–HCl buffer (pH 7.2) and concentrated 14-fold against Aquacide I [13].

Anion-exchange FPLC

This preparative-scale separation was performed with a 53-ml Q-Sepharose FastFlow column (analytical scale, Mono Q HR 5/5) using a combined pH–NaCl gradient in 0.02 *M* Tris–HCl with two slopes: from 0 to 0.25 *M* in 200 min, then to 1 *M* in 25 min at a flow-rate of 2 ml/min, with a pH of 7.2 at 0 *M* NaCl and a pH of 7.7 at 1 *M* NaCl. The sample volume was 5 ml, and eluted proteins were collected in 10-ml fractions. ATA-active fractions were pooled and concentrated 40-fold by vacuum dialysis against the buffer of the next purification step.

Size-exclusion (SEC) FPLC

For each batch we applied 0.2 ml of sample to a Superose 12 column (HR 10/30), equilibrated with 0.1 *M* NaCl and 0.05 *M* Tris–HCl (pH 7.7) at a flow-rate of 0.2 ml/min to separate each ATA pool from accompanying proteins of different size (APM, DPP IV, GGT).

Preparative electrophoresis

Native samples were applied to laboratory-cast discontinuous polyacrylamide gradient gels (3–20% T) in a Desaphor VA chamber (System Havana; Desaga, Heidelberg, Germany), and separated for 7 h at 500 V and 250 mA [15]. Thin lanes of the run gel were stained for enzyme activity (ATA, APM, DPP IV, GGT) to locate separated proteins. The ATA band was excised and then homogenized by squeezing it vigorously through a syringe.

Polyclonal antibodies (PAB)

The homogenized gel band was suspended in ABM-ZK adjuvant and injected (intramuscular, subcutaneous) into rabbits (White New Zealand strain, fed tap water and standard food *ad libitum*). Twelve days after each of six immunizations up to 40 ml of blood were taken and the rabbit was finally bled out. A crude IgG fraction was obtained by serum precipitation with caprylic acid [16], followed by dialysis and concentration of the soluble fraction against PBS in a Minitan ultrafiltration unit (exclusion size 30 kilodalton) (Millipore, Eschborn i. Ts., Germany).

Determination of enzyme activities

For quantitative determination, individual peak fractions were assayed kinetically in a PM-4 spectrophotometer (Zeiss, Oberkochen, Germany) at 405 nm and 37°C. A 1-ml volume of ATA assay medium contained 1.66 m*M*/l substrate (L-α-Glu-pNA), 20 μ*M* Tris–HCl buffer (pH 7.7), 25 μ*M* Ca^{2+} and 0.1 ml of the sample. One enzyme unit was defined as the amount of enzyme catalysing the formation of 1 μ*M*/min of pNA (37°C). For the APM assay the same buffer (without Ca^{2+}, pH 8.0) and 1.66 m*M*/l L-Ala-pNA were used. DPP IV was determined with 5 m*M*/l Gly–Pro-pNA and 20 μ*M* Tris–HCl (pH 8.5), whereas GGT was assayed with 2.9 m*M*/l L-γ-Glu-3-carboxy-4-NA (Monotest new, Boehringer, Mannheim, Germany). Fast semi-quantitative screening of chromatographic fractions of ATA, APM, DPP IV and GGT was performed in flat-bottomed microtitre plates (Nunc, Wiesbaden, Germany) with a plate photometer (Flow, Meckenheim, Germany) at 405 nm and room temperature, using the same assay media as mentioned above. Michaelis constants were determined for ATA I and II with L-α-Glu-pNA according to standard methods; each point on the Lineweaver–Burk plot was the mean of three values.

Protein determination

Total protein was determined at 562 nm according to the method of Smith *et al.* [17] using bicinchoninic acid (Sigma) and BSA as a standard.

Electrophoretic analyses

Native polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulphate (SDS) PAGE, titration curve analysis (TCA) and isoelectric focusing (IEF) were performed using the PHAST system equipment (Pharmacia) according to the PHAST Application Files Nos. 100 and 110. For native PAGE, 1-μl samples and PHAST native buffer strips were applied to PHAST 8–25% gradient gels and run at 400 V, 10 mA, 300 V h and 15°C. SDS-PAGE was carried out in the previously described way [18]. Run gels were developed by either the sensitive silver staining method (Application File No. 210) or by staining for ATA activity [14].

