Journal of Chromatography, 521 (1990) 199–210
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1983

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

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

ABSTRACT

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

INTRODUCTION

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

0021-9673/90/$03.50

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

EXPERIMENTAL

Materials

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

Animals

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

Preparation of brush border membrane vesicles from rabbit small intestine

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

Purification of the intestinal peptide transport protein

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

Solubilization of brush border membrane vesicles

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

WGA–agarose chromatography

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

Hydrophobic interaction chromatography

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

Gel filtration

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

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

Hydroxyapatite chromatography

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

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

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

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

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

Photoaffinity labelling

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

SDS-PAGE

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

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

Two-dimensional gel electrophoresis

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

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

Fluorography

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

RESULTS AND DISCUSSION

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

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

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

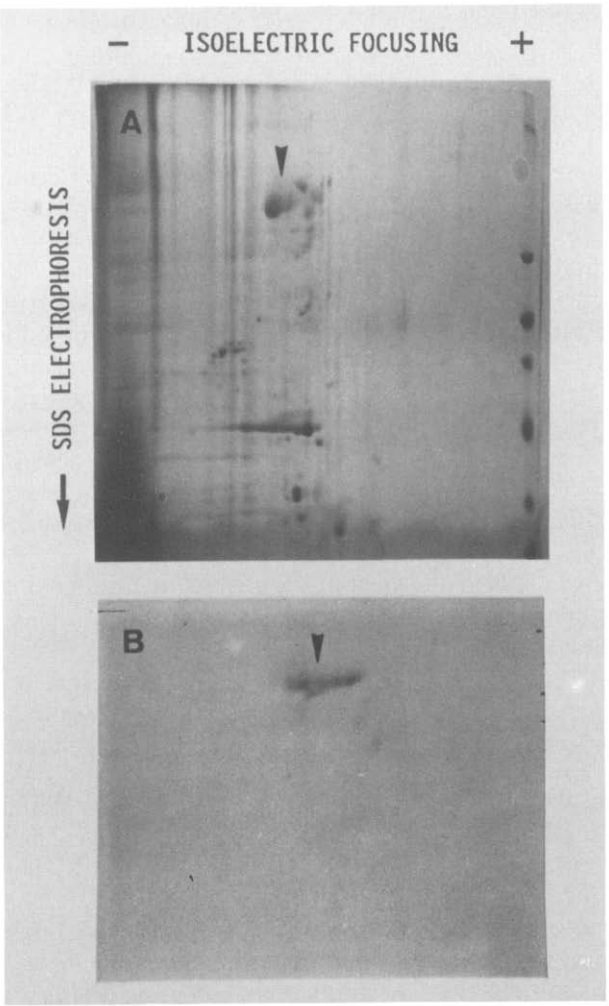

Fig. 1. Two-dimensional electrophoresis of rabbit small intestinal brush border membrane vesicles after photoaffinity labelling with [^{3}H]benzylpenicillin. A 1.5-mg amount of brush border membrane vesicle protein was photoaffinity labelled with 1.6 μM (15 μCi) [^{3}H]benzylpenicillin. After washing,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

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

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

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

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

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

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

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

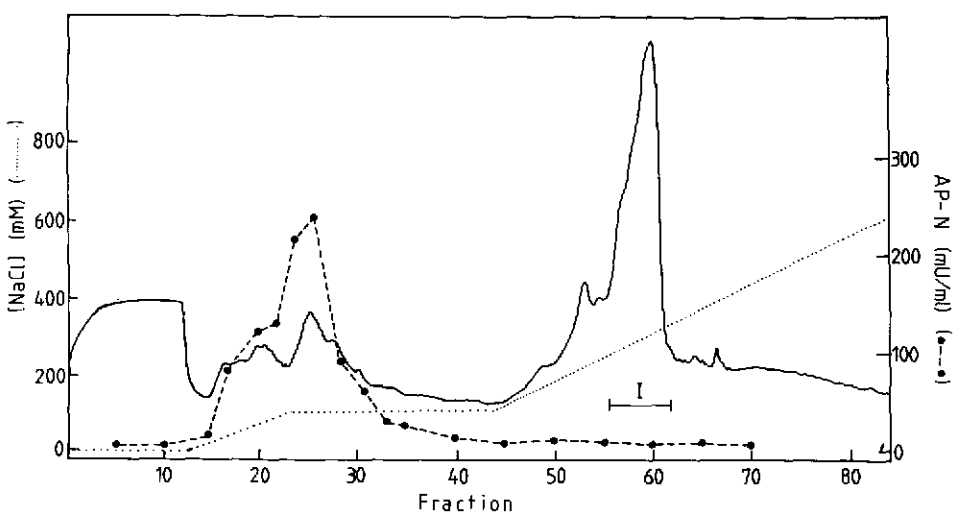

Fig. 2. Chromatography of the eluate from WGA–agarose chromatography on a Mono S HR 5/5 column with *n*-octylglucoside as detergent. The eluate from wheat germ lectin chromatography was fractionated on a Mono S HR 5/5 column using 20 m*M* sodium acetate buffer (pH 4.5)–0.7% *n*-octylglucoside as buffer.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

