Biochem. J. (1983) **213**, 193–200
Printed in Great Britain

Confirmation of direct angiotensin formation by kallikrein

Haruo MARUTA and Kikuo ARAKAWA
Department of Internal Medicine, Fukuoka University, School of Medicine, Fukuoka 814-01, Japan

(*Received 8 November 1982/Accepted 14 March 1983*)

This study was undertaken to confirm our previous preliminary observation that hog pancreas kallikrein (EC 3.4.21.35) directly liberated an angiotensin-like substance from human plasma protein Cohn fraction IV-4 at an acidic pH of 4.0–5.0. First, the possibility of proangiotensin or des-Asp^1-angiotensin being the pressor substance was ruled out by t.l.c. Secondly, the pressor substance was purified by Sephadex G-25 and Bio-Gel P-2 gel filtration, and finally by high-performance liquid chromatography. The amino acid composition of the isolated pressor substance (residues/mol) was: Asp, 1.03; Val, 1.03; Ile, 1.00; Tyr, 0.69; Phe, 1.04; His, 0.91; Arg, 0.86; Pro, 0.86. This composition was identical with that of angiotensin. Since the reaction mixture was not contaminated with common proteolytic enzymes, such as trypsin, chymotrypsin, renin, cathepsin D and proangiotensin-converting enzyme, and other enzymes activated by kallikrein, it is clear that hog kallikrein directly produces angiotensin *in vitro*.

Pancreas kallikrein and trypsin are known to liberate kallidin (Habermann & Blennemann, 1964) and bradykinin (Elliot *et al.*, 1961) from kininogen in alkaline conditions.

To our surprise, however, trypsin generated [Ile^5]angiotensin from human plasma protein at pH 6.0 (Arakawa *et al.*, 1976, 1980). Similarly, hog pancreas kallikrein also generated an angiotensin-like pressor substance from human plasma protein at pH 4.0–5.0 (Arakawa & Maruta, 1980). It seemed that hog pancreas kallikrein directly acted on the substrate and liberated the pressor substance, since acid- and alkali-treated Cohn fraction IV-4 did not contain renin, kallikrein, trypsin-activatable renin, cathepsin D or proangiotensin-converting enzyme and since pepstatin and captopril had no influence on the pressor-substance formation (Arakawa & Maruta, 1980).

There remained the possibility, however, that some unknown enzyme that was activated by pancreas kallikrein and inhibited by aprotinin might participate in the pressor-substance formation.

The pressor substance is similar to [Ile^5]angiotensin, but the possibility of the pressor substance being des-Asp^1-[Ile^5]angiotensin or des-Asp^1,Arg^2-[Ile^5]angiotensin (Bumpus *et al.*, 1961; Schwyzer, 1963; Campbell & Pettinger, 1976) needs to be ruled out, because the pressor activities of these peptides are also not inhibited by proangiotensin-converting enzyme inhibitor, but are inhibited by [Sar^1,Ile^8]-angiotensin (Campbell & Schmitz, 1979; Sexton *et al.*, 1979). Furthermore, anti-angiotensin antibody also cross-reacts with them (Cain *et al.*, 1969; Emanuel *et al.*, 1973; Lijnen *et al.*, 1978).

To solve these problems, we investigated the mechanism of the pressor-substance formation in detail, as well as purifying it to homogeneity and analysing for amino acid composition.

Abbreviations used: Bz, benzoyl; ONap, α-naphthyl ester; OEt, ethyl ester; h.p.l.c., high-performance liquid chromatography.

Materials

Human plasma protein Cohn fraction IV-4 was obtained from Midori-Juji (Osaka, Japan). Authentic [Ile^5]proangiotensin and [Ile^5]angiotensin, des-Asp^1-[Ile^5]angiotensin, Bz-Gly-His-Leu and Bz-Tyr-OEt were purchased from the Protein Research Foundation (Osaka, Japan). Pure hog pancreas kallikrein (specific activity 1800 μg of bradykinin equivalent/min per mg at pH 8.0) and Pro-Phe-Arg-ONap were obtained from Sanwa Chemicals (Nagoya, Japan). Crystalline bovine trypsin (EC 3.4.21.4) (3000 NF units) was from Miles Laboratories (Slough, Berks., U.K.), α-chymotrypsin (EC 3.4.21.1) (3 × crystallized) was from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.), bovine haemoglobin, EDTA disodium salt (Na_2EDTA), 8-hydroxyquinoline sulphate, 2,3-dimercaptopropan-1-ol, acetic acid, NaOH, H_3BO_3, H_3PO_4 and ethanol were purchased from Nakarai Chemicals (Kyoto, Japan). Sephadex G-25 and activated CH-Sepharose 4B were from Pharmacia

(Uppsala, Sweden) and Bio-Gel P-2 (100–200 mesh) was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Avicel SF Cellulose thin-layer plates (10cm × 10cm) were from Funakoshi Chemicals (Tokyo, Japan). The h.p.l.c. apparatus (model ALC/GPC 240) and μ-Bondapak C_{18} column (3.9mm × 30cm) were from Waters Associates (Milford, MA, U.S.A.). The methanol used in h.p.l.c. was spectrophotometric grade and other chemicals used were of guaranteed reagent grade.

