Isolation and Identification of a Pepsitensin

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1. From ox plasma incubated with pepsin a highly purified pepsitensin has been isolated by fractional precipitation, solvent extraction, column chromatography, countercurrent distribution and paper chromatography. 2. Comparison of the properties of this substance with those of synthetic valyl-5 angiotensin I showed: (a) the same specific pressor activity; (b) the same amino acid composition; (c) identical paper-electrophoretic mobilities at various pH values. 3. *N*- and *C*-Terminal studies carried out on the intact polypeptide and on the products of chymotrypsin digestion established the following sequence for the amino acids present in the molecule: Asp-Arg-Val-Tyr-Val-His-Pro-Phe-His-Leu. This structure is identical with that of valyl-5 angiotensin I.

The kidney enzyme renin acts on a plasma globulin to produce angiotensin, a powerful pressor polypeptide (Braun Menéndez, Fasciolo, Leloir & Muñoz, 1939; Page & Helmer, 1940). Skeggs and his collaborators (Skeggs, Marsh, Kahn & Shumway, 1954*a,b*, 1955; Skeggs, Kahn & Shumway, 1956; Skeggs, Lentz, Kahn, Shumway & Woods, 1956; Lentz, Skeggs, Woods, Kahn & Shumway, 1956) showed that renin liberates an inactive decapeptide, angiotensin I, which is immediately transformed into the active octapeptide, angiotensin II, by a plasma enzyme. These workers isolated and established the structure of both horse angiotensins. Almost simultaneously, Peart (1955, 1956) isolated ox angiotensin I. The amino acid sequence in this molecule was determined by Elliott & Peart (1956, 1957) soon thereafter.

Horse and ox angiotensins are identical except in position 5, where the isoleucine residue in the horse peptide is replaced by a valine residue. Hog angiotensin is reported to be identical with that of horse origin (Page & Bumpus, 1961). Angiotensins from other animal species have not been studied.

Trypsin acting on horse globulins releases a tetradecapeptide, which yields angiotensin I when incubated with renin (Skeggs, Kahn, Lentz & Shumway, 1957). The structure of this polypeptide renin substrate was confirmed by synthesis (Skeggs, Lentz, Kahn & Shumway, 1958). This remarkable investigation proved that renin releases angiotensin I from its mother molecule by splitting a Leu-Leu bond.

Chemical synthesis has been accomplished for isoleucyl-5 and valyl-5 angiotensin octa- and decapeptides (Schwyzer *et al.* 1958*a,b*; Schwarz, Bumpus

& Page, 1957; Bumpus, Schwarz & Page, 1957). Several structural requirements for pressor activity were shown by the comparative study of their biological activities (Schwyzer, 1963).

Croxatto & Croxatto (1942) found that plasma globulins incubated with pepsin gave a product with pressor and vasoconstrictor properties. The active material was named pepsitensin and had many pharmacological properties in common with angiotensin (Alonso, Croxatto & Croxatto, 1943). Paiva (1954) and Paiva, Bandiera & Prado (1954) showed that angiotensin and pepsitensin have identical actions on isolated guinea-pig ileum and could not be separated by paper chromatography in a butan-1-ol-acetic acid system. However, by zone electrophoresis at pH 2.5 they succeeded in fractionating a mixture of both substances. Dengler (1956) found 13 different amino acids in pepsitensin but was unable to identify phenylalanine among them. The significance of this fact is open to question since the preparation was very impure.

Recently Blair (1962) prepared pepsitensin from horse globulins and crystalline pepsin. The crude material was partially purified and its pharmacological properties were carefully compared with those of vasopressin (Pitressin), synthetic asparaginyl-1 valyl-5 angiotensin II and natural isoleucyl-5 angiotensin II (75% pure). Blair (1962) found that the pharmacological activities of pepsitensin and angiotensin are very similar, although minor differences were observed in their action on guinea-pig ileum and rat uterus.