Semi-dry electroblotting

After native or SDS-PAGE, gels were placed in the PhastTransfer apparatus (Pharmacia), connected to a PHAST electrophoresis unit, and separated proteins were transferred to an Immobilon P membrane (blotting time 1 h; Milipore). Free binding sites on the membrane were blocked by incubation for 1 h in buffer containing 20 m*M* Tris–HCl (pH 7.5), 0.2 *M* NaCl and 5% BSA. Labelling of blotted protein bands with diluted PAB (anti-ATA I 1:3000, anti-ATA II 1:2000) took place overnight at 4°C. Rabbit antibodies were detected with POD-conjugated goat anti-rabbit antibodies, followed by incubation with AEC substrate, by the red colour produced.

Double immunodiffusion

In gels of 1% agarose, cast on glass plates, seven wells (one in the centre) were punched to take samples of 20 μl each. The centre well was filled with purified antiserum to ATA I and II, whereas the outer wells were supplied with various antigen solutions. After incubation overnight in a humid chamber at 37°C, the gels were rinsed with isotonic NaCl solution three times for 2 h each to remove unbound protein and then dried in an oven at 45°C. The dried gels were subsequently stained for ATA activity to reveal the precipitation lines.

Immunohistochemistry

Acetone-dried cryosections (6–7 μm) of normal kidney tissue were incubated for 1 h in a humid chamber at 25°C with 100–150 μl of anti-ATA I or II PAB diluted 500-, 1000- and 2000-fold. Between the incubation steps, slices were rinsed with phosphate-buffered saline (PBS) for 10 min. For detection, goat anti-rabbit IgG antibody was used, which

TABLE I

PURIFICATION OF ANGIOTENSINASE A ISOFORMS I AND II FROM HUMAN KIDNEY

Fraction	Total protein (mg)	Total activity (U)	Total volume (ml)	Specific activity (U/mg)	Yield (%)	Total purification (-fold)
Tissue homogenate	40 749	1281	1400	0.031	100	1
Bromelain supernatant	13 073	1022	1260	0.078	79.7	2.5
80% ammonium sulphate sediment	2160	786	67.5	0.364	61.3	11.7
Phenyl-Sepharose	142	363.4	238	2.56	28.4	82.5
2nd phenyl-Sepharose	53.1	265.7	103	5.0	20.7	161.3
Dialysed and concentrated	51.9	319.8	7.5	6.16	24.9	198.7
Q-Sepharose: ATA I/ATA II	37.1 /3.1	102.1/34.9	3.6/1.4	2.75/11.42	10.7	88.7/368.4
Superose 12	8.15/1.01	53.9/19.27	17.2/10.5	6.61/19.1	5.7	213.2/615.5
Preparative native PAGE[a]	4[a]/0.5[a]	n.d.[b]	n.d.	12[a]/40[a]	n.d.	400[a]/1200[a]

[a] Approximate values.
[b] Not determined.

was either labelled with carboxymethylindocyanine (Cy3) for immunofluorescence or peroxidase-conjugated with AEC substrate. In the latter instance the cryosections were counterstained with haematoxyline.

RESULTS AND DISCUSSION

Purification of ATA isoforms

Results of the purification procedure are summarized in Table I. The first two purification steps, solubilization by bromelain and fractional ammonium sulphate precipitation, reduced the total protein to 5.3% of the initial protein content, while the ATA activity remained at 61.3%. To use the hydrophobic interactions under high-salt conditions between the gel matrix and the protein mixture, phenyl-Sepharose CL-4B and phenyl-Superose were chosen as separation media for the next chromatographic step (Fig. 1). Owing to broad and overlapping enzyme peaks, a complete separation of ATA from other peptidases was not possible, but the pro-