Methods

Bioassay

Pressor bioassay was carried out by the rat-blood-pressure method as described previously (Arakawa & Maruta, 1980).

Radioimmunoassay

Radioimmunoassay was performed by the method of Haber *et al.* (1969) for proangiotensin and Lijnen *et al.* (1978) for angiotensin. Anti-angiotensin antibody completely cross-reacted with des-Asp^1-angiotensin and des-Asp^1,Arg^2-angiotensin.

Preparation of substrate

Human plasma Cohn fraction IV-4, which was rich in angiotensinogen, was used as substrate. To eliminate any possible prorenin and renin present, the fraction was pretreated before use with acid and alkali by the methods described previously (Arakawa *et al.*, 1975; Yokosawa *et al.*, 1979).

Purity of hog pancreas kallikrein

α-Chymotrypsin, trypsin and pancreas kallikrein (50μg each) were subjected to electrophoresis on 7.0% (w/v) polyacrylamide gel at pH9.5 (Davis, 1964). The gels were stained with Coomassie Brilliant Blue G-250. Zymogram of pancreas kallikrein was prepared after disc-gel electrophoresis by the method of Hitomi *et al.* (1980), with slight modifications using Pro-Phe-Arg-ONap as substrate and Fast Red ITR as dye.

Enzyme assay

Renin was determined by measurement of pro-angiotensin liberated. Chymotryptic activity was determined by using Bz-Tyr-OEt as substrate (Hummel, 1959). Cathepsin D activity was determined by using denatured bovine haemoglobin as substrate (Barrett, 1967). Proangiotensin-converting enzyme activity was detected by the method of Cushman & Cheung (1971).

The mechanism of the pressor-substance formation

Pancreas kallikrein was coupled to activated CH-Sepharose 4B according to the manufacturer's instructions. About 10mg of kallikrein per ml of settled Sepharose 4B was obtained.

Acid- and alkali-treated Cohn fraction IV-4 (10mg) was pre-incubated with or without 0.5ml of settled kallikrein–Sepharose 4B in 2.0ml of Briton–Robbinson buffer, pH4.0 (Briton & Robbinson, 1931), containing 200μl of 0.1M-Na_2EDTA, 20μl of 0.45M-8-hydroxyquinoline sulphate and 5μl of 10% (v/v) 2,3-dimercaptopropan-1-ol as angiotensinase inhibitors, at 37°C for 0, 1, 2, 4 and 18h. After the pre-incubation, kallikrein–Sepharose 4B was removed by filtration on a glass filter. A portion (100μl) of the filtrate was mixed with ethanol and boiled to stop reaction. To the rest of the filtrate, 10mg of Cohn fraction IV-4 in 2ml of Briton–Robbinson buffer, pH4.0, containing angiotensinase inhibitors, was added and the mixture was incubated again at 37°C for 18h. Reaction was stopped as described above. The ethanol extract was evaporated to dryness, and bioassayed for the pressor activity and radioimmunoassayed for [Ile^5]-proangiotensin and [Ile^5]angiotensin.

Preparation and purification of the pressor substance by gel filtration

Acid- and alkali-treated Cohn fraction IV-4 (10g) and 10mg of hog pancreas kallikrein were incubated in 200ml of Briton–Robbinson buffer, pH4.0, containing 20ml of 0.1M-Na_2EDTA, 2ml of 0.45M-8-hydroxyquinoline sulphate and 0.5ml of 10% (v/v) 2,3-dimercaptopropan-1-ol at 37°C for 18h. The pressor substance was extracted from the incubation mixture with 2 litres of hot ethanol. The extract was evaporated to dryness under reduced pressure at 45°C. The residue was dissolved in 10ml of 0.05M-acetic acid and applied on a Sephadex G-25 column (3.9cm × 100cm) equilibrated in 0.05M-acetic acid, eluted with the same buffer at a flow rate of 40ml/h and collected in fractions of 12ml each. Fractions containing the pressor substance were combined and freeze-dried. The freeze-dried material was dissolved in 1.5ml of 0.05M-acetic acid. A second gel filtration was carried out on a Bio-Gel P-2 column (1.2cm × 90cm) equilibrated with 0.05M-acetic acid and 1.25ml fractions were collected. Fractions containing the pressor substance were combined and freeze-dried. The freeze-dried material was resuspended in equilibrating buffer for reversed-phase h.p.l.c.