Braun Menéndez & Paladini (1958) have established that a given amount of angiotensinogen yields equivalent amounts of pressor activity when

treated with renin or pepsin. Further, the same authors confirmed the fact, already pointed out by Braun Menéndez, Fasciolo, Leloir, Muñoz & Taquini (1943), that no pressor activity is released by pepsin from angiotensinogen that has been incubated with renin.

All the experimental facts mentioned above point to a close chemical similarity between pepsitensin and angiotensin, but the proof of their possible identity must necessarily rest on the study of pure pepsitensin. This has been the objective of the present work. A preliminary report of this work has been given (Paladini, Franze de Fernández & Delius, 1964).

EXPERIMENTAL

Materials

Fresh ox plasma was obtained by centrifugation of citrate-treated blood collected at a nearby slaughterhouse. Crystalline pepsin and di-isopropyl phosphorofluoridate-treated carboxypeptidase were obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.); 1:10000 pepsin (U.S.A. National Formulary) was from Inorp (Buenos Aires, Argentina); crystalline α -chymotrypsin was from Sigma Chemical Co. (St Louis, Mo., U.S.A.); valyl-5 angiotensin I, asparaginyl-1 valyl-5 angiotensin II and aspartyl-1 valyl-5 angiotensin II were pure synthetic products obtained from Ciba Ltd. (Basle, Switzerland).

All solvents were redistilled in glass. The ammonium acetate used in the volatile buffers was prepared from gaseous NH₃ and distilled acetic acid.

Methods

Measurement of the pressor activity. This was performed by the technique of Paladini, Braun Menéndez, del Frade & Massani (1959). One rat unit is equivalent to 20 μ g. of asparaginyl-1 valyl-5 angiotensin II.

Nitrogen determination. This was done by the micro-Kjeldahl technique of Shepherd *et al.* (1954). The specific activity of the different pepsitensin preparations was usually expressed in rat units/mg. of nitrogen.

Protein determination. This was done by the procedure of Lowry, Rosebrough, Farr & Randall (1951).

Concentration of solutions. This was carried out by vacuum distillation at below 45°, unless specified otherwise.

Preparation of crude pepsitensin. A 20l. portion of plasma was adjusted to pH 6.0 and incubated for 10 min. at 47° with 6g. of 1:10000 pepsin/l. The reaction was stopped by lowering the pH to 4.2 and heating for 5 min. at 90°. The coagulated proteins were separated by filtration and washed with 10l. of distilled water. The clear filtrate and washings were concentrated to one-tenth of the original volume of plasma.

The whole procedure repeated 25 times yielded a crude extract with a total pressor activity equivalent to 6.4×10^6 rat units.

First stages of purification. A 50l. portion of crude pepsitensin was adjusted to pH 5.0 and mixed with 200l. of ethanol. The precipitate was filtered off and the solution was concentrated to the starting volume. Extraction of

Table 1. *Biological activity of the product at successive stages of purification during the isolation of a pepsitensin*

	Activity	
	(10 ⁻⁶ × rat units)	(rat units/ mg. of N)
Incubation mixture (500l. of ox plasma)	6.4	6.4
↓		
Ethanol precipitation and ether extraction	6.0	12.0
↓		
Butan-1-ol extraction; 0.01N-HCl extraction	4.0	23.0
↓		
Preparative countercurrent distribution	2.5	138
↓		
Salt precipitation; acetic acid extraction	2.4	235
↓		
CM-cellulose chromatography	2.2	3900
↓		
Countercurrent distributions		
↓		
(System 1)		
Minor peak Major peak	1.5	43000
↓		
(System 2)		
↓		
Fractions I and II	0.68	—
↓		
Paper chromatography		
↓		
Pepsitensin	0.62	280000

this liquid with 1vol. of diethyl ether at pH1 eliminated a great proportion of the lipids and coloured substances present. Pepsitensin was next extracted with 2vol. of butan-1-ol, at the same pH. When the butan-1-ol extract was stirred with 2vol. of 10mN-HCl all the pressor activity was recovered in the aqueous phase; it was neutralized and concentrated to a volume of 8.1l., with a total pressor activity equivalent to 4.0×10^6 rat units.