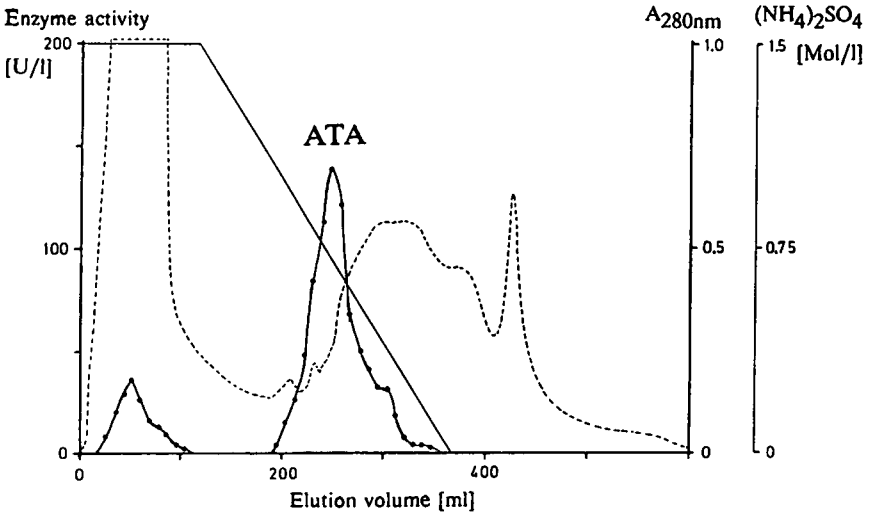

Fig. 1. Hydrophobic interaction chromatography (HIC) of the fraction obtained from $(NH_4)_2SO_4$ precipitation (60 and 80% saturation). Column, XK 26/20 laboratory packed with 67 ml of phenyl-Sepharose CL-4B and equilibrated with 1.5 mol/l $(NH_4)_2SO_4$ plus 50 mmol/l Tris–HCl (pH 7.7). Elution conditions: sample size, 15 ml; fraction size, 10 ml; flow-rate, 2 ml/min; column back-pressure, 0.5 MPa. Solid line = salt gradient; dashed line = protein absorbance; ● = ATA activity.

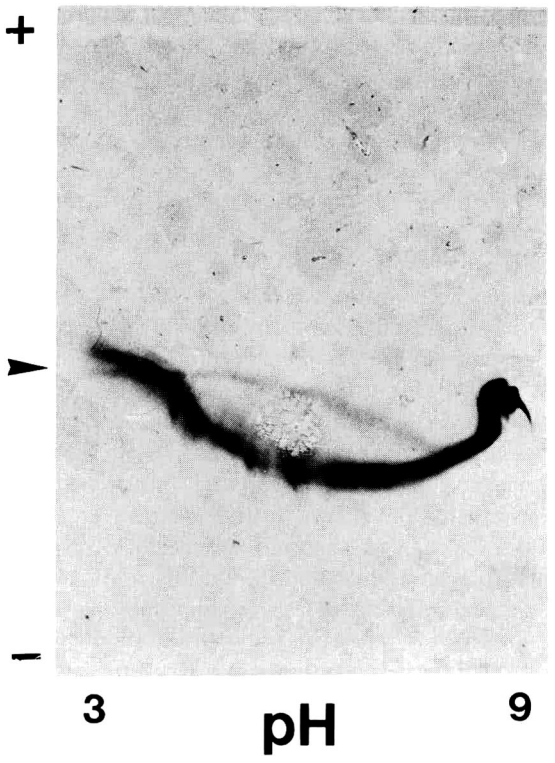

Fig. 2. Electrophoretic titration curve of the sample from Fig. 1. The curve was generated by prefocusing the ampholytes across a Phast IEF gel and subsequent electrophoretic separation of the sample components perpendicular to the pH gradient. Each component migrates towards the anode or cathode owing to its net charge at any particular pH. The arrowhead indicates the sample application line. The gel was stained for ATA activity.

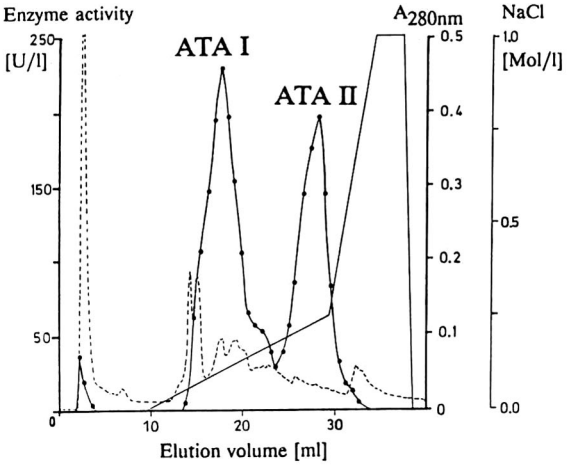

Fig. 3. Anion-exchange chromatography of pooled ATA fractions obtained from HIC. Column, HR 5/5 prepacked with 1 ml of Mono Q and equilibrated with 20 mmol/l Tris–HCl (pH 7.2) (buffer A). Elution conditions: buffer B, 20 mmol/l Tris–HCl (pH 7.7)–1 mol/l NaCl; sample size, 0.1 ml; fraction size, 500 μl; flow-rate, 1 ml/min; column back-pressure, 1.25 MPa. Solid line = salt gradient; dashed line = protein absorbance; ● = ATA activity revealing two peaks (designated ATA I and ATA II).

tein content was diminished to 0.12% of the starting material after rechromatography on phenyl-Sepharose. Removal of ammonium sulphate and any other inhibitors present by dialysis in Visking tubing (exclusion limit, 10 kilodalton) against Tris–HCl buffer (20 m*M*, pH 7.7) increased the enzyme activity and yield of ATA.