Isolation of the pressor substance by h.p.l.c.

A Waters Associates model 204 liquid chromatograph equipped with two pumps (model 600A), a programmer for gradient elution (model 660), a universal liquid-chromatography injector (model U6K) and an absorbance detector (model 440) were used. The chromatography column (3.9mm internal diameter × 30cm) used was a Waters Associates

μ-Bondapak C_{18}. Deionized and distilled water was used and solvents were filtered through 0.45μm Millipore filters before use.

Usually u.v. absorbance at short wavelength, i.e. 214nm or 217nm, is suitable for the detection of peptide. However, it is too sensitive to monitor a large amount of peptide, and moreover acetic acid and methanol interfere at these wavelengths.

To circumvent these inconveniences, monitoring at this stage was done at 254nm continuously. The first chromatography system was performed by the method of Tonnaer *et al.* (1980) with slight modifications. Ammonium acetate (0.01M), which was adjusted to pH4.15 with acetic acid (X), and methanol containing 1.5ml of acetic acid per litre (Y) were used as the solvent elution system. The composition of the initial solvent elution system (A) was X/Y (4:1, v/v) and the final composition (B) was X/Y (1:3, v/v). Elution (1.5ml/min) was carried out at room temperature with a 60min linear elution gradient of solvent A to solvent B. Fractions were collected at 1min intervals and bioassayed for the pressor activity. Fractions containing the pressor activity were combined and freeze-dried. Peptide content of sample was determined as described below. In a second chromatography system, isocratic elution was carried out with a solvent A/solvent B (1:1, v/v) mixture. Elution flow rate was 1ml/min, and fractions were collected at 0.4min intervals. The last chromatography step was performed under the same conditions as the second.

T.l.c.

Homogeneities of the samples were checked by t.l.c. on Avicel-SF cellulose plates (10cm × 10cm) using the ascending technique. The solvent systems were: (1), isopentanol/pyridine/water (7:7:6, by vol); (2), butan-1-ol/acetic acid/water (3:1:1, by vol). Ninhydrin solution (0.2%) in butan-1-ol saturated with water was used for visual detection.

Determination of protein or peptide

The quantity of protein or peptide was determined by spectrophotometric analysis at either 280nm or 210nm for column chromatography and 254nm for h.p.l.c. Peptide content of sample after h.p.l.c. was determined at 210nm by using authentic [Ile^5]angiotensin as standard ($A^{1\%}_{1cm,210}$418).

Specific activity

The ratios of the pressor activity to quantities of proteins or peptides obtained from each purification step were calculated and used as a measure of specific activity.

Amino acid composition analysis

Amino acid analysis was performed after hydrolysing peptide samples with 6M-HCl at 110°C for 20h in a sealed ampoule in the presence of N_2 gas. The amino acid composition was determined by using a model 835 Hitachi (Tokyo, Japan) high-speed amino acid analyser.

Results

Purified pancreas kallikrein showed a single band with a mobility markedly different from those of trypsin and chymotrypsin on alkaline disc-gel electrophoresis (Fig. 1*a*). The band also showed Pro-Phe-Arg-ONap hydrolytic activity (Fig. 1*b*). Moreover, the kallikrein preparation did not hydrolyse Bz-Tyr-OEt, denatured bovine haemoglobin or Bz-Gly-His-Leu, indicating that it was free from proteolytic enzymes such as trypsin, chymotrypsin, cathepsin D and proangiotensin-converting enzymes. The substrate, acid- and alkali-treated Cohn fraction IV-4, also had no detectable contaminating activities of these enzymes.

