Journal of Chromatography, 521 (1990) 279–289
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1997

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

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

ABSTRACT

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

INTRODUCTION

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

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

0021-9673/90/$03.50

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

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

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

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

EXPERIMENTAL

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

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

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

Lectin affinity chromatography

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

Conventional gel filtration

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

Fast protein liquid chromatography

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

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

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

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

Enzyme determination

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

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

Protein determination

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

Electrophoretic analysis

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

RESULTS AND DISCUSSION

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

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

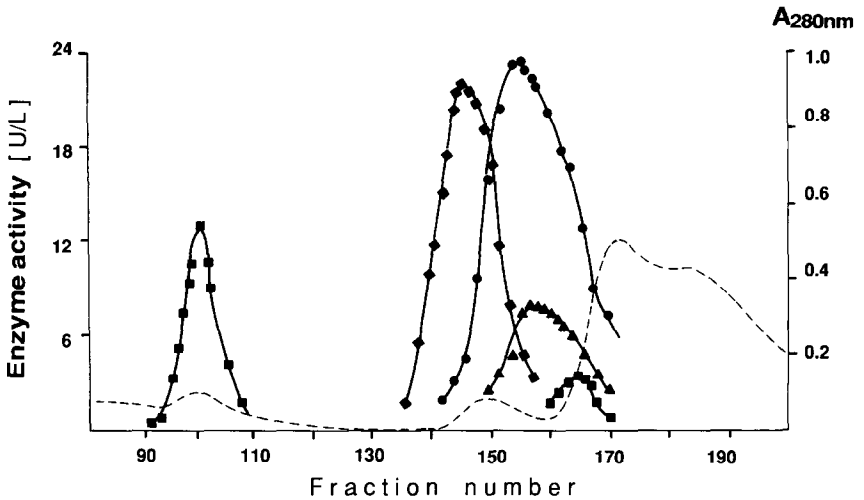

Fig. 1. Gel filtration (Sephacryl 300) of pooled urine concentrate from patients with tubular damage. Elution profiles of protein and kidney marker enzymes γ-glutamyl transpeptidase (γ-GT), dipeptidyl aminopeptidase IV (DAP IV), angiotensinase A (ATA) and aminopeptidase M (APM). Protein absorbance recorded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

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

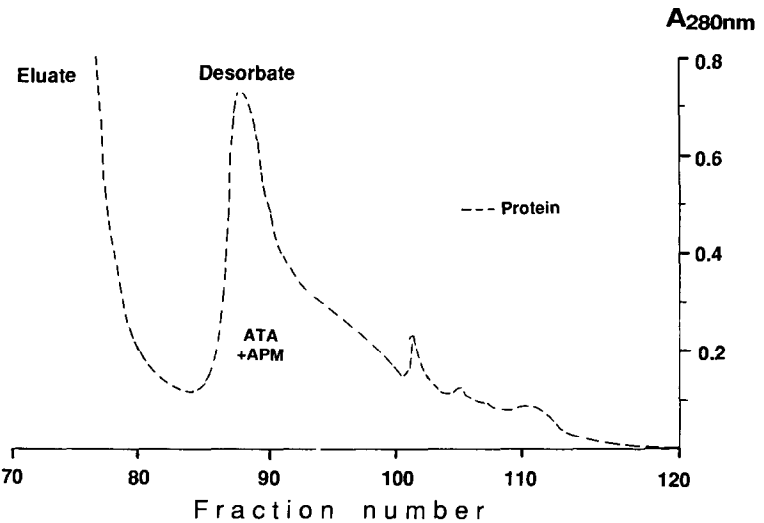

Fig. 2. Wheat germ lectin affinity chromatography of urine from patients with renal disease (tubular damage). ATA and APM excreted in urine were biospecifically bound to the gel matrix and then desorbed (desorbate) with 0.05 *M* Tris–0.2 *M* NaCl + 0.01 *M* $CaCl_2$ buffer containing 60 m*M* N-acetyl-D-glucosamine.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

TABLE I

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

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

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

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

[c] Molecular weights as determined by native PAGE.

