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

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[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 Human kidney ATA contains N-acetyl-α-D-glucosamine to bind specifically to wheatgerm agglutinin (unpublished results).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.

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$_4$)$_2$SO$_4$] 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 mM/l substrate (L-α-Glu-pNA), 20 μM Tris–HCl buffer (pH 7.7), 25 μM Ca^{2+} and 0.1 ml of the sample. One enzyme unit was defined as the amount of enzyme catalysing the formation of 1 μM/min of pNA (37°C). For the APM assay the same buffer (without Ca^{2+}, pH 8.0) and 1.66 mM/l L-Ala-pNA were used. DPP IV was determined with 5 mM/l Gly–Pro-pNA and 20 μM Tris–HCl (pH 8.5), whereas GGT was assayed with 2.9 mM/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 mM 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-
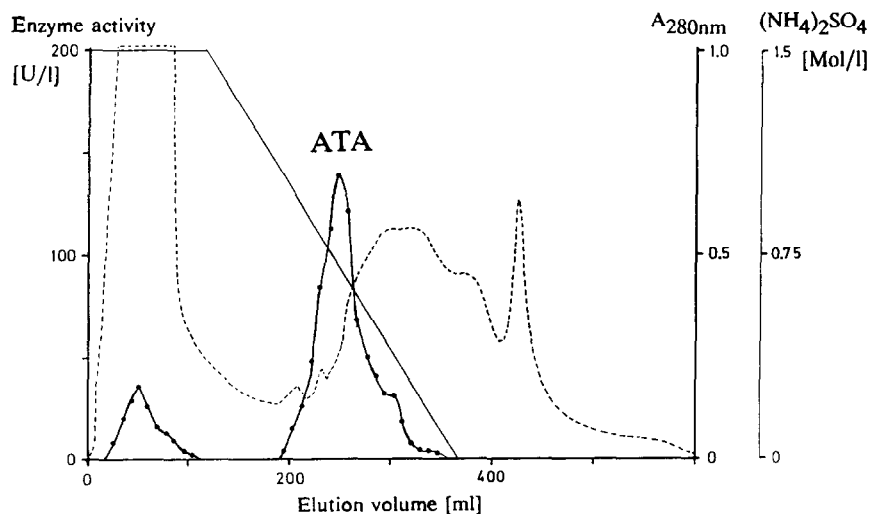

Fig. 1. Hydrophobic interaction chromatography (HIC) of the fraction obtained from $(NH_4)_2SO_4$ precipitation (60 and 80% saturation). Column, XK 26/20 laboratory packed with 67 ml of phenyl-Sepharose CL-4B and equilibrated with 1.5 mol/l $(NH_4)_2SO_4$ plus 50 mmol/l Tris–HCl (pH 7.7). Elution conditions: 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.
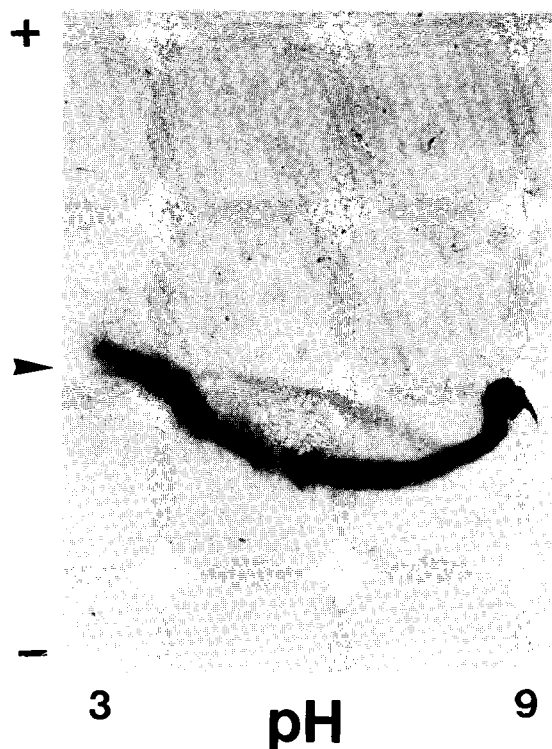
Fig. 2. Electrophoretic titration curve of the sample from Fig. 1. The curve was generated by prefocusing the ampholytes across a Phast IEF gel and subsequent electrophoretic separation of the sample components perpendicular to the pH gradient. Each component migrates towards the anode or cathode owing to its net charge at any particular pH. The arrowhead indicates the sample application line. The gel was stained for ATA activity.
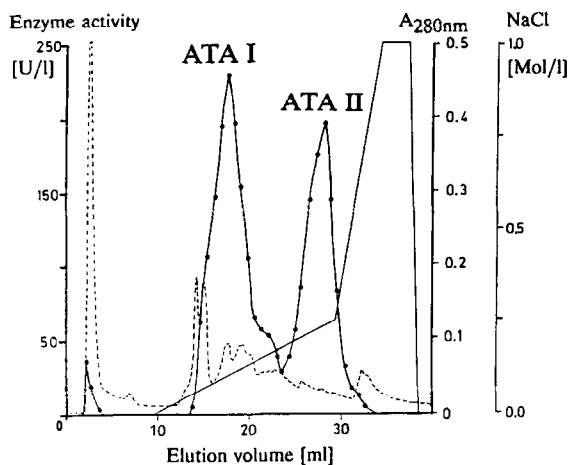