Proof that pancreas kallikrein directly liberated the pressor substance is shown in Table 1. In the experiment involving 0h pre-incubation and an additional 18h incubation, no pressor substance, such as proangiotensin and angiotensin, was formed without kallikrein, indicating that acid- and alkali-treated Cohn fraction IV-4 did not contain any angiotensin-forming enzymes. Kallikrein alone with-

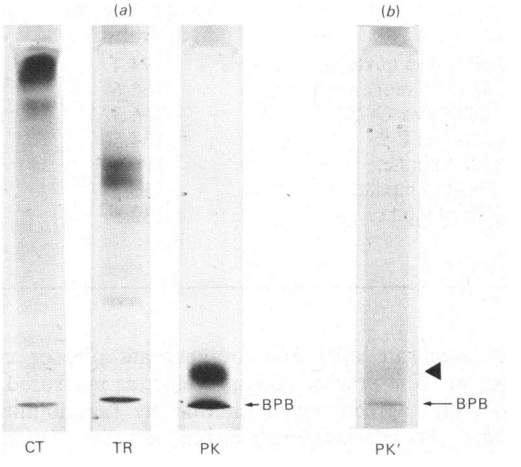

Fig. 1. *Electrophoretic analysis of purified pancreas kallikrein*

(*a*) Comparison of hog pancreas kallikrein (PK) with trypsin (TR) and chymotrypsin (CT) by disc-gel electrophoresis on 0.7% polyacrylamide gels at pH9.5. (*b*) Zymogram of hog pancreas kallikrein (PK') after disc-gel electrophoresis was carried out with Pro-Phe-Arg-ONap as substrate and Fast Red ITR as dye. Abbreviation used: BPB, electrophoresis front marked with Bromophenol Blue; ◀, Pro-Phe-Arg-ONap hydrolysis activity.

Table 1. *Evidence for kallikrein directly forming pressor substance*

Acid- and alkali-treated Cohn fraction IV-4 (10mg) was pre-incubated with 0.5ml of settled kallikrein–Sepharose 4B (about 5mg of kallikrein) in 2.0ml of Briton–Robbinson buffer, pH4.0, containing 200μl of 0.1M-Na_2EDTA, 20μl of 0.45M-8-hydroxyquinoline sulphate and 5μl of 10% 2,3-dimercaptopropan-1-ol as angiotensinase inhibitors, at 37°C for 0, 1, 4 and 18h. After pre-incubation, kallikrein–Sepharose 4B was removed by filtration on a glass filter. A portion (100μl) of the filtrate was taken for measurement of the pressor activity and radioimmunoassay for proangiotensin and angiotensin. To the rest of the filtrate, 10mg of Cohn fraction IV-4 in the same buffer as that for pre-incubation was added and the mixture was incubated at 37°C for 18h. Bioassay and radioimmunoassay for proangiotensin and angiotensin were then performed again.

	Total amount of pressor substance formed per tube (ng)					
	Pre-incubation with kallikrein			Additional 18h incubation with pre-incubation filtrate and 10mg of new substrate		
		Radioimmunoassay			Radioimmunoassay	
Pre-incubation time (h)	Bioassay for angiotensin	Angiotensin	Proangiotensin	Bioassay for angiotensin	Angiotensin	Proangiotensin
0	0.0	0.02	4.0	0.0	0.02	6.0
1	553.6	543.2	12.0	539.0	546.4	8.0
2	1068.0	1102.0	8.0	1054.0	1077.0	8.0
4	1124.0	1106.0	6.0	1160.0	1145.5	8.0
18	1856.0	1892.0	12.0	1848.0	1780.0	10.0
Without substrate	—	—	—	0.0	0.0	0.0

Table 2. *Summary of the purification procedure, recoveries and specific activities*

The specific activity at each step of the purification of the pressor substance generated by pancreas kallikrein was calculated from the ratio of pressor activity to the total absorbance determined at 280nm or 210nm.

	Pressor activity (μg of angiotensin equivalent)	Recovery of pressor activity (%)	Total		Specific activity
			A_{280}	A_{210}	
1. Reaction mixture	274.4	—	17685.3	—	0.016
2. Hot ethanol extraction	265.6	96.8	696.96	—	0.38
3. Sephadex G-25	216.3	81.5	85.67	—	2.524
4. Bio-Gel P-2	184.5	67.2	31.29	375.10	5.896
5. First h.p.l.c.	176.4	64.2	—	21.85	96.94
6. Second h.p.l.c.	156.7	57.1	—	9.85	191.8
7. Third h.p.l.c.	132.8	48.4	—	5.71	278.71

out substrate also did not generate pressor substances. The pressor activity value, as measured by bioassay, agreed well with that by radioimmunoassay for [Ile^5]angiotensin but not for [Ile^5]proangiotensin.

If any enzymes were activated by kallikrein during pre-incubation and thereby generated pressor substances, additional pressor formation would be expected during subsequent incubation with newly added substrate, but this was not observed.

The purification procedures for the pressor substance are summarized in Table 2, along with recoveries and specific activities of it. Hot ethanol extraction was found to be suitable for efficient purification of the pressor substance, with a good recovery. The gel-filtration profile of the pressor substance on Sephadex G-25 is shown in Fig. 2.