The next purification step was a countercurrent distribution in the system butan-1-ol-0.1M-potassium phosphate buffer, pH 7.50 (1:1, v/v). The 32-tube machine used was a modified version of that devised by Verzele (1953), and had a capacity of 700ml./phase in each tube. To 2.7l. of the preparation, solid KH₂PO₄ was added until the salt concentration was 0.1M; the pH was adjusted to 7.50 with 15N-KOH, and butan-1-ol added until the solution was saturated. This liquid was placed in the first four tubes of the distribution train as the lower phase. Equal volumes of fresh upper and lower phases were added to fill the machine and the distribution was performed as follows: 28 transfers were done in the usual way followed by 13 transfers by recycling and 55 transfers by single withdrawal. The distribution of the material was followed by measurements

Fig. 1. Preparative countercurrent distribution of crude pepsitensin in the system butan-1-ol-0.1M-potassium phosphate buffer, pH 7.50 (1:1, v/v). A 32-tube machine of 700 ml. capacity was used. A 2.7 l. portion of pepsitensin solution (1.4×10^6 rat units; 60 g. of N) was distributed as follows: 28 transfers by the usual procedure, followed by 13 transfers by recycling and 55 transfers by single withdrawal. The distribution was analysed by measurements of pressor activity (●) and total nitrogen (○). The fractions indicated (↔) were pooled and subjected to further purification (see the text).

of pressor activity and total nitrogen (Fig. 1). Three experiments were necessary to process all the preparation. The fractions with highest pressor activity were pooled and concentrated to a volume of 6 l. The pepsitensin was precipitated by the addition of 320 g. of NaCl/l. at pH 7.50. The precipitate was filtered off and dried *in vacuo* over silica gel. The pepsitensin present in the dry powder was extracted with acetic acid, and the filtered acid extract was evaporated at below 30°. The residue was dissolved in 6 l. of distilled water. The total pressor activity at this stage was equivalent to 2.4×10^6 rat units.

Chromatography on CM-cellulose. The impure pepsitensin obtained in the previous step was next chromatographed on a column (9 cm. $\times$ 26 cm.) of CM-cellulose (Serva, Heidelberg; 0.55 m-equiv./g.) equilibrated with 2.0 mM-ammonium acetate buffer, pH 5.80. A column of this size accepts up to 1 l. of impure pepsitensin adjusted to pH 5.80 with 3 N-NH₃. After a rinse with 19 l. of 20 mM-ammonium acetate buffer, pH 5.80, the pressor activity was eluted with 0.1 M-ammonium acetate buffer, pH 5.80. The rate of flow through the column was always kept at 23 ml./min. The effluent collected in 200 ml. fractions was analysed for pressor activity and extinction at 280 m μ (Fig. 2). The fractions with highest biological activity were pooled and evaporated at below 30°. The ammonium acetate was removed by sublimation in a freeze-drying apparatus. The remaining solid was dissolved in 40 ml. of distilled water. This step was repeated six times to process all the material. The total pressor activity recovered was equivalent to 2.2×10^6 rat units.