Fig. 3. Anion-exchange chromatography of pooled ATA fractions obtained from HIC. Column, HR 5/5 prepacked with 1 ml of Mono Q and equilibrated with 20 mmol/l Tris–HCl (pH 7.2) (buffer A). Elution conditions: buffer B, 20 mmol/l Tris–HCl (pH 7.7)–1 mol/l NaCl; sample size, 0.1 ml; fraction size, 500 μl; 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 mM, 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 mM NaCl, followed by APM, while isoform II left the column at 240 mM 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).
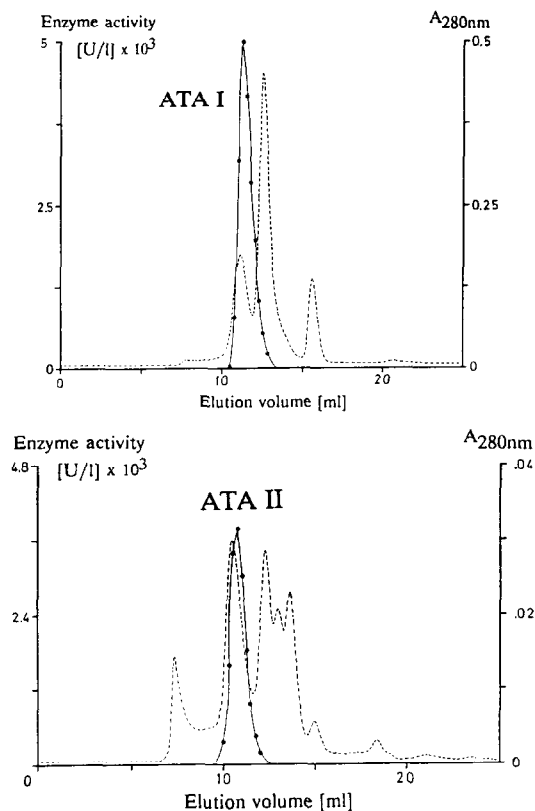
The rabbit antibodies obtained showed a major