Fig. 3 shows that the pressor substance was eluted on Bio-Gel P-2 in similar fractions to authentic [Ile^5]angiotensin. However, [Ile^5]proangiotensin, [Ile^5]angiotensin and des-Asp^1-[Ile^5]angiotensin were not completely separated in this system. The crude pressor substance at this step contained three major and several minor components on t.l.c. One of the major components showed the migration distance, which was equal to that of authentic [Ile^5]angiotensin (R_F0.42), and no components migrated to the position of [Ile^5]proangiotensin and des-Asp^1-[Ile^5]-angiotensin (Fig. 4*a*).

Authentic samples of [Ile^5]proangiotensin, [Ile^5]-

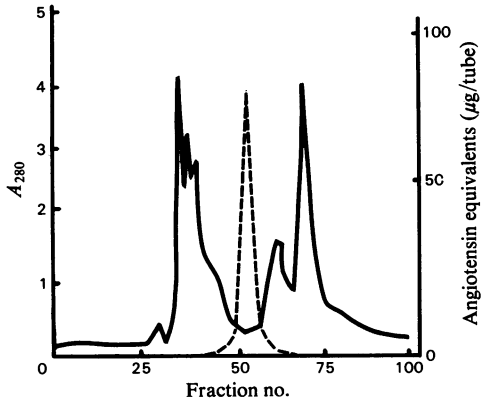

Fig. 2. *Gel filtration of pressor substance on Sephadex G-25*

Pressor substance after ethanol extractions was applied to a column (3.9cm × 100cm) of Sephadex G-25 equilibrated with 0.05M-acetic acid. Elution was carried out with the same buffer at a flow rate of 40ml/h, collecting 12ml fractions, ——, A_{280}; ----, μg of angiotensin equivalent/tube.

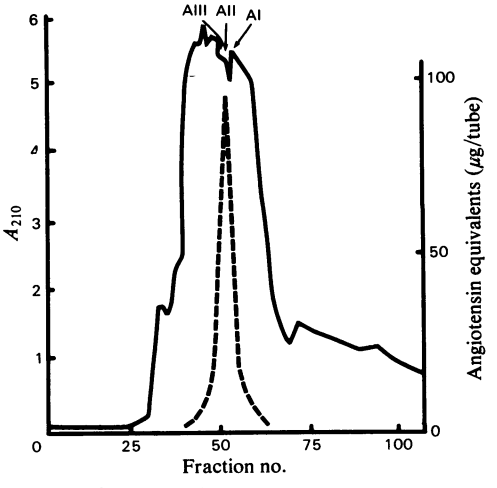

Fig. 3. *Gel filtration of pressor substance on Bio-Gel P-2 column*

After gel filtration on Sephadex G-25, a portion was applied to a Bio-Gel P-2 column (1.2cm × 90cm) equilibrated with 0.05M-acetic acid, collecting 1.25ml fractions. The column was calibrated with [Ile^5]proangiotensin (AI), [Ile^5]angiotensin (AII) and des-Asp^1-[Ile^5]angiotensin (AIII). ——, A_{210}; ----, μg of angiotensin equivalents/tube.

angiotensin, des-Asp^1-[Ile^5]angiotensin and the pressor substance generated by kallikrein were chromatographed under the condition of the first

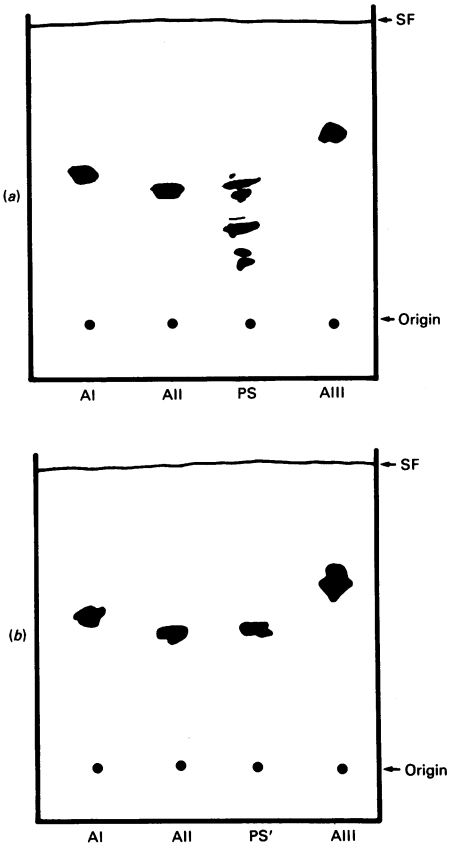

Fig. 4. *T.l.c. of isolated pressor substance and authentic [Ile^5] proangiotensin (AI), [Ile^5]angiotensin (AII) des-Asp^1[Ile^5]angiotensin (AIII)*