After titration-curve analysis (Fig. 2) in the pH range 3–9, we decided to perform anion-exchange chromatography on the Mono Q column under the previously described conditions. ATA isoform I was eluted at 110 m*M* NaCl, followed by APM, while isoform II left the column at 240 m*M* NaCl (Fig. 3). This step was scaled up on a 53-ml Q-Sepharose column (not shown) with similar resolution compared with Mono Q. Further purification of both isoforms was achieved by applying size-exclusion chromatography on Superose 12 (Fig. 4). This step accomplished adequate separation from accompanying GGT, especially for ATA II. The resolution of this method was too low to separate proteins of similar molecular size (APM, DPP IV, ATA) from each other.

However, after Superose 12 FPLC, the ATA I pool contained 54 U of ATA, but still 58 U of APM, 145 U of DPP IV and 7 U of GGT, while the ATA II pool contained 19.3 U of ATA, 2.6 U of APM, 1.2 U of DPP IV and 1.4 U of GGT. Even preparative native PAGE could not completely remove ATA from the other peptidases, because the protein bands were broad and overlapping (Fig. 5, right). Final data for the last separation step are approximate values (Table I), because the ATA activity and total protein were not quantified, but ATA active gel material was injected into rabbits for immunization. The remaining specific activity was approximately estimated, referring to the finally Coomassie Blue-stained preparative gel lane (not shown).

The rabbit antibodies obtained showed a major

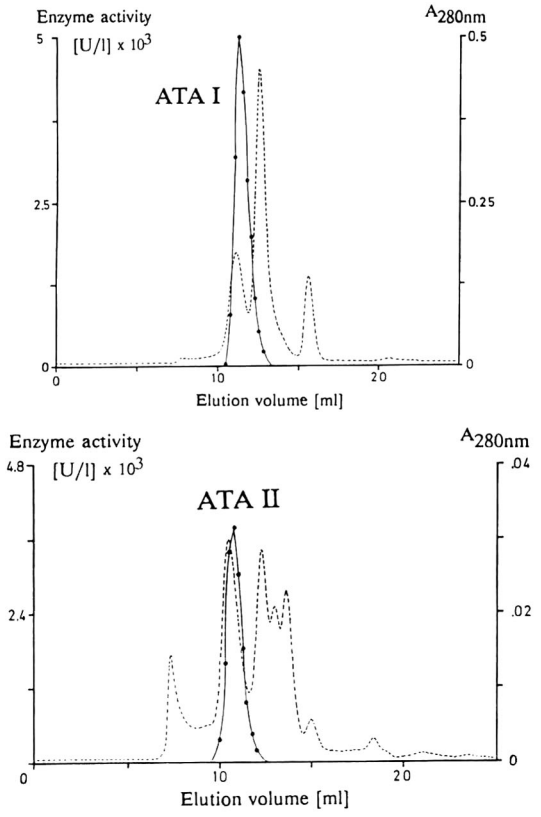

Fig. 4. Size-exclusion chromatography of pooled ATA I (top) and ATA II (bottom) fractions on a Superose 12 HR 10/30 column (23.5 ml). Elution conditions: eluent, 50 mmol/l Tris–HCl (pH 7.7)–0.1 mol/l NaCl; sample size, 0.2 ml; flow-rate, 0.2 ml/min; column back-pressure, 2 MPa. Dashed line = protein absorbance; ● = ATA activity.

immunoreactivity against ATA, but additional faint precipitates were found against APM and DPP IV (not shown), indicating some impurities of the ATA fraction used for immunisation. Silver-stained SDS-PAGE of ATA II revealed one band of 117 and one of 127.5 kilodalton (Fig. 6, lane 1), of which the smaller one was recognized by ATA I antibodies (lane 1′) whereas the larger one (127.5 kilodalton) was recognized by ATA II antibodies (lane 1″) as observed by immunoblotting. Data obtained by immunoblotting using *native* polyacrylamide gel electrophoresis is shown in Fig. 7. Antibodies against both isoforms of ATA precipitated urinary ATA from patients with tubular proteinuria after acute renal failure (Fig. 8), indicating a high degree of structural homogeneity between the two proteins. Nevertheless, determination of Michaelis constants disclosed a difference between ATA I and II: K_M (ATA I) = 2.05 mM, K_M (ATA II) = 0.87 mM. Isoelectric focusing (Phastgel pH 4–6.5) revealed another difference between the isoforms: pI_{ATAI} = 4.81, pI_{ATAI} = 4.69. Neuraminidase treatment showed no change in pI for ATA isoform I, b but a slight shift to 4.73 for ATA isoform II.