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

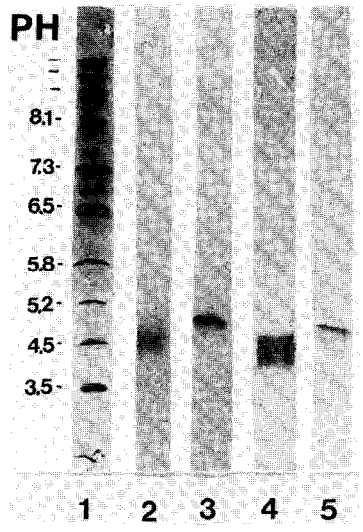

Fig. 3. Isoelectric focusing of urine protein fractions using PHASTGel p*I* 3–9. (1) Marker proteins for calibration of p*I*; (2) ATA solubilized from kidney tissue particles (p*I* 4.3–4.6); (3) ATA urine (p*I* 4.85); (4) APM solubilized from human kidney particles (p*I* 4.0–4.6); (5) APM urine (p*I*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

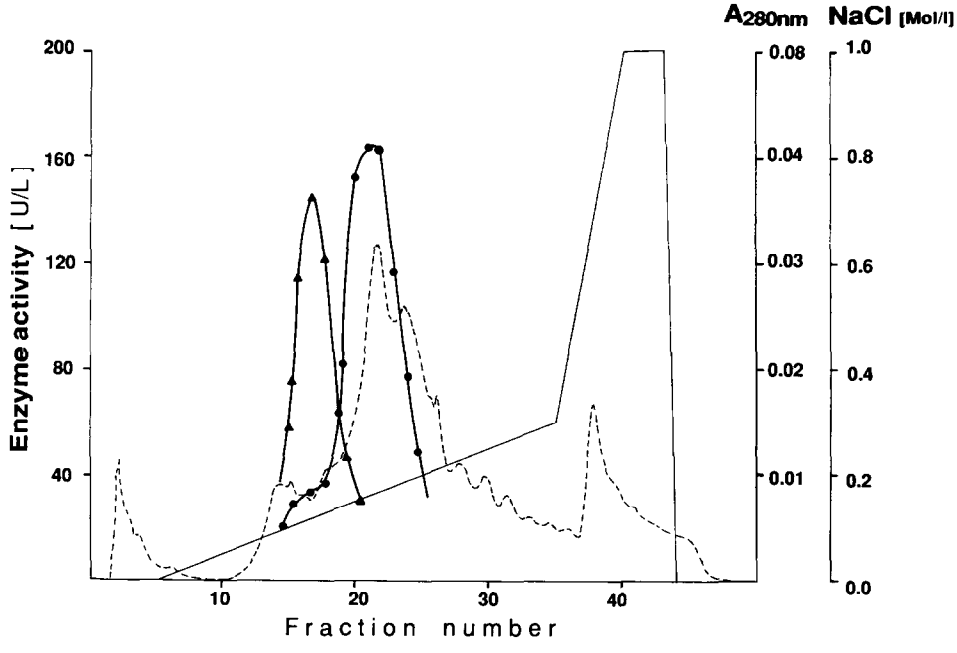

Fig. 4. Anion-exchange chromatography of urinary proteins (see above) using a Mono Q HR 5/5 column. FPLC: flow-rate, 2 ml/min; pressure, 2 MPa; sample volume, 100–500 μl; 20 m*M* Tris–HCl buffer (pH 7.2), fraction size, 1 ml. Using an NaCl gradient from 0 to 1 *M* ATA and APM activities are eluted in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

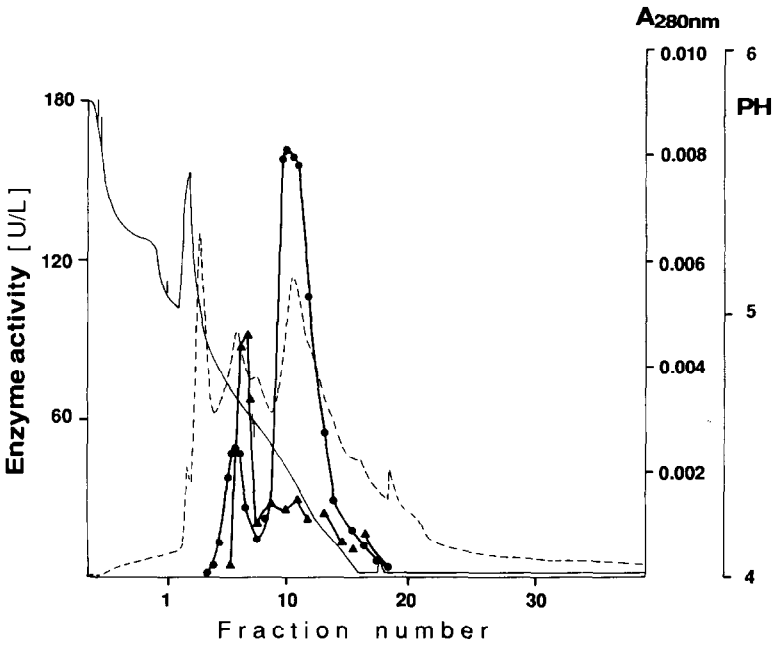

Fig. 5. FPLC chromatofocusing of urine proteins (see above) applying a Mono P HR 5/20 column. Precolumn procedure as described in the text (buffer equilibration/PD-10 Sephadex). Buffer A, 0.025 *M* methylpiperazin (pH 5.7); buffer B, Polybuffer 74; pH gradient from 5 to 4 (elution range of proteins);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