T.l.c. was performed with solvent system (1) on Avicel-SF cellulose plates (10cm × 10cm), after Bio-Gel P-2 (*a*) and the third h.p.l.c. step (*b*). Abbreviations used: PS, the pressor substance after gel filtration on Bio-Gel P-2 column; PS', the pressor substance after the third h.p.l.c. step; SF, solvent front.

h.p.l.c. system (Fig. 5). Retention times of these materials were 46.3min, 31.4min, 28.7min and 31–32min respectively. Most contaminants in the pressor substance were removed by the first h.p.l.c. system.

In the second h.p.l.c. experiments, the retention time of the pressor substance was 13.4min, which was the same as that of authentic [Ile^5]angiotensin (Figs. 6*a* and 6*b*), and differed from that of des-Asp^1-[Ile^5]angiotensin, i.e. 8.2min (Fig. 6*c*). [Ile^5]Proangiotensin was not eluted during 30min under the second h.p.l.c. condition, until solvent B was used as eluent.

The h.p.l.c. was repeated for the third time to further purify the pressor substance and a single

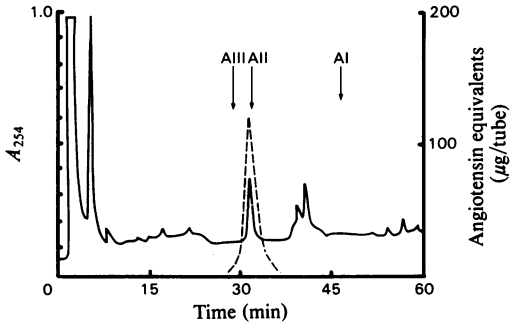

Fig. 5. *The first h.p.l.c. step*
The first h.p.l.c. step was carried out on a column (3.9mm internal diameter × 30cm) of μ-Bondapak C_{18} with a 60min linear gradient of from solvent A to solvent B at a flow rate of 1.5ml/min. Abbreviations are as defined in the legend to Fig. 3. ——, A_{254}; ----, μg of angiotensin equivalents/tube.

Table 3. *Amino acid composition of purified pressor substance*
The residue weight and content are means from four experiments and are from 20 acid hydrolysate. In each experiment, the content of any amino acid other than the eight amino acids shown was negligible. The value of each amino acid is given as a molar ratio of each amino acid to isoleucine.

	Purified pressor substance		Authentic
Amino acid	Average residue wt. (%)	Molar ratio	[Ile^5]angiotensin molar ratio
Asp	12.11	1.03	1.14
Val	10.59	1.03	1.10
Ile	11.62	(1.00)	(1.00)
Tyr	11.06	0.69	0.82
Phe	15.05	1.04	1.07
His	12.46	0.91	0.96
Arg	13.12	0.86	1.07
Pro	8.71	0.86	1.01

peak with pressor activity was finally obtained that coincided with authentic angiotensin (Fig. 6*d*). Also, on analysis by t.l.c., with solvent system (1), the pressor substance showed a single spot with R_F 0.42, which is equal to that of authentic [Ile^5]angiotensin (Fig. 4*b*). This coincidence of migration was also found for another solvent system (2). On the basis of the specific absorption of authentic [Ile^5]angiotensin at 210nm, the finally isolated pressor substance from the third h.p.l.c. step was pure. The amino acid composition of the purified pressor substance was identical with that of authentic [Ile^5]angiotensin (Table 3).

