THE ACTION OF CRYSTALLINE PROTEOLYTIC ENZYMES ON ANGIOTONIN

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Angiotonin, a pressor substance resulting from the interaction of renin and its specific substrate, α_2-globulin, appears to participate in the mechanisms of renal hypertension. Knowledge of its structure would be desirable if the search for therapeutic agents which inactivate it is to proceed in an orderly manner and on sure grounds. Further, the search for chemical analytical methods to determine its concentration in blood and other body fluids will be haphazard as long as its structure remains unknown. Upon such methods may depend the delineation of the rôle angiotonin may play in arterial hypertension in man.

Since angiotonin is one of the products of the enzymatic splitting of blood proteins it would be safe to assume that it contains polypeptide linkages or perhaps it is itself a polypeptide without other nitrogen-containing prosthetic groups. If it contains polypeptide linkages these will be hydrolyzed under the influence of proteolytic enzymes with consequent loss of physiological activity. Intestinal amino peptidase (1), yeast amino peptidase (2), trypsin and pepsin (3–6) have all been reported to destroy angiotonin *in vitro*, but none of these enzymes has been used in chemically pure form. Therefore it is difficult to interpret the results in terms of original structure of the substrate.

The hydrolysis of a polypeptide by a proteolytic enzyme seems to be dependent on the structural detail of the substrate, a relationship generally but paradoxically regarded as the "specificity" of the enzyme. These enzymes will attack a substrate only if this substrate contains one or more peptide linkages resulting from the requisite and specific combination of amino acids.

Enzymes which attack a substrate centrally in its molecule are called "endopeptidases" and since they are capable of hydrolyzing not only peptides but also proteins they are sometimes referred to as proteinases. Their specificity lies in the nature of the amino acid residues on either side of the sensitive peptide linkage and does not call for a free functional group.

Another group of proteolytic enzymes attack the substrate peripherally and are called "exopeptidases." The best known representatives of this group are aminopeptidase and carboxypeptidase. The former, as its name implies, will only attack a peptide which contains a free amino group while the latter (carboxypeptidase) requires a free carboxyl group in the substrate molecule in order to be effective.

Bergmann and his associates have made a penetrating study of the exact structural detail of substrates for crystalline carboxypeptidase (7), trypsin (8, 9), chymotrypsin (10), and pepsin (11). We have adopted their methods and conclusions. With these four enzymes as *reagents*, their action on angiotonin was investigated in the hope that some information might be obtained concerning the structure and arrangement of the angiotonin molecule itself.

Methods and Results

Angiotonin.—Angiotonin was prepared as previously described (19). Alcohol or heat denaturization was used for the removal of proteins after incubation of renin-substrate with renin. A list of analytical findings is given in Table I from which it can be seen that heat denaturization of the mixture gave an angiotonin solution with

TABLE I

Analytical Constants of Several Angiotonin Samples

1	2	3	4	5	6	7
Sample No.	Pressor units per cc.	Mixture denatured by	Total N (Kjeldahl)	NH_2-N	N-COOH	Pressor units per mg. NH_2-N
			mg./cc	*mg./cc.*	*mg./cc.*	
P-50-S	10	Alcohol	0.220	0.187	0.061	80
P-62-69	22	Alcohol	0.261	—	0.065	—
P-74-1	25	Heat	0.507	0.155	0.047	230
P-75-1	23	Heat	0.778	0.173	0.057	198

Column 5 shows the amino nitrogen as determined by the method of Van Slyke and is therefore a measure of the total number of amino groups in the sample. Amino nitrogen attributable to free amino acids (column 6) has to be subtracted from the total amino nitrogen in order to arrive at the correct figure for "Pressor units per mg. NH_2-N"(column 7) since free amino acids may be assumed to be devoid of any pressor activity.

low amino nitrogen content per unit of physiological activity. Sample P-50-S was arbitrarily chosen as a standard containing 10 units per cc. A unit is defined as the quantity necessary to raise the arterial pressure of a pithed cat 30 to 50 mm. Hg; the pressor strengths of the other samples were expressed in units relative to the standard. Total nitrogen content was determined by the Kjeldahl method. Amino acid carboxyl was estimated by the ninhydrin method of Van Slyke, Dillon, MacFadyen, and Hamilton (12).