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 IgG₁ 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
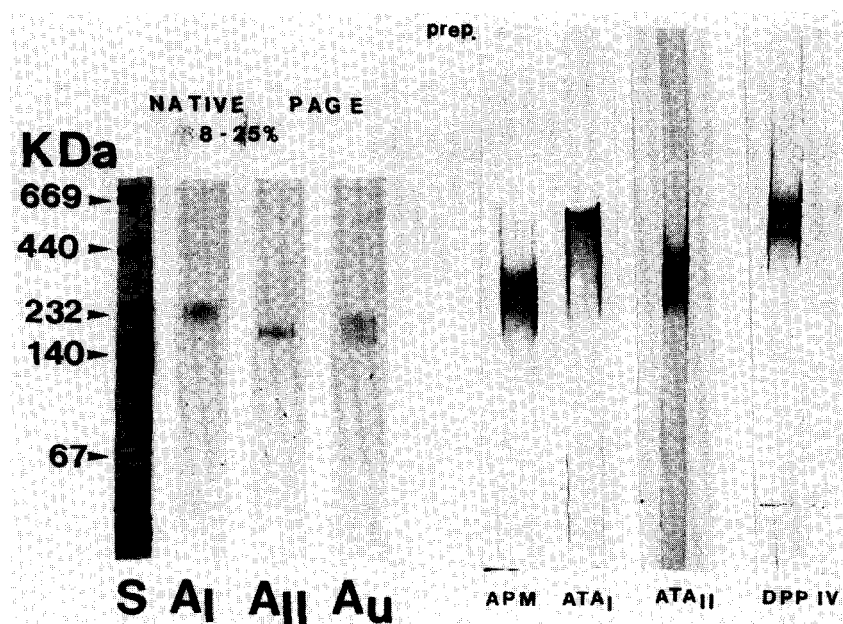
Fig. 5. Native PAGE of purified fractions using the Phast system (left) and Havana system (Desaga, 3–20% T, laboratory-cast preparative gel) (right). S = High-molecular-mass markers (Pharmacia); A_I and A_{II} = ATA I and ATA II Mono Q fractions; A_U = purified ATA fraction from human urine. Lanes on 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	SDS-PAGE	19
		270/350–400	GF[b]	
Intestinal mucosa	Pig	120	SDS-PAGE	20
		247	Ultracentrifugation	
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	GF	30
		191	Native PAGE	
Reproductive tissue	Boar, bull	n.d.[a]	n.d.	25
	Gerbil, human			
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.
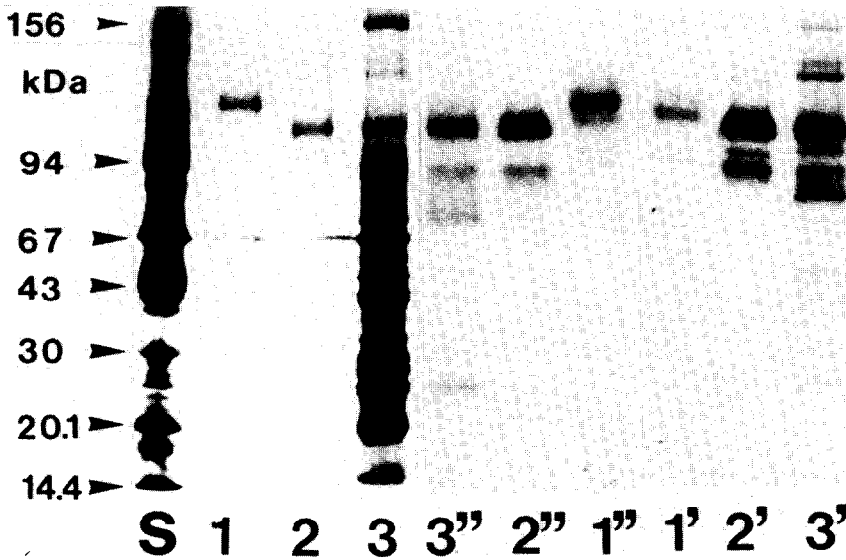
Fig. 6. Semi-dry immunoblot of fractions of different purification steps after SDS-PAGE. S = Low-molecular-mass markers (Pharmacia), plus monomeric IgG (156 kilodalton). 1, 1′, 1″ = ATA II peak fraction after SEC; 2, 2′, 2″ = APM peak fraction after SEC; 3, 3′, 3″ = fraction of 60–80% $(NH_4)_2SO_4$ 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.
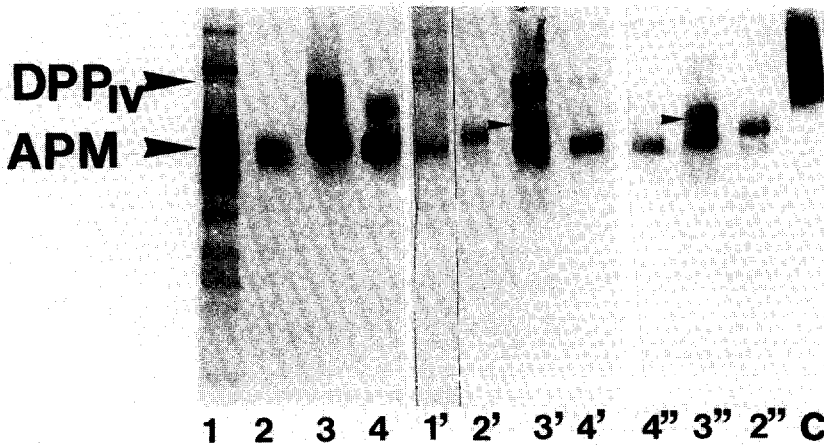
Fig. 7. Semi-dry immunoblot of purified fractions after native PAGE. 1, 1′ = Fraction of 60–80% $(NH_4)_2SO_4$ precipitation; 2, 2′, 2″ = ATA II peak fraction after SEC; 3, 3′, 3″ = ATA I fraction after SEC; 4, 4′, 4″ = APM peak fraction after SEC; C = rabbit serum immunoglobulin as a control. Lanes 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.
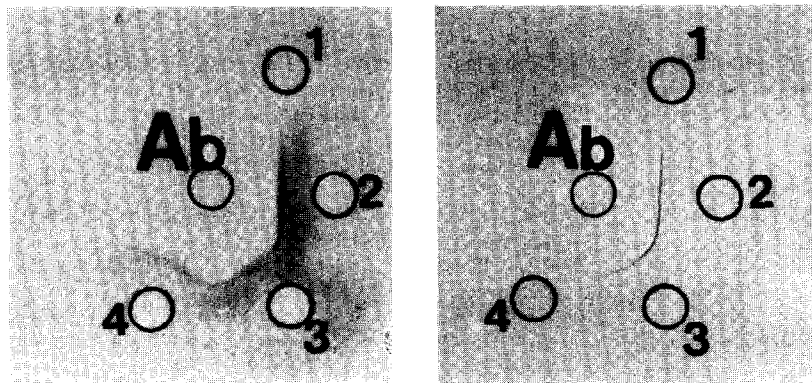
Fig. 8. Double-immunodiffusion assay. Centre wells (Ab) = ATA I antiserum (left) and ATA II antiserum (right), respectively; outer wells (clockwise, starting from 1) = APM, ATA I, ATA II, urinary ATA; staining for ATA activity. Faint precipitation line between ATA II antiserum and urinary ATA.

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.

ACKNOWLEDGEMENTS

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