Furthermore, immunohistology of kidney cryosections showed different distribution patterns for ATA I and ATA II: while the luminal portions of proximal tubule epithelia disclosed immunoreactivity to both ATA I and ATA II, the glomerular tuft, recognized strongly by antibodies against ATA II, was negative or labelled only very faintly by antibodies against the ATA I isoform. However, ATA is present in many tissues and body fluids of various species (Table II), and determinations of molecular mass reveal different values, ranging from 45 (subunit) to 600 kilodalton (polymeric). Apart from this, in biophysical properties ATA is similar to the more abundant APM, and the separation of the two proteins is the major problem in its purification.

Kidney brush border peptidases, including ATA, are excreted into urine at an increased rate in patients with renal cell damage [11,29], representing another valuable source for their isolation and characterization [14,30]. Further, a murine monoclonal antibody of the IgG_1 subclass, recently generated against mouse ATA, caused severe albuminuria and immuno-complex deposits in the capillary wall after *in vivo* administration to mice [31]. This indicates a possible role of ATA as a pathogen, and further studies are in progress to evaluate the autoimmunological potential of the human ATA isoforms described here.

CONCLUSIONS

Protease-solubilized angiotensinase A was purified from human kidney cortex using fractional ammonium sulphate precipitation, hydrophobic interaction FPLC, anion-exchange FPLC, size-exclusion FPLC and preparative gel electrophoresis. ATA could not be separated completely from accompanying peptidases owing to the heterogeneity caused by bromelaine digestion. Despite this, it was possible to separate two isoforms of ATA which

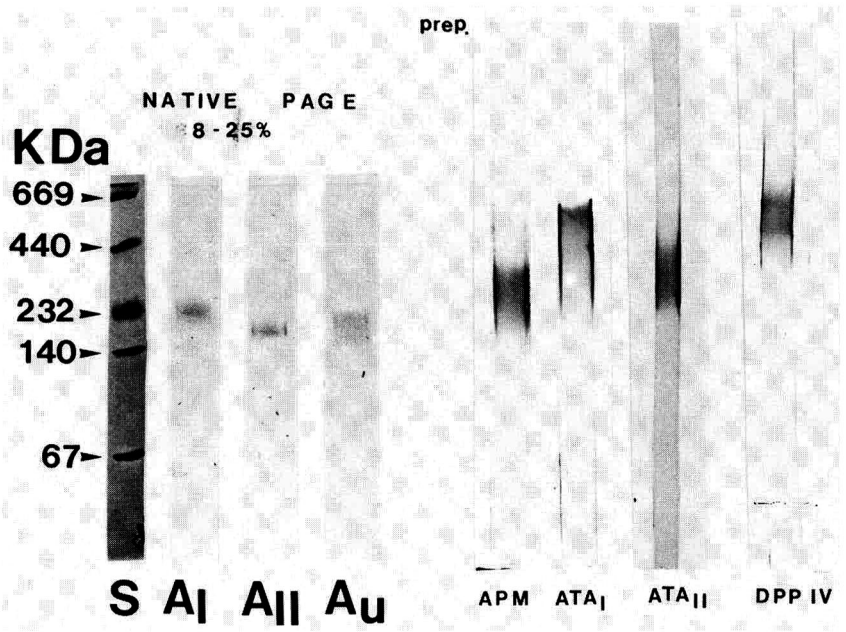

Fig. 5. Native PAGE of purified fractions using the Phast system (left) and Havana system (Desaga, 3–20% T, laboratory-cast preparative gel) (right). S = High-molecular-mass markers (Pharmacia); A_I and A_{II} = ATA I and ATA II Mono Q fractions; A_U = purified ATA fraction from human urine. Lanes on the right are fractions from size-exclusion chromatography stained for the indicated enzyme activity. KDa = kilodaltons.

TABLE II

SURVEY OF HITHERTO ISOLATED AND CHARACTERIZED ANGIOTENSINASE A (AMINOPEPTIDASE A) FROM VARIOUS SOURCES

M_r = molecular mass. Analytical ultracentrifugation according to Yphantis [32].

Source	Species	M_r (kilodalton)	Method	Ref.
Kidney cortex	Pig	45/90/110/155 270/350–400	SDS-PAGE GF[b]	19
Intestinal mucosa	Pig	120 247	SDS-PAGE Ultracentrifugation	20
Serum	Human	190	GF	21
Maternal serum	Human	260	GF	22
Serum	Human	210	GF	23
Kidney	Pig	300	GF	
Placenta	Human	600, polymeric	GF	24
Urine	Human	151 191	GF Native PAGE	30
Reproductive tissue	Boar, bull Gerbil, human	n.d.[a]	n.d.	25
Small intestine	Rabbit	n.d.	n.d.	26
Adrenal cortex capillaries	Rat	n.d.	n.d.	27
Cerebral micro-vessels	Pig	n.d.	n.d.	28

[a] No data.
[b] Gel filtration.