The standard angiotonin solution was used for most experiments with enzymes. The results were controlled with one of the other samples. No significant differences were found if enzyme controls were run for each incubation experiment. This was necessary since the stability of different angiotonin preparations varies slightly with the pH of the incubation mixture.

Crystalline Enzymes.—Carboxypeptidase was prepared according to Anson (13) and recrystallized two to four times. Pepsin, trypsin, and chymotrypsinogen and chymotrypsin were prepared according to the classical procedures of Northrop (14).

TABLE II

The Action of Crystalline Enzymes on Synthetic Substrate

Substrate	Enzyme	Protein N per cc. test solution	pH	K	C
		mg.			
Carbobenzoxy-*l*-glutamyl-*l*-tyrosine (15).........	Pepsin	1.54	4.2	7.7×10^{-4}	4.92×10^{-4}
Carbobenzoxy-*l*-glutamyl-*l*-tyrosine (15).........	Carboxypeptidase	0.000307	7.4	1.35×10^{-3}	4.40
Carbobenzoxy-glycyl-*l*-phenylalanine (10).....	Carboxypeptidase	0.00063	7.6	1.4×10^{-2}	22
Phenylalanineamide (10)..	Chymotrypsin	1.30	7.7	4.2×10^{-3}	3.2×10^{-3}
Benzoyl-*l*-arginine-amide (8, 9)................	Trypsin	0.25	7.7	8.56×10^{-3}	3.44×10^{-2}

The proper acidity was obtained by use of M/10 phosphate or citrate buffers. The course of hydrolysis was followed by amino nitrogen or amino acid determinations. Reported K values represent average of three determinations. Incubation temperature 37°C.

The number after the name of each compound designates the authors to whom reference is made.

TABLE III

Action of Crystalline Enzymes on Angiotonin

Enzyme preparation	Protein N per cc. test solution	pH	K	C_A
	mg.			
Carboxypeptidase..............	0.0030	7.4	0.0152	5.05
Carboxypeptidase..............	0.0060	7.4	0.0300	5.00
Chymotrypsin..................	0.0048	7.1	0.0186	3.87
Chymotrypsin..................	0.0097	7.1	0.0365	3.76
Chymotrypsinogen..............	0.3700	7.3	—	—
Pepsin........................	0.0246	5.5	0.0308	1.25
Pepsin........................	0.0246	3.3	0.00045	0.018
Trypsin.......................	0.00018	7.2	0.0158	86.0

Incubation temperature was 37°C. M/10 phosphate or citrate buffers were used to ensure the correct acidity. For explanation of K and C_A see text.

All enzyme preparations were tested for their activity using synthetic substrates and their activity (proteolytic coefficient) compared to the values reported in the literature (7–11). The results are given in Table II.

The synthetic substrates used for this purpose were carbobenzoxy-glycyl-*l*-phenylalanine, carbobenzoxy-*l*-glutamyl-*l*-phenylalanine, *l*-phenylalanineamide, and benzoyl-*l*-arginineamide.

