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SERUM AMINOPEPTIDASES, "ANGIOTENSINASE," AND HYPERTENSION—I.

DEGRADATION OF ANGIOTENSIN II BY HUMAN SERUM*

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Abstract—The enzymic hydrolysis of the naturally occurring octapeptide angiotensin II by human serum has characteristics distinct from those of enzymes hydrolyzing the N-terminal amino acid from the synthetic analogue, angiotensin II amide. Enzymic release of the N-terminal aspartic acid from angiotensin II appears to be catalyzed by an enzyme or enzymes similar to aminopeptidase A, a mammalian peptidase having a specificity for N-terminal L-dicarboxylic amino acids.

RECENT reports have shown that the naturally occurring vasopressor octapeptide angiotensin II, Asp^1-Arg^2-Val^3-Tyr^4-Val^5-His^6-Pro^7-Phe^8, is inactivated in a variety of mammalian tissues and body fluids by enzymic degradation involving predominantly sequential hydrolysis of N-terminal amino acids, i.e., by aminopeptidase activity.[1–4] The heptapeptide which is formed upon removal of the terminal aspartic acid residue from angiotensin II displays one half the pressor effect of angiotensin II, while the hexapeptide resulting from arginine release exhibits essentially none of the pressor effect of the parent octapeptide.[3, 5] It is likely that the enzymic mechanism responsible for the *in vivo* release of aspartic acid may be rate limiting in the further degradation and loss of activity of this vasopressor polypeptide and, hence, may be of physiologic importance.

The finding in human tissues of an enzyme, aminopeptidase A, which has a specificity for N-terminal L-dicarboxylic amino acid residues[6] has prompted us to investigate the possibility that a similar or identical enzyme exists in human serum.

As a corollary to this study and in view of the widespread use of angiotensin II amide (α-L-asparaginyl^1-angiotensin II, Hypertensin-CIBA) in clinical studies of angiotensin II degradation,[1, 2, 7] it was deemed necessary to investigate the mechanism of angiotensin II amide degradation by human serum.

MATERIALS AND METHODS

Serum obtained from normal human volunteers was dialyzed against distilled water for 14 hr, lyophilized, and reconstituted to a third of its initial volume with distilled

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water. This procedure was employed in order to eliminate the high amino acid blank values of undialyzed serum and to reduce incubation time by increasing enzyme concentration.

The substrates, α-L-aspartyl[1]-valyl[5]-angiotensin II, β-L-aspartyl[1]-valyl[5]-angiotensin II, and α-L-asparaginyl[1]-valyl[5]-angiotensin II (angiotensin II amide), were obtained through the generosity of Dr. R. Schwyzer, Research Laboratories, CIBA Ltd., Basle, Switzerland. The dipeptide fragment α-L-aspartyl-L-arginine was a gift from Dr. D. F. Elliott, Research Division, CIBA Ltd., Horsham, England. β-L-Aspartyl-L-arginine was prepared without further isolation from a trypsin digest of β-aspartyl-angiotensin II.

Incubation conditions employed for octapepetide degradation studies were as follows: 0·04 ml concentrated serum, 0·2 μmole substrate made up to a total of 0·125 ml with 0·1 M Tris–maleate buffer (pH 7·0) at 37°. Calcium chloride dihydrate, 1·5 μmoles, was incorporated in all incubating media except those used for activation and inhibition experiments. All incubations were performed for 4 hr with the exception of those for time-course studies. The reaction was stopped by the addition of 0·02 ml of 50% trichloroacetic acid, and the inactivated incubating mixture was centrifuged at 2,000 rpm for 20 min. Aliquots of the supernatant fluid (15 μliters) and serum control were applied to Whatman 1 paper. One-dimensional ascending chromatography was performed with the solvent system acetonitrile:urea:water:triethylamine (60 ml: 1 g: 40 ml: 2 ml), equilibrated for 14 hr prior to use. The chromatograms were developed with 0·2% ninhydrin in ethanol:glacial acetic acid (100 ml:1 ml). The amino acid degradation products resulting from incubation of the substrates were identified by a comparison of their R_f values in the presence of trichloroacetic acid-inactivated serum with those of the initial substrates, their amino acid constituents, and peptide fragments (Table 1 and Fig. 1). A modified Sakaguchi reaction was also used to identify the initial substrates, arginine, and arginyl peptide fragments.

TABLE 1. R_F VALUES OF ANGIOTENSIN II, ITS AMIDE AND AMINO ACID CONSTITUENTS

Asp. A	Arg	Val	Tyr	His	Pro	Phe	Aspara	Angio II	Angio II Amide
0·22	0·16	0·52	0·52	0·36	*	0·59	0·33	0·50	0·50

Solvent phase: acetonitrile: urea: water: triethylamine (60 ml: 1 g: 40 ml: 2 ml); chromatographed on Whatman 1 paper.