Countercurrent distribution. To purify the material further, two countercurrent distribution steps were performed. System 1 was prepared with butan-1-ol-benzene-0.3 N-NH₃ (17:3:20, by vol.), adjusted to pH 7.50 with acetic acid. To 80 ml. of material from the previous step 6 N-NH₃

Fig. 2. Chromatography of impure pepsitensin on a column (9 cm. $\times$ 26 cm.) of CM-cellulose equilibrated with 20 mM-ammonium acetate buffer, pH 5.80. A 1.0 l. portion of pepsitensin solution (3.7×10^5 rat units; 1.6 g. of N), adjusted to pH 5.80, was applied to the column. After a wash with 19 l. of 20 mM-ammonium acetate buffer, pH 5.80, the active material was eluted with 0.1 M-ammonium acetate buffer, pH 5.80. The effluent flow rate was 23 ml./min. The effluent was analysed by measurements of pressor activity (●) and extinction at 280 m μ (○). The fractions indicated (↔) were pooled and subjected to further purification (see the text).

Fig. 3. Countercurrent distribution of impure pepsitensin in the system butan-1-ol-benzene-0.3 N-NH₃ (17:3:20, by vol.), adjusted to pH 7.50 with acetic acid. An 80 ml. sample of pepsitensin solution (7.0×10^5 rat units; 180 mg. of N) was submitted to 942 transfers in an automatic 1000-tube machine. The distribution was analysed by measurements of pressor activity (●) and extinction at 280 m μ (○). The fractions indicated (↔) were pooled and subjected to further purification (see the text).

was added to give a final concentration of 0.3 N; the pH was adjusted to 7.50 with acetic acid and the solution was saturated with butan-1-ol-benzene (17:3, v/v) mixture and then equilibrated with an equal volume of the upper phase of system 1. This sample was placed in the first 40 tubes of an automatic machine (Craig, Hausmann, Ahrens &

Harfenist, 1951) with 1000 tubes of 3 ml. capacity. After 942 transfers the analysis for pressor activity disclosed a minor peak in tubes 50–100 and a major peak in tubes 150–300 (Fig. 3). Both fractions were recovered, the solvents were evaporated at below 30° and the remaining ammonium acetate was removed by sublimation in a freeze-drying apparatus.

The major peak was submitted to a second counter-current distribution in system 2 composed of butan-1-ol-0.3N-NH₃ (1:1, v/v), adjusted to pH 9.0 with acetic acid. The usual procedure was applied up to 1000 transfers. The distributions of pressor activity and protein indicated inactive material in tubes 0–375 and 675–1000. The solvent in these tubes was replaced with fresh system and the distribution carried on to 2653 transfers by the recycling procedure. Analysis of the distribution then showed the presence of one major pressor component (Fig. 4). The contents of tubes 76–125 (fraction I) and 150–199 (fraction II) were separately pooled and processed as described above to recover the active material therein.

Paper chromatography. Fractions I and II were further purified by paper chromatography on Whatman 3MM paper (45 cm. × 12 cm. strips) developed for 24 hr. by the ascending technique with the solvent butan-1-ol-acetic acid-water (7:1:2, by vol.). The material was applied in a concentration of approx. 3000 rat units/cm. After the run the paper was dried at room temperature in the dark. A 3 mm. strip cut longitudinally from the middle of the paper was first developed with ninhydrin (0.2%, w/v, in acetone) and then with Sakaguchi reagent, as described by Jepson & Smith (1953). By using the stained strip as a guide, the main portions of the chromatogram were cut out and eluted with ethanol-acetic acid-water (35:7:65, by vol.).

The activities at different stages of the preparation are shown in Table 1.

Fig. 4. Countercurrent distribution of impure pepsitensin in the system butan-1-ol-0.3N-NH₃ (1:1, v/v), adjusted to pH 9.0 with acetic acid. The sample was 4.25×10^5 rat units (10 mg. of N) of pepsitensin from the major peak of the previous distribution (Fig. 3). After 1000 transfers the contents of tubes 0–375 and 675–1000 were replaced by fresh system and the distribution was carried on to 2653 transfers by the recycling procedure. Analysis was done by measurements of pressor activity (●). The broken curve (Δ) indicates the theoretical distribution of a pure compound. The fractions indicated (↔) were pooled and subjected to further purification (see the text).