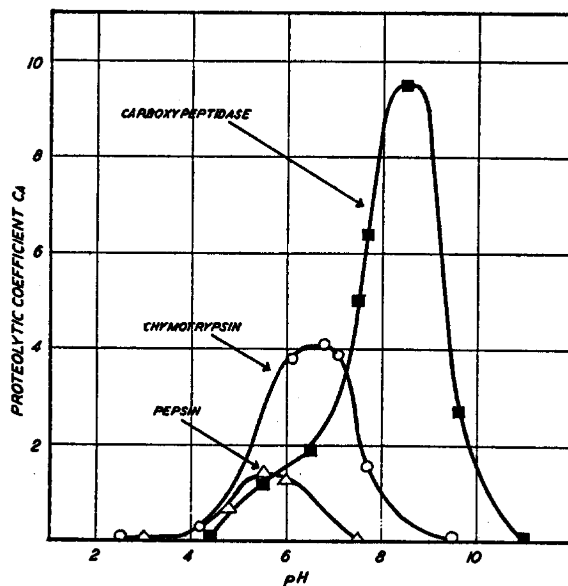


FIG. 1. Proteolytic coefficients C_A as a function of pH obtained by allowing the crystalline enzymes carboxypeptidase, chymotrypsin, and pepsin to act on angiotonin. Incubation temperature in all experiments was 37°C. The hydrogen ion concentration was maintained at the values indicated by the use of citrate or phosphate buffers.

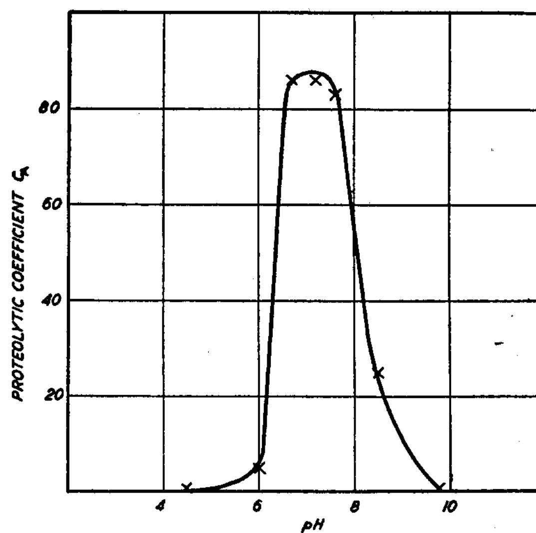


FIG. 2. Proteolytic coefficient C_A as a function of pH using crystalline trypsin as reagent. Experimental conditions are the same as described in the legend to Fig. 1.

The course of the hydrolysis of synthetic substrates was followed either by Van Slyke's amino nitrogen determinations or the ninhydrin method of Van Slyke, Dillon, MacFadyen, and Hamilton (12) whichever method seemed more suitable. For each experiment the first order reaction constant was determined $K = 1/t \log a/a - x$ and the average K value used for the determination of the proteolytic coefficient (C value). These coefficients were calculated by dividing K by the milligrams protein nitrogen per cubic centimeter of the test solution (17).

Numerous attempts were made to follow the course of angiotonin hydrolysis by measuring the increase in amino nitrogen or amino acid nitrogen (ninhydrin method) but the difference even under optimal conditions was so small that no quantitative interpretation of such data could be given.[1] Hence the course of the hydrolysis was followed by bioassay of angiotonin using a pithed cat as test animal. First order constants were determined as described for the assay of angiotonase (16). Dividing this constant by the concentration of the enzyme expressed in milligrams proteins nitrogen per cubic centimeter test solution, gave the proteolytic coefficient C_A.

The results of the experiments involving angiotonin as substrate are given in graphic form in Figs. 1 and 2. The proteolytic coefficient C_A is plotted against the pH of the incubation mixture.

DISCUSSION

As mentioned in the introduction, the literature contains several references to the inactivation of angiotonin by proteolytic enzymes. Muñoz, Braun-Menendez, Fasciolo, and Leloir (3) showed that angiotonin (hypertensin) is destroyed by commercial pepsin and trypsin and this was confirmed by Edman, von Euler, Jorpes, and Sjöstrand (6). Neither of these two groups of investigators gave detailed information concerning the purity of the enzyme preparation used nor are any other experimental data given. Such findings are of limited value since commercial preparations of trypsin and pepsin usually contain other pancreatic enzymes as *e.g.* carboxypeptidase and chymotrypsin. It would therefore be impossible to ascertain to which of these enzymes the destruction of angiotonin could be attributed.

For this reason, we employed enzymes recrystallized three times. It was found (Table III) that crystalline chymotrypsin, carboxypeptidase, trypsin, and pepsin all hydrolyze angiotonin at a very appreciable rate.