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

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

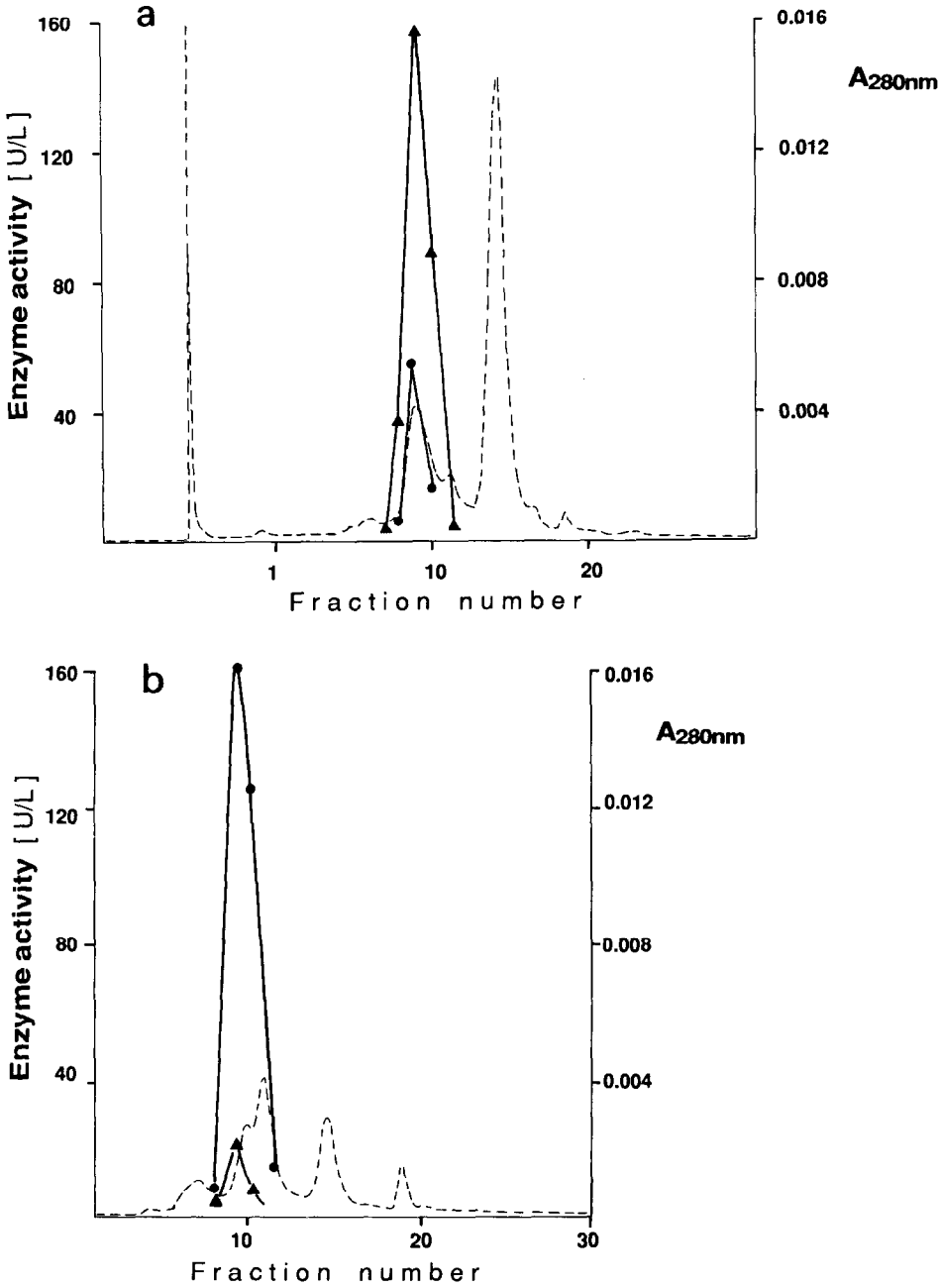

Fig. 6. FPLC gel permeation of urine proteins using a Superose 12 HR 10/30 column. Pooled peak fractions of either (a) ATA or (b) APM activity after anion-exchange FPLC (Mono Q, Fig. 3) were applied to the gel and eluted with 0.15 *M* NaCl–0.05 *M* Tris–HCl buffer (pH 7.6). Fraction size, 1 ml; sample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