* No reactive spot for proline was given by ninhydrin with this system.

Aspartic acid, arginine, and asparagine were determined quantitatively by a modified Moore and Stein method.[8] Absorbancies of the eluted amino acids were obtained in a Beckman DU spectrophotometer at 580, 405, and 340 mμ for aspartic acid, arginine, and asparagine respectively. Chromatographic recovery of these amino acids after incubation with concentrated serum and in the presence of calcium and nickel salts was determined. The effect of calcium on asparagine[1] release from angiotensin II amide was also evaluated by means of an automatic amino acid analyzer.[9]

The hydrolysis of the α-L-aspartyl, α-L-glutamyl, L-leucyl, and benzoyl-DL-arginine

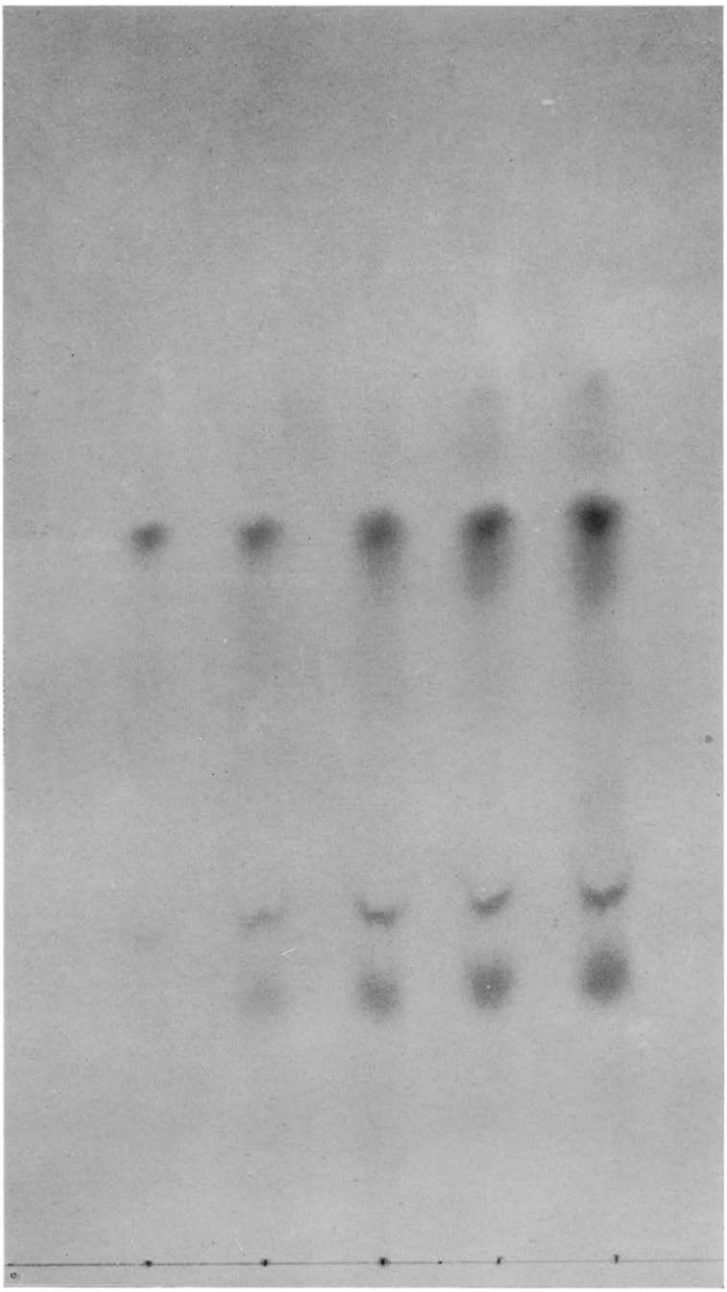

FIG. 1. Chromatograph of hydrolysis products of angiotensin II after serum incubation at (from left to right) zero time, 1·5, 3·5, 5·5, and 7 hr. Low spot, arginine; middle, aspartic acid; top, angiotensin II and other fragments.

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β-naphthylamides was measured in the presence and absence of 0·01 M calcium chloride by means of a colorimetric method with a Beckman DU spectrophotometer[6] and a fluorometric method[10] with an Aminco-Bowman spectrophotometer. The deviation in values obtained by the two methods was ±0·4%. The α-L-aspartyl and α-L-glutamyl β-naphthylamides were synthesized in our laboratory. The L-leucine and benzoyl-DL-arginine β-naphthylamide derivatives were obtained from Mann Research Laboratories, New York, N.Y.

RESULTS

Time course of hydrolysis

The enzymic release of aspartic acids from angiotensin II and asparagine[1] from angiotensin II amide was linear and followed zero-order kinetics for a period of 5 hr (Figs. 2 and 3). Although the rate of release of arginine[2] from angiotensin II amide was