The rate at which proteolytic enzymes act upon a substrate of known structure is dependent upon the nature of the substrate and the enzyme (17). The absolute value of the proteolytic coefficient is therefore a characteristic of both but of itself permits no further interpretation. However, some indication of the nature of the various peptide linkages can be obtained by comparing the C values for the four enzymes involved. They are of approximately

[1] In one instance, the increase in amino nitrogen of a triple strength angiotonin sample after consecutive chymotrypsin, trypsin, and carboxypeptidase digestion amounted to 0.006 mg. NH_2-N per cc.

the expected magnitude for typical substrates; the proteolytic coefficients for typical carboxypeptidase substrates are high compared to those for chymotrypsin and these, in turn are very much greater than the *C* values for the best pepsin substrates. Trypsin represents an exception and is discussed in detail below.

A slight deviation from the usual pH optima for all four enzymes is not unusual, since this too seems to be a characteristic of both substrate and enzymes. For instance, Fruton and Bergmann (11) found the pH maximum for hydrolysis of carbobenzoxy-*l*-glutamyl-*l*-tyrosine by crystalline pepsin to be about 4.0 while the closely related carbobenzoxy-*l*-glutamyl-*l*-phenylalanine is optimally hydrolyzed at pH 4.5. The highest *C* value using angiotonin was obtained at pH 5.5. Usually chymotrypsin is most effective at pH 7.7 but for the hydrolysis of angiotonin pH 6.5–7.0 was found to give the highest proteolytic coefficient. Similarly, carboxypeptidase usually has a pH maximum at pH 7.6–7.8 while angiotonin is optimally hydrolyzed by it at pH 8.5.

Proper interpretation of these facts might give some information concerning the minimum number and nature of amino acids necessary to fulfill the condition of angiotonin hydrolysis. Such a study cannot be taken as offering proof of the structure of the substrate for the latter is not as yet available in crystalline condition. However the results can be of great help in the subsequent isolation and identification of degradation products of it. If a pure starting material becomes available, crystalline enzymes can be used as reagents in order to accomplish splitting and degradation at any desired position in the molecule.

Croxatto and Croxatto (2) reported that angiotonin is rapidly destroyed by yeast amino peptidase (method of Johnson (18)) and hence concluded that angiotonin contains a free amino group. This is in agreement with the observation of Fasciolo, Leloir, Muñoz, and Braun-Menendez (1) who found that intestinal mucosa is the richest source of "hypertensinase," *i.e.*, angiotonase. It would seem likely that this "hypertensinase" is in reality intestinal amino peptidase. Although the enzyme employed by Croxatto and Croxatto was not crystalline, it was, nevertheless, free from endopeptidase and their results may therefore be taken as indicative, if not conclusive evidence that angiotonin contains a free terminal amino group.

An important conclusion from our experimental data can be used to support the hypothesis of a free amino group. Fruton and Bergmann (10) showed that chymotrypsin exhibits the phenomenon of multiple specificity, one specificity being that of a carbonyl proteinase (endopeptidase) and the other is that of an amino peptidase (exopeptidase) both requiring an aromatic amino acid residue in the peptide linkage involved. Neglecting for the moment the former specificity, chymotrypsin, like amino peptidase (intestinal or yeast), should destroy angiotonin quite rapidly. In view of the fact that both specifici-

ties of chymotrypsin are unique in that they require aromatic amino acid residues in the substrate in order to be effective, the terminal amino group in angiotonin must be part of a tyrosine or phenylalanine molecule. However, this process may be superimposed upon endopeptidase activity. The breaking of one peptide linkage in the angiotonin molecule apparently is sufficient to cause complete inactivation, hence if the two specificities of chymotrypsin are superimposed we have only measured the faster of the two reactions, *i.e.*, the rate determining step. The rate of the second reaction may be so low by comparison that to demonstrate its presence or course would be impossible with the analytical method at our disposal.

Fruton and Bergmann consider the possibility that there might be a third specificity of chymotrypsin in view of the fact that chymotrypsin hydrolyzes glycyl-glycineamide at an appreciable rate. They expressed doubt whether this splitting can be attributed to the same action as the splitting of glycyl-*l*-tyrosineamide or glycyl-*l*-phenylalanineamide. It is therefore conceivable that the proteolytic action of chymotrypsin on angiotonin might involve a glycine residue.