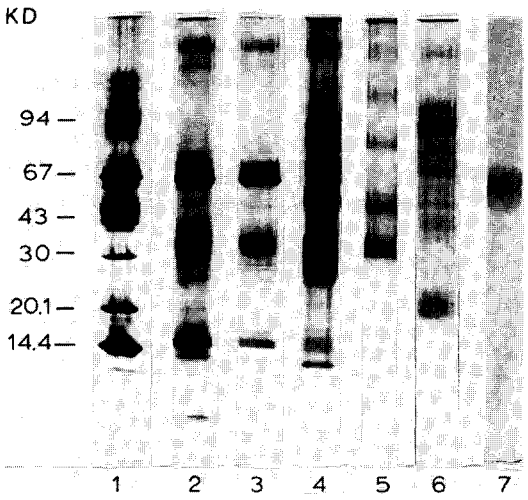

Fig. 7. SDS–PAGE of urinary protein fractions with a PHAST system. 1 = LMW marker; 2 = urine pool used for the separation of ATA and APM; 3 = WGA eluate (unbound proteins); 4 = WGA desorbate (adsorbed glycoproteins); 5 = APM peak fractions (Mono Q column); 6 = ATA peak fractions (Mono Q); 7 = native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

CONCLUSIONS

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

ACKNOWLEDGEMENTS

The authors thank Mrs. Marianne Haimerl and Mrs. Margret Lindermann-Rosemann for skilful technical assistance. This work was supported by a grant from the Dr. Cilli Weil and W. Klein foundation, Frankfurt am Main, to J.E.S. and by a BGA/BMFT grant, Berlin/Bonn "Forschungsschwerpunkt AIDS"(No. I-B4; "HIV-Infektion und Niere") to W.S. and J.E.S.

REFERENCES

1 H. Mattenheimer, H. Grötsch, J. Froelke *et al.*, *J. Clin. Chem. Clin. Biochem.*, 26 (1988) 635–644.
2 P. Kugler, *Histochemistry*, 74 (1982) 229–245.
3 P. Kugler, *Histochemistry*, 74 (1982) 247–261.
4 P. Kugler, G. Wolf and J. E. Scherberich, *Histochemistry*, 83 (1985) 337–342.
5 G. Wolf, J. E. Scherberich and W. Schoeppe, *Fresenius' Z. Anal. Chem.*, 330 (1988) 429–430.
6 J. E. Scherberich, G. Wolf, C. Stuckhardt, P. Kugler and W. Schoeppe, *Adv. Exp. Med. Biol.*, 240 (1989) 275–282.
7 G. Wolf, J. E. Scherberich, C. Stuckhardt and W. Schoeppe, *Protides Biol. Fluids*, 35 (1987) 289–292.
8 I. Nagatsu, T. Nagatsu, T. Yamamoto, G. G. Glenner and J. W. Mehl, *Biochim. Biophys. Acta*, 198 (1970) 255–270.
9 K. Lalu, S. Lampelo and T. Vanha-Perttula, *Int. J. Biochem.*, 17 (1985) 1227–1235.
10 H. Tobe, F. Kojima, T. Aoyagi and H. Umezawa, *Biochim. Biophys. Acta*, 613 (1980) 459–468.
11 G.-J. Sanderink, Y. Artur and G. Siest, *J. Clin. Chem. Clin. Biochem.*, 26 (1988) 795–807.
12 J. E. Scherberich, G. Wolf, C. Albers, A. Nowack, C. Stuckhardt and W. Schoeppe, *Kidney Int.*, 36, Suppl. 27 (1989) 38–51.
13 J. E. Scherberich, P. Fischer, A. Bigalke, P. Stang, G. Wolf, M. Haimerl and W. Schoeppe, *Electrophoresis*, 10 (1989) 58–62.
14 G. Wolf, J. E. Scherberich, P. Fischer and W. Schoeppe, *Clin. Chim. Acta*, 179 (1989) 61–72.
15 J. E. Scherberich, *Clin. Chim. Acta*, 185 (1989) 271–282.
16 G. Wolf, J. E. Scherberich and W. Schoeppe, *Cell. Mol. Biol.*, 34 (1988) 311–321.