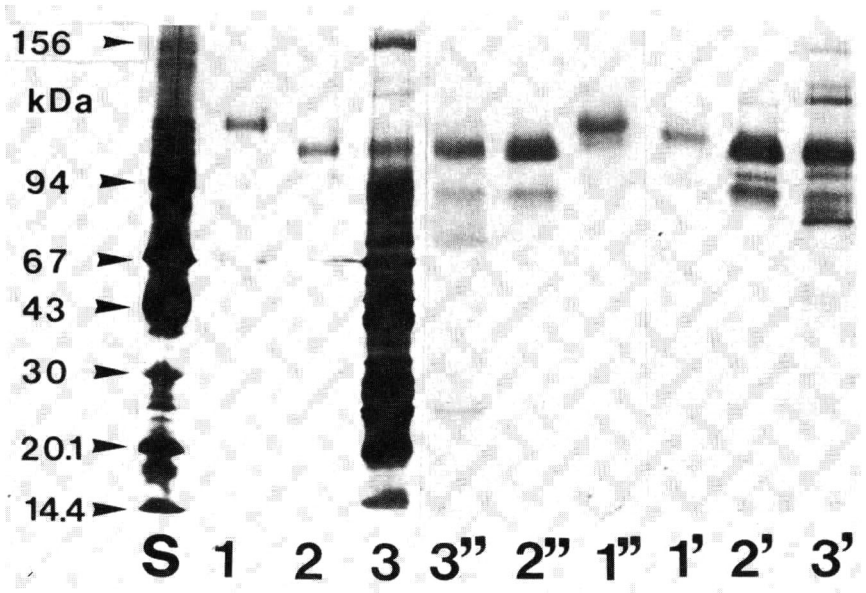

Fig. 6. Semi-dry immunoblot of fractions of different purification steps after SDS-PAGE. S = Low-molecular-mass markers (Pharmacia), plus monomeric IgG (156 kilodalton). 1, 1′, 1″ = ATA II peak fraction after SEC; 2, 2′, 2″ = APM peak fraction after SEC; 3, 3′, 3″ = fraction of 60–80% $(NH_4)_2SO_4$ precipitation. Lanes S, 1, 2 and 3 are silver-stained (SDS gel), lanes 1′, 2′ and 3′ are incubated with anti-APM and lanes 1″, 2″ and 3″ are incubated with anti-ATA II (Fraction 3″–3′ were blotted on Immobilon P membranes, second antibody goat anti-rabbit POD conjugated, revealed with AEC substrate). kDa = kilodaltons.

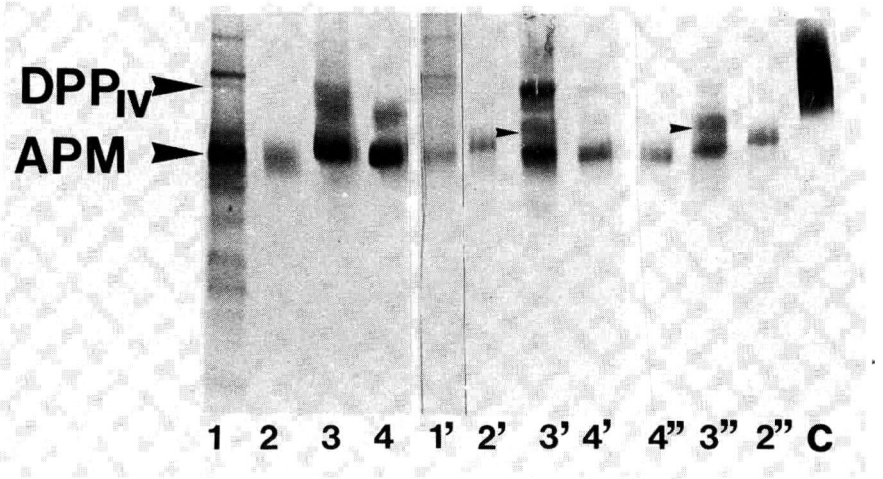

Fig. 7. Semi-dry immunoblot of purified fractions after native PAGE. 1, 1′ = Fraction of 60–80% $(NH_4)_2SO_4$ precipitation; 2, 2′, 2″ = ATA II peak fraction after SEC; 3, 3′, 3″ = ATA I fraction after SEC; 4, 4′, 4″ = APM peak fraction after SEC; C = rabbit serum immunoglobulin as a control. Lanes 1–4 are incubated with anti-APM, lanes 1′–4′ are incubated with anti-ATA I and lanes 4″, 3″ and 2″ are incubated with anti-ATA II (Immobilon P membranes, second antibody goat anti-rabbit POD conjugated, revealed with AEC substrate). The small arrows indicate ATA isoform I.