Paper electrophoresis. This was performed on Whatman 3MM paper (10 cm. × 26.4 cm. strips) as described by Kunkel & Tiselius (1951). The sample was applied as a narrow band of length between 0.3 cm. and 6.0 cm. according to the amount of material (0.02–0.7 mg.). The potential gradient along the paper was usually 16 v/cm. The buffers used for pH 3.20 and pH 3.50 were prepared by mixing 4.00 ml. of pyridine with 6.20 ml. or 3.80 ml. of 99% formic acid respectively and diluting to 1 l. with distilled water. For the buffer of pH 5.05, 6.05 ml. of pyridine and 1.91 ml. of 99% formic acid were mixed and diluted to 1 l. with distilled water. The buffers of pH 6.50, pH 7.15 and pH 7.70 were prepared with 50 mM-NaH₂PO₄ adjusted to the corresponding pH with 50 mM-Na₂HPO₄; the buffer of pH 9.80 was obtained by mixing 16.1 ml. of dimethylamino-ethanol with 4.0 ml. of acetic acid and diluting to 1 l. with distilled water; the buffer of pH 11.55 was 0.53% Na₂CO₃. Peptides were detected by the ninhydrin and Sakaguchi reactions, as described above. Electroendosmosis was followed by the movement of glucose, located by reaction with benzidine (Horrocks, 1949).

Quantitative amino acid analysis. This was performed by ion-exchange chromatography by the procedure of González Cadavid & Paladini (1964). Peptides were hydrolysed in small sealed tubes with 6N-HCl for 24 hr. at 110°; phenol was added to improve the recovery of tyrosine (Sanger & Thompson, 1963).

N-Terminal amino acids. These were detected by the method of Sanger & Thompson (1953). The DNP-amino acid was identified by thin-layer chromatography as described by Brenner, Niederwieser & Pataki (1961).

Enzymic hydrolysis. Carboxypeptidase A was incubated at 25° with various amounts (0.05–0.25 μmole) of peptide dissolved in 0.1–0.25 ml. of 0.1% NaHCO₃. The enzyme:substrate molar ratio was kept between 1:200 and 1:300. Samples of the incubation mixture, taken at different times, were mixed with 1 ml. of 0.25N-HCl to stop the reaction. The amino acids liberated were measured by the method of González Cadavid & Paladini (1964).

The experiments with α-chymotrypsin were done in 50 mM-ammonium carbonate-ammonium acetate buffer, pH 7.90 (Elliott & Peart, 1957). The enzyme (0.025–0.125 mg.) was added to the peptide (0.1–0.5 μmole) dissolved in 0.1–0.5 ml. of buffer. After 2 hr. at 37° the reaction was stopped by the addition of 10–50 μl. of acetic acid and the solution was evaporated at below 30°. The solid residue was kept for 24 hr. *in vacuo* over NaOH pellets and conc. H₂SO₄. The products of these hydrolyses were analysed by paper electrophoresis.

RESULTS AND DISCUSSION

Preparation of pepsitensin. To find the experimental conditions for the maximum yield of pepsitensin several variables were studied: pH, temperature and time of incubation, ratio of pepsin to plasma proteins, and different ways of stopping the enzymic reactions. The best results were obtained by incubating ox plasma at pH 6.0 with 6 mg. of 1:10000 pepsin/ml. for 10 min. at 47°, then lowering the pH to 4.2 and heating the mixture for 5 min. at 90°. Under these conditions 10–15 rat units of pressor activity/ml. of plasma

were usually obtained. The yield did not improve substantially when crystalline pepsin was used instead of 1:10 000 pepsin. No pressor activity could be detected in control experiments where pepsin was not added to the plasma.

Purification of pepsitensin. Table 1 shows the results of the successive steps of purification.