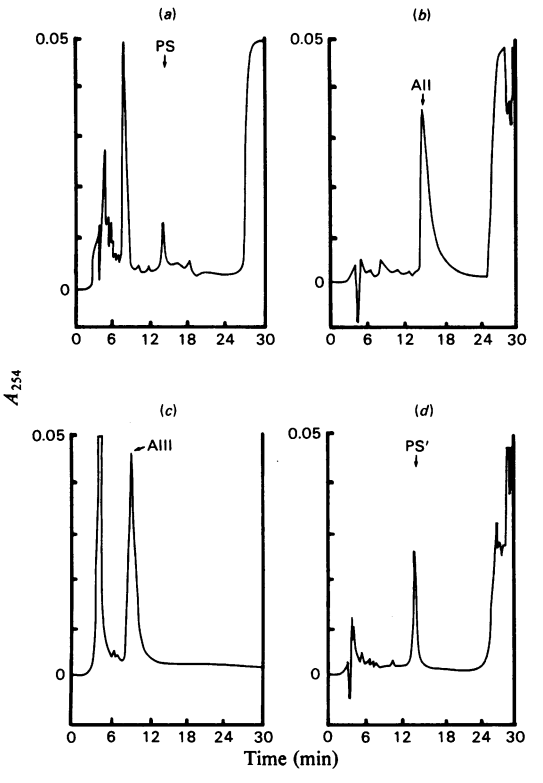

Fig. 6. *The second and the third h.p.l.c. steps*
The chromatography column was the same as for the first h.p.l.c. step. Chromatography was performed with isocratic elution of solvent A and solvent B (1:1, v/v) at a flow rate of 1ml/min. (*a*) Elution profile of the pressor substance (PS) after the first h.p.l.c. step; (*b*) elution profile of authentic [Ile^5]-angiotensin (AII); (*c*) elution profile of authentic des-Asp^1-[Ile^5]angiotensin (AIII); (*d*) elution profile of the pressor substance PS′ after the second h.p.l.c. step. The arrow marks the elution position of the indicated substances.

Discussion

The renin–angiotensin system is representative of the whole angiotensin-forming system, which begins with renin release and ends with conversion of proangiotensin into angiotensin by proangiotensin-converting enzyme.

However, trypsin, a well known bradykinin-forming enzyme, was found by us to generate angiotensin directly from human plasma protein at pH6.0 (Arakawa *et al.*, 1976, 1980). Subsequently, kallikrein of hog pancreas was also found to liberate the pressor substance from human protein at pH4.0–5.0. No enzymes other than kallikrein (such as prorenin, renin, cathepsin D, angiotensin-converting enzyme, trypsin and chymotrypsin) par-

ticipated in this reaction. Our previous study (Arakawa & Maruta, 1980) suggested that the substance was angiotensin-like, since its pressor effect was inhibited by [Sar^1,Ile^8]angiotensin, but not by captopril, and since the bioassay value of the pressor activity was coincident with the radioimmunoassay value for [Ile^5]angiotensin. However, these criteria did not exclude des-Asp^1-[Ile^5]angiotensin and des-Asp^1,Arg^2-[Ile^5]angiotensin, whose pressor activities were similarly antagonized by [Sar^1,Ile^8]angiotensin but not inhibited by proangiotensin-converting enzyme inhibitor (Bumpus *et al.*, 1961; Schwyzer, 1963; Campbell & Pettinger, 1976; Campbell & Schmitz, 1979; Sexton *et al.*, 1979). They also cross-reacted with anti-angiotensin antibody (Cain *et al.*, 1969; Emanuel *et al.*, 1973; Lijnen *et al.*, 1978). However, in the present study we confirmed that the pressor substance generated by kallikrein was [Ile^5]angiotensin.

Trypsin and pancreas kallikrein are well known kinin-forming enzymes, the former liberating bradykinin and the latter generating kallidin in weak alkaline conditions (Elliot *et al.*, 1961; Habermann & Blennemann, 1964). Bradykinin and kallidin are vasodepressor peptides that may play roles in blood-pressure control and hypertension (Margolius *et al.*, 1974).

It would be more correct if the term 'kinin(s)' was restricted to vasodepressor peptide(s) and the term 'tensin(s)' to vasopressor peptide(s). Based on this nomenclature we proposed a 'kinin–tensin enzyme system' in which two different activities of opposite direction, i.e. both depressor and pressor, were generated by one enzyme and the direction of the reaction was determined only by the condition of incubation, the optimal pH for depressor formation being weak alkaline pH and that for pressor formation weak acidic pH (Arakawa & Maruta, 1980). In the present paper we have proved that, like trypsin, kallikrein also belongs to this system.

Other unique angiotensin-forming systems, i.e. tonin from rat salivary gland (Boucher *et al.*, 1974; Grisé *et al.*, 1981; Thibault & Genest, 1981) and human neutrophil lysosomal proteinase (Wintroub *et al.*, 1974, 1981a,*b*; Seidah *et al.*, 1978; Coblyn *et al.*, 1979), have been reported. Both of these enzymes liberated angiotensin directly from renin substrate, whereas neutrophil lysosomal proteinase did not generate any kinin when incubated in neutral-pH conditions (Wintroub *et al.*, 1974). Whether these angiotensin-forming systems really do not generate kinin at acidic pH, unlike the kinin–tensin system, awaits further examination.