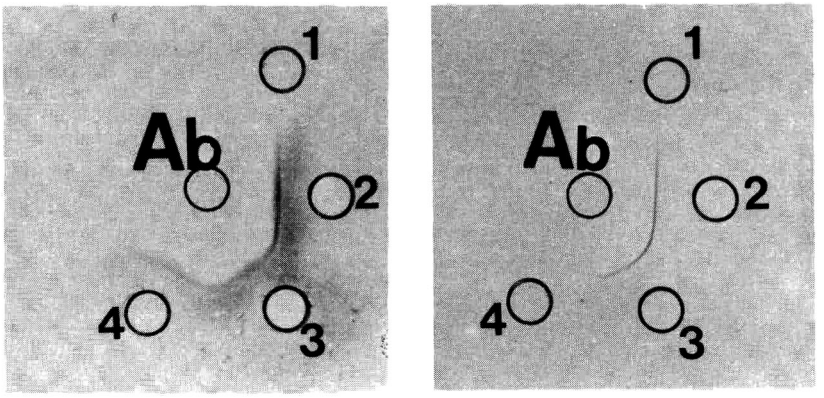

Fig. 8. Double-immunodiffusion assay. Centre wells (Ab) = ATA I antiserum (left) and ATA II antiserum (right), respectively; outer wells (clockwise, starting from 1) = APM, ATA I, ATA II, urinary ATA; staining for ATA activity. Faint precipitation line between ATA II antiserum and urinary ATA.

TABLE III

COMPARISON OF PROPERTIES OF ANGIOTENSINASE A I AND II PREPARED FROM HUMAN KIDNEY CORTEX

Immunoreactivity as revealed by immunohistology (POD technique) and immunofluorescent labelling (Cy3) on frozen kidney sections (6 μm).

Property	ATA I	ATA II
M_r (kilodalton) (native PAGE)	212	180
M_r (kilodalton (SDS-PAGE)	117	127
pI (IEF, pH 4–6.5)	4.81	4.69
K_M (mM)	2.05	0.87
Immunoreactivity in glomeruli	Negative (0/+)[a]	Positive (+ + +)[a]
Immunoreactivity in proximal tubules	Positive (+ + +)[a]	Positive (+ + +)[a]

[a] Grading: 0 = negative, + = faint, + + + = strongly positive.

differed in several respects from each other (Table III). In addition, polyclonal antibodies were raised against each isoform (anti-ATA I, anti-ATA II) and used to distinguish ATA I from ATA II in human tissue sections.

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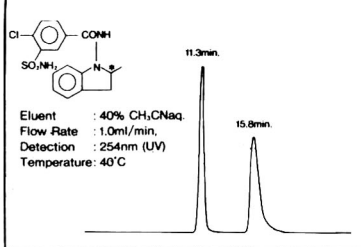
Reversed Phase CHIRAL HPLC Column

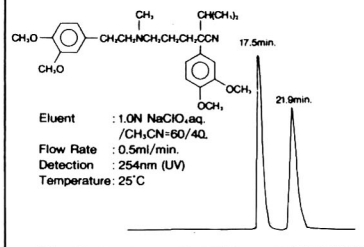
NEW CHIRALCEL® OD-R

Indapamide



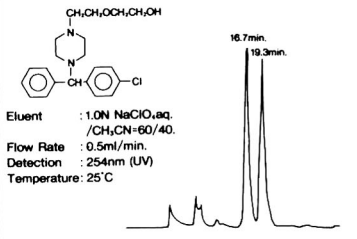
Eluent : 40% CH_3CNaq.
Flow Rate : 1.0ml/min.
Detection : 254nm (UV)
Temperature: 40°C

Verapamil



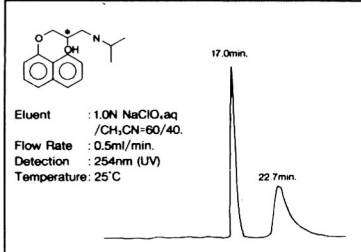
Eluent : 1.0N $NaClO_4$aq. /CH_3CN=60/40.
Flow Rate : 0.5ml/min.
Detection : 254nm (UV)
Temperature: 25°C