The filtrate from the incubation mixture was precipitated with ethanol and the supernatant extracted with diethyl ether to remove lipids and coloured materials. The pepsitensin present in the clear remaining solution was extracted first with butan-1-ol and then, from the organic solvent, with 10M-N-hydrochloric acid; at this point a 3.6-fold purification was achieved, with a recovery of 62.5% of the initial activity.

The acid solution was concentrated at low temperature and submitted to a countercurrent distribution in a butan-1-ol-potassium phosphate buffer system (Fig. 1). This step led to a 6-fold purification, with a recovery of 63% of the starting pressor material.

The precipitation of pepsitensin solutions by sodium chloride and its extraction from the dry solids by acetic acid gave a product, practically free from salt, suitable for column chromatography on CM-cellulose, on which pepsitensin is strongly held while most of the inactive materials run through the column. With 0.1 M-ammonium acetate, pepsitensin emerges as a very sharp peak (Fig. 2). A 17-fold purification was achieved, with 92% recovery of the pressor activity. The use of the 1000-tube countercurrent distribution machine for its further purification followed. The first run was done at pH 7.50 in a butan-1-ol-benzene-ammonium acetate system (Fig. 3). After 942 transfers two peaks of pressor activity were clearly separated. The minor one, representing 10% of the total pressor activity in the sample, was not further purified. The major peak was recovered and distributed in the system butan-1-ol-ammonium acetate-aq. ammonia, pH 9.00. At 2653 transfers the active pressor material was spread in more than 300 tubes and its distribution did not follow the curve predicted by the theory applying to a pure substance (Fig. 4). The active material was recovered in two separate fractions. When they were analysed by paper chromatography in a butan-1-ol-acetic acid solvent, both gave an identical pattern of ninhydrin-positive spots. Of these, only one, with R_f 0.20, reacted with Sakaguchi reagent and contained all the pressor activity in the sample. The material eluted from this region in the chromatograms of both fractions gave values for specific activity (Table 2) and amino acid composition (Table 3) that were indistinguishable and that were comparable, within experimental error, with those obtained with synthetic valyl-5

Table 2. *Specific pressor activity of pepsitensin and synthetic valyl-5 angiotensin I*

The absolute amount (in μ moles) of peptide was estimated by amino acid analysis.

Expt. no.	Specific pressor activity (rat units/ μ mole)		
	Pepsitensin from fraction I	Pepsitensin from fraction II	Angiotensin I
1	70 000	64 000	66 000
2	69 000	61 000	60 000
3	—	63 000	70 000
4	—	70 000	—

Table 3. *Quantitative amino acid analysis of pure pepsitensin and synthetic valyl-5 angiotensin I*

Amino acid compositions are expressed as molar proportions. The value 1.00 was arbitrarily assigned to arginine.

Amino acid	Amino acid composition		
	Pepsitensin from fraction I	Pepsitensin from fraction II	Angiotensin I
Asp	1.03	1.00	0.99
Arg	(1.00)	(1.00)	(1.00)
Phe	0.98	1.02	0.97
His	1.74	1.81	1.85
Leu	1.10	1.03	0.95
Pro	1.10	1.06	1.00
Tyr	0.89	0.97	0.90
Val	1.79	1.76	1.71

angiotensin I. This substance is referred to as pepsitensin and the total amount obtained represents approx. 10% of the active material in the initial incubation mixture, purified 44 000-fold.

A possible explanation for the non-theoretical curve obtained in the last countercurrent distribution (Fig. 4) may be found in the high number of transfers applied and the small amount of active material present: the apparent partition coefficient of the substance sought may be affected by the distribution of the impurities carried along with it, as well as by small mechanical errors accumulated in a run of this length.

Paper electrophoresis. The electrophoretic mobilities of pepsitensin at several pH values were identical with those of valyl-5 angiotensin I under similar conditions (Fig. 5).

N-Terminal group. Aspartic acid was the only N-terminal amino acid revealed by the application of the method of Sanger & Thompson (1953) to pepsitensin.