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

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

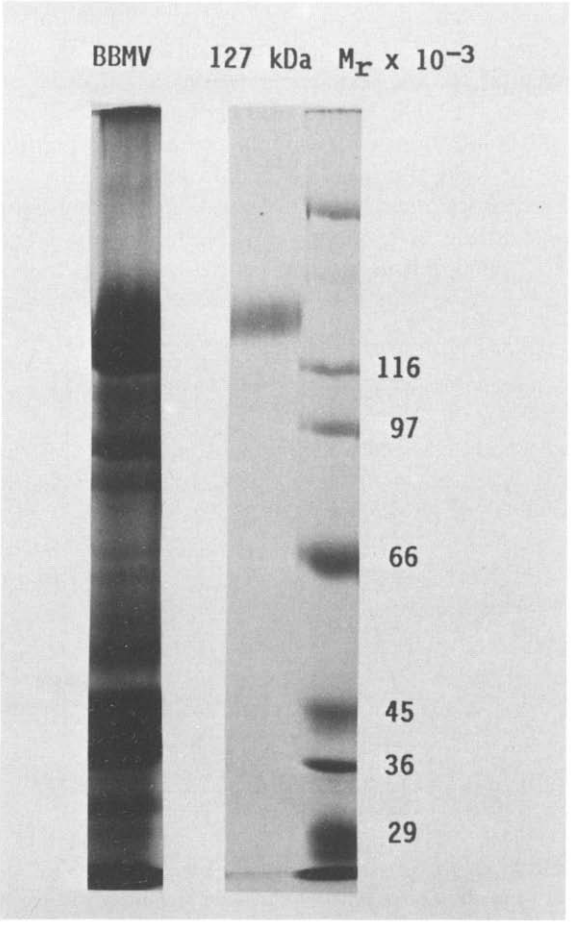

Fig. 3. SDS-PAGE of the putative intestinal peptide transporter purified by FPLC. Solubilized brush border membrane proteins from rabbit small intestine (BBMV) and the purified putative peptide transporter of M_r 127 000 were submitted to SDS-PAGE on a 7.5% gel. The numbers indicate the molecular masses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

TABLE I

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

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

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

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

REFERENCES

1 E. Nakashima, A. Tsuji, H. Mizuno and T. Yamana, *Biochem. Pharmacol.*, 33 (1984) 3345–3352.
2 T. Kimura, H. Endo, M. Yoshihawa, S. Muranishi and K. Sezaki, *J. Pharmacobiodyn.*, 1 (1978) 262 267.
3 T. Okano, K. Inui, M. Takano and R. Hori, *Biochem. Pharmacol.*, 35 (1986) 1781–1786.
4 W. Kramer, F. Girbig, I. Leipe and E. Petzoldt, *Biochem. Pharmacol.*, 37 (1988) 2427–2435.
5 V. Ganapathy and F. H. Leibach, *Am. J. Physiol.*, 249 (1985) G153 G160.
6 W. Kramer, F. Girbig, E. Petzoldt and I. Leipe, *Biochim. Biophys. Acta*, 943 (1988) 288–296.
7 W. Kramer, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 361 (1987) R46.
8 W. Kramer, *Biol. Chem. Hoppe-Seyler*, 368 (1987) 1261.
9 W. Kramer, *Biochim. Biophys. Acta*, 905 (1987) 65–74.
10 G. Burckhardt, W. Kramer, G. Kurz and F. A. Wilson, *J. Biol. Chem.*, 258 (1983) 3618 3622.
11 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
12 W. Kramer, I. Leipe, E. Petzoldt and F. Girbig, *Biochim. Biophys. Acta*, 939 (1988) 167–172.
13 D. Wessel and U. J. Flügge, *Anal. Biochem.*, 138 (1984) 141–143.
14 W. Kramer, G. Burckhardt, F. A. Wilson and G. Kurz, *J. Biol. Chem.*, 258 (1983) 3623–3628.
15 J. P. Chamberlain, *Anal. Biochem.*, 98 (1979) 132–135.

16 R. A. Laskey and A. D. Mills, *Eur. J. Biochem.*, 56 (1975) 335–341.
17 W. M. Bonner and R. A. Laskey, *Eur. J. Biochem.*, 46 (1974) 83–88.
18 W. Kramer, *XXXI International Congress of Physiological Sciences, July 9–14, 1989, Helsinki, Finland*, Abstr. S 3074.
19 W. Kramer and W. Schiebler, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 340 (1989) R78.
20 W. Kramer, *Biol. Chem. Hoppe-Seyler*, 370 (1989) 624.
21 M. R. Fogel and S. A. Adibi, *J. Lab. Clin. Med.*, 84 (1974) 327–333.
22 K. W. Smithson and G. M. Gray, *J. Clin. Invest.*, 60 (1977) 665–674.
23 D. Louvard, M. Semeriva and S. Maroux, *J. Mol. Biol.*, 106 (1976) 1023–1040.
24 D. Gratecos, L. Varesi, M. Kniebühler and M. Semeriva, *Biochim. Biophys. Acta*, 705 (1982) 218–227.
25 M. Semeriva, L. Varesi and D. Gratecos, *Eur. J. Biochem.*, 122 (1982) 619–626.
26 W. Kramer, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 339 (1989) R42.
27 S. D. Carson and W. H. Konigsberg, *Anal. Biochem.*, 116 (1981) 398–401.