Hydroxyzine



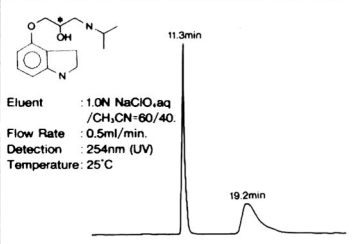
Eluent : 1.0N $NaClO_4$aq. /CH_3CN=60/40.
Flow Rate : 0.5ml/min.
Detection : 254nm (UV)
Temperature: 25°C

Propranolol



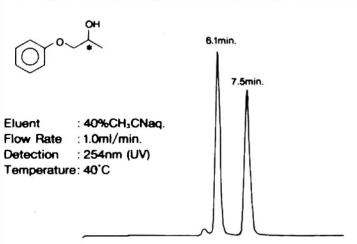
Eluent : 1.0N $NaClO_4$aq /CH_3CN=60/40.
Flow Rate : 0.5ml/min.
Detection : 254nm (UV)
Temperature: 25°C

Pindolol



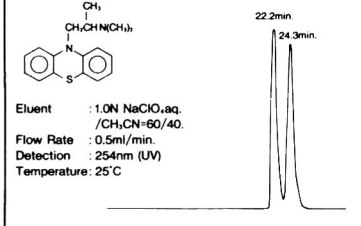
Eluent : 1.0N $NaClO_4$aq /CH_3CN=60/40.
Flow Rate : 0.5ml/min.
Detection : 254nm (UV)
Temperature: 25°C

1-Phenoxy-2-Propanol



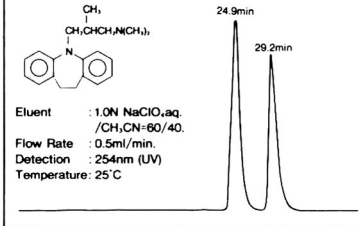
Eluent : 40%CH_3CNaq.
Flow Rate : 1.0ml/min.
Detection : 254nm (UV)
Temperature: 40°C

Alimemazine



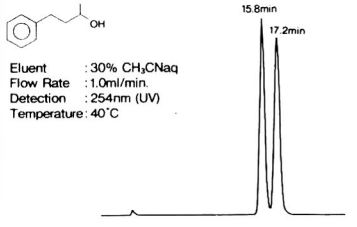
Eluent : 1.0N $NaClO_4$aq. /CH_3CN=60/40.
Flow Rate : 0.5ml/min.
Detection : 254nm (UV)
Temperature: 25°C

Trimepramine



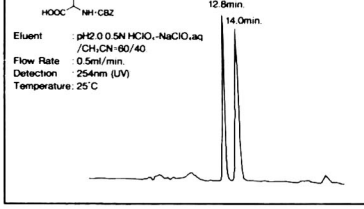
Eluent : 1.0N $NaClO_4$aq. /CH_3CN=60/40.
Flow Rate : 0.5ml/min.
Detection : 254nm (UV)
Temperature: 25°C

4-Pheny-2-butanol



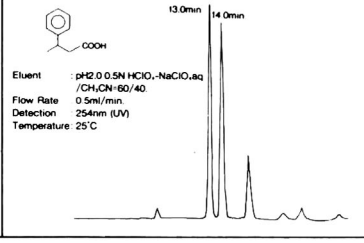
Eluent : 30% CH_3CNaq
Flow Rate : 1.0ml/min.
Detection : 254nm (UV)
Temperature: 40°C

CBZ-Val.



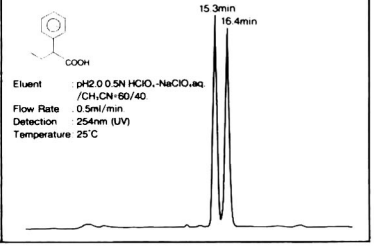
Eluent : pH2.0 0.5N $HClO_4$-$NaClO_4$aq /CH_3CN=60/40
Flow Rate : 0.5ml/min.
Detection : 254nm (UV)
Temperature: 25°C

3-Phenylbutyric acid



Eluent : pH2.0 0.5N $HClO_4$-$NaClO_4$aq /CH_3CN=60/40.
Flow Rate : 0.5ml/min.
Detection : 254nm (UV)
Temperature: 25°C

2-Phenylbutyric acid



Eluent : pH2.0 0.5N $HClO_4$-$NaClO_4$aq. /CH_3CN=60/40.
Flow Rate : 0.5ml/min
Detection : 254nm (UV)
Temperature: 25°C

For more information about CHIRALCEL OD-R column, please give us a call.

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