Fig. 5. Paper electrophoresis, showing the movement of pepsitensin (O) and valyl-5 angiotensin I (Δ), relative to glucose, at various pH values. Electrophoresis was carried out for 3 hr. at 16 v/cm.

Fig. 6. Rate of release of amino acids from pepsitensin by the action of carboxypeptidase A, measured by ion-exchange chromatography: ●, leucine; ○, histidine; Δ, phenylalanine.

C-Terminal group. This information was obtained by incubating pepsitensin with carboxypeptidase A. The results (Fig. 6) indicate the sequence: Phe-His-Leu. No further liberation of other amino acids was obtained on prolonged incubation. In this experiment the pressor activity of the incubation mixture shows an initial abrupt decrease followed by a transitory rise. This characteristic variation was described by Lentz *et al.* (1956) for angiotensin I, and tentatively explained by assuming that the inactive nonapeptide remaining after liberation of C-terminal leucine is resistant to the action of the plasma converting enzyme, whereas the octapeptide arising when carboxypeptidase liberates histidine is angiotensin II. The activity falls to zero when phenylalanine is removed.

Chymotrypsin action. Since the molecule of angiotensin I has two bonds sensitive to chymotrypsin it gives rise to three different peptides on incubation with this enzyme (Elliott & Peart, 1957). Under similar conditions pepsitensin was also fragmented into three peptides with electrophoretic mobilities identical with those shown by

Fig. 7. Electrophoresis of chymotryptic digests of angiotensin I (A) and pepsitensin (P) at pH 3.20. Electrophoresis was carried out for 2 hr. at 16 v/cm. Spots were located by reaction with ninhydrin.

Table 4. *Peptides isolated by electrophoresis of a chymotryptic digest of pepsitensin (see Fig. 7)*

Amino acid compositions are expressed as molar proportions. The value 1.00 was arbitrarily assigned to valine for peptides 1 and 2 and to leucine for peptide 3.

Peptide	Amino acid composition	C-Terminal sequence	N-Terminal residue	Structure
1	Asp: 1.00 Arg: 0.80 Val: (1.00) Tyr: 0.79	Val-Tyr	—	Asp-Arg-Val-Tyr
2	Val: (1.00) His: 1.00 Pro: 1.10 Phe: 1.10	—	Val	Val-His-Pro-Phe
3	His: 1.05 Leu: (1.00)	—	—	His-Leu

the peptides originating from angiotensin (Fig. 7). This experiment could be easily scaled up, and the three peptides were recovered by elution of the electrophoretogram. Amino acid analysis, terminal group determination and degradative studies were performed on these materials (Table 4).

Since the C-terminal sequence of pepsitensin is Phe-His-Leu, peptide 3 clearly is: His-Leu, and

peptide 2 must have phenylalanine in the C-terminus. The amino acid next to phenylalanine in pepsitensin forms a peptide bond not split by carboxypeptidase A (Fig. 6). Peptide 2 contains this region of the molecule and, from its amino acid composition, proline must be responsible for this behaviour. Since valine is the N-terminal amino acid in this peptide, its sequence is established as: Val-His-Pro-Phe. The presence of aspartic acid in peptide 1 indicates its origin from the N-terminal region of pepsitensin; this fact, together with the C-terminal sequence found, establishes on a firm basis the formula Asp-Arg-Val-Tyr for this peptide.

When all the information obtained on peptides 1, 2 and 3 is put together the sequence of the ten amino acids present in pepsitensin must be:

This structure is identical with that established by Elliott & Peart (1957) for ox angiotensin I and accounts for all the facts described in the present work. One may conclude, then, that the principal component of the mixture of active pressor polypeptides formed by the action of pepsin on ox plasma is valyl-5 angiotensin I.

The minor active component found in the counter-current distribution at pH 7.50 was not identified, but its partition coefficients in the solvent systems 1 and 2 (Table 1) were identical with those of valyl-5 angiotensin II.

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