This study was supported in part by grants-in-aid from the Japanese Ministry of Education (grant no. B-00548200) and from the Fukuoka University Research Institute. We thank Midori-Juji, Osaka, Japan, for preparing human Cohn fraction IV-4 and Sanwa Chemicals, Nagoya, Japan, for hog pancreas kallikrein. The skilful assistance of Miss Yukiko Taniguchi and Miss Mikiko Arimizu is greatly appreciated.

References

Arakawa, K. & Maruta, H. (1980) *Nature (London)* **288**, 705–706

Arakawa, K., Minohara, A., Uemura, N., Sakai, T. & Ikeda, M. (1975) *Endocrinol. Jpn.* **22**, 427–432

Arakawa, K., Ikeda, M., Fukuyama, J. & Sakai, T. (1976) *J. Clin. Endocrinol. Metab.* **42**, 599–602

Arakawa, K., Yuki, M. & Ikeda, M. (1980) *Biochem. J.* **187**, 647–653

Barrett, A. J. (1967) *Biochem. J.* **104**, 601–608

Boucher, R., Asselin, J. & Genest, J. (1974) *Circ. Res. Suppl. I* **34/35**, 203—209

Briton, H. T. S. & Robbinson, R. A. (1931) *J. Chem. Soc.* 1456–1462

Bumpus, F. M., Khairallah, P. A., Arakawa, K., Page, I. H. & Smeby, R. R. (1961) *Biochim. Biophys. Acta* **46**, 38–44

Cain, M. D., Catt, K. J. & Caghlan, J. P. (1969) *J. Clin. Endocrinol. Metab.* **29**, 1639–1643

Campbell, W. B. & Pettinger, W. A. (1976) *J. Pharmacol. Exp. Ther.* **198**, 450–456

Campbell, W. B. & Schmitz, J. M. (1979) *Eur. J. Pharmacol.* **54**, 209–216

Coblyn, J. S., Austen, K. F. & Wintroub, B. U. (1979) *J. Clin. Invest.* **63**, 998–1005

Cushman, D. W. & Cheung, H. S. (1971) *Biochem. Pharmacol.* **20**, 1637–1648

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427

Elliot, D. F., Horton, E. W. & Lewis, G. P. (1961) *Biochem. J.* **78**, 60–65

Emanuel, R. L., Cain, J. P. & Williams, G. H. (1973) *J. Lab. Clin. Med.* **81**, 632–640

Grisé, C., Boucher, R., Thibault, G. & Genest, J. (1981) *Can. J. Biochem.* **59**, 250–255

Haber, E., Koerner, T., Page, L. B., Kliman, B. & Purnode, A. (1969) *J. Clin. Endocrinol.* **29**, 1349–1355

Habermann, E. & Blennemann, C. T. (1964) *Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharmakol.* **249**, 353–373

Hitomi, Y., Minobe, M. & Fujii, S. (1980) *Clin. Chim. Acta* **100**, 275–283

Hummel, B. C. W. (1959) *Can. J. Biochem. Physiol.* **37**, 1393–1399

Lijnen, P. J., Amery, A. K., Fagard, R. H. & Katz, F. H. (1978) *Clin. Chim. Acta* **88**, 403–412

Margolius, H. S., Morwita, D., Gelber, R. G., Alexander, R. W., Gill, J. R., Pisano, J. J. & Keiser, H. R. (1974) *Circ. Res.* **35**, 812–819

Schwyzer, R. (1963) *Pure Appl. Chem.* **6**, 265–295

Seidah, N. G., Routhier, R., Caron, M., Chrétien, M., Demassieux, S., Boucher, R. & Genest, J. (1978) *Can. J. Biochem.* **56**, 920–925

Sexton, J. M., Britton, S. L., Beierwaltes, W. H., Fiksen-Olsen, M. J. & Romero, J. C. (1979) *Am. J. Physiol.* **237**, H218–H223

Thibault, G. & Genest, J. (1981) *Biochim. Biophys. Acta* **660**, 23–29
Tonnaer, J. A. D. M., Verhoef, J., Wiegant, V. M. & de Jong, W. (1980) *J. Chromatogr.* **183**, 303–309
Wintroub, B. U., Goetzl, E. J. & Austen, K. F. (1974) *J. Exp. Med.* **140**, 812–824
Wintroub, B. U., Klichstein, L. B., Kaempfer, C. E. & Austen, K. F. (1981*a*) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1204–1208
Wintroub, B. U., Klickstein, L. B. & Watt, K. W. K. (1981*b*) *J. Clin. Invest.* **68**, 484–490
Yokosawa, N., Takahashi, N., Inagami, T. & Page, D. L. (1979) *Biochim. Biophys. Acta* **569**, 211–219