As will be seen later, the simplest combination of amino acids satisfying the results of proteolysis would require five amino acid residues for endopeptidase splitting by chymotrypsin, while exopeptidase splitting by the same enzyme calls for only four amino acids. If both specificities are effective, five amino acids are necessary.

The only other exopeptidase available in crystalline condition is carboxypeptidase. Since it inactivates angiotonin (Fig. 1) angiotonin must contain the necessary structural detail, *i.e.*, a free carboxyl group flanked by a peptide bond (7). The amino acid radical need not be of any special nature although there is a wide variation in the magnitude of the proteolytic coefficients of various synthetic substrates satisfying these requirements. Carbobenzoxy-glycyl-*l*-phenylalanine, recommended as the most satisfactory synthetic substrate, when hydrolyzed by carboxypeptidase gives a maximum proteolytic coefficient of 22 at pH 7.6 and 37° compared to 9.0 for angiotonin at pH 8.5.

Since the hydrolysis of synthetic substrates by carboxypeptidase can be inhibited by some of their split products (7), *e.g.* inhibition by chloracetate ion or formaldehyde, the action of this enzyme on angiotonin was investigated from a kinetic point of view. First order kinetics were obtained (Table III) which tend to show that the substrate has nothing unusual in its structure.

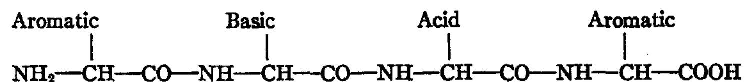
The fact that angiotonin is inactivated by trypsin at a rate quite appreciably greater than that of benzoylarginineamide (9) (Fig. 2) indicates that angiotonin contains at least one basic amino or guanidino group in central position. Its structural arrangement must be unusually favorable for tryptic hydrolysis since the proteolytic coefficient is the highest yet obtained for any substrate.

The previous report by Page and Helmer (19) that angiotonin gives a positive Sakaguchi reaction may be suggestive of a guanidino group (*i.e.*, arginine) but is not proof since a positive reaction can be elicited by heat denaturization of renin solutions without renin substrate. Furthermore, the reaction does not parallel presssor activity. Since all angiotonin preparations up to the present contain a small amount of free amino acids as determined by the ninhydrin method (Table I) it is possible that arginine may constitute part of them.

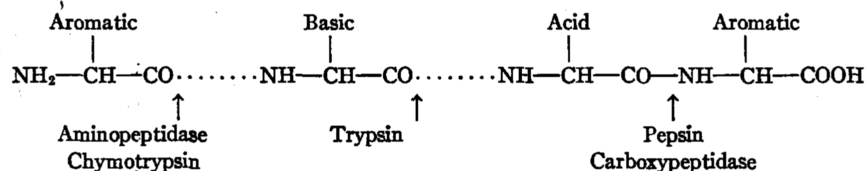
Amino nitrogen determinations on angiotonin samples acidified with hydrochloric acid to pH 4.0 were found to be non-reproducible (Table I, sample P-62–69). The values were as much as four or five times higher than when the same samples were acidified with glacial acetic acid. Such behavior is characteristic of arginine. Since the values were many times greater than the total (free) amino acid nitrogen the evidence is strongly in favor of arginine as part of the angiotonin molecule rather than as free amino acid.

The specificity of pepsin has been investigated by Fruton and Bergmann (11) who found that the sensitivity of a peptide bond toward pepsin depends not on one but on both amino acid residues involved. One of these must contain a free carboxyl while the other must be aromatic such as tyrosine or phenylalanine. Since the hydrolysis of angiotonin by pepsin proceeds at a very much greater rate than carbobenzoxy-*l*-glutamyl-*l*-tyrosine it would be safe to assume that angiotonin contains at least one such arrangement. Indeed, its hydrolysis is sufficiently fast to allow the assumption that the aromatic amino acid residue is terminal in the angiotonin molecule, a hypothesis not in conflict with the conditions outlined for the hydrolysis by carboxypeptidase. As can be seen from Table II, carbobenzoxy-*l*-glutamyl-*l*-tyrosine is hydrolyzed by carboxypeptidase at a rate almost comparable to the rate of hydrolysis of angiotonin.

Summarizing the evidence we venture to speculate as to the possible structure of angiotonin. The compound having the necessary structural details must contain at least three peptide linkages and contain (1) a free terminal amino group, (2) a free terminal carboxyl group, (3) one basic amino acid residue which may be terminal but its carboxyl must be involved in a peptide linkage, (4) one central dibasic amino residue in combination with an aromatic amino acid residue, (5) an aromatic amino acid residue which may be part of (4) and, if not part of (4) must be terminal with its carboxyl group in peptide linkage. The simplest possible compound which answers this description would have the following structure:—



and angiotonin must contain the same arrangement except that any number of inert amino acid residues or other types of organic bases might have to be inserted where the peptide bond is indicated by a dotted line



SUMMARY

Angiotonin was subjected to enzymatic digestion by crystalline carboxypeptidase, chymotrypsin, trypsin, and pepsin. These enzymes were found to destroy it *in vitro*. Hydrogen ion optima and proteolytic coefficients for these reactions were determined and were found to be of approximately the expected magnitude for typical substrates.

Regarding the purified crystalline enzymes as reagents, the experimental findings were interpreted on the basis of Bergmann's specificity studies. We were thus directed to the conclusion that angiotonin contains (1) a free terminal amino group, (2) a free terminal carboxyl group, (3) one basic amino acid residue which may be terminal but its carboxyl must be united in a peptide linkage, (4) a central dibasic amino acid residue in combination with an aromatic amino acid residue, (5) an aromatic amino acid residue which may be part of (4) and, if not part of (4) must be terminal with its carboxyl group in peptide linkage. The simplest compound satisfying these conditions is tyrosyl-arginyl-glutamyl-phenylalanine or a combination of amino acids with similar general characteristics.

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BIBLIOGRAPHY

1. Fasciolo, J. C., Leloir, L. F., Muñoz, J. M., and Braun-Menendez, S., *Rev. Soc. argent. biol.*, 1940, **16,** 643.
2. Croxatto, H., and Croxatto, R., *Science*, 1942, **96,** 519.
3. Muñoz, J. M., Braun-Menendez, E., Fasciolo, J. C., and Leloir, L. F., *Am. J. Med. Sc.*, 1940, **200,** 608.
4. Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., and Muñoz, J. M., *J. Physiol.*, 1940, **98,** 283.
5. Alouso, O., Croxatto, R., and Croxatto, H., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52,** 61.
6. Edman, P., von Euler, U. S., Jorpes, E., and Sjöstrand, O. T., *J. Physiol.*, 1942, **101,** 284.
7. Hoffmann, K., and Bergmann, M., *J. Biol. Chem.*, 1940, **134,** 225.

8. Bergmann, M., Fruton, J. S., and Pollok, H., *J. Biol. Chem.*, 1939, **127**, 643.
9. Hoffmann, K., and Bergmann, M., *J. Biol. Chem.*, 1939, **130**, 81.
10. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, 1942, **145**, 253.
11. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, 1939, **127**, 627.
12. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, 1941, **141**, 627.
13. Anson, M. L., *J. Gen. Physiol.*, 1937, **20**, 663.
14. Northrop, J. H., Crystalline enzymes, New York, Columbia University Press, 1940.
15. Bergmann, M., and Zervas, L., *Z. physiol. Chem.*, 1934, **224**, 1.
16. Plentl, A. A., and Page, I. H., *J. Exp. Med.*, 1943, **78**, 367.
17. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, 1942, **145**, 247.
18. Johnson, M. L., *J. Biol. Chem.*, 1941, **137**, 575.
19. Page, I. H., and Helmer, O. M., *J. Exp. Med.*, 1940, **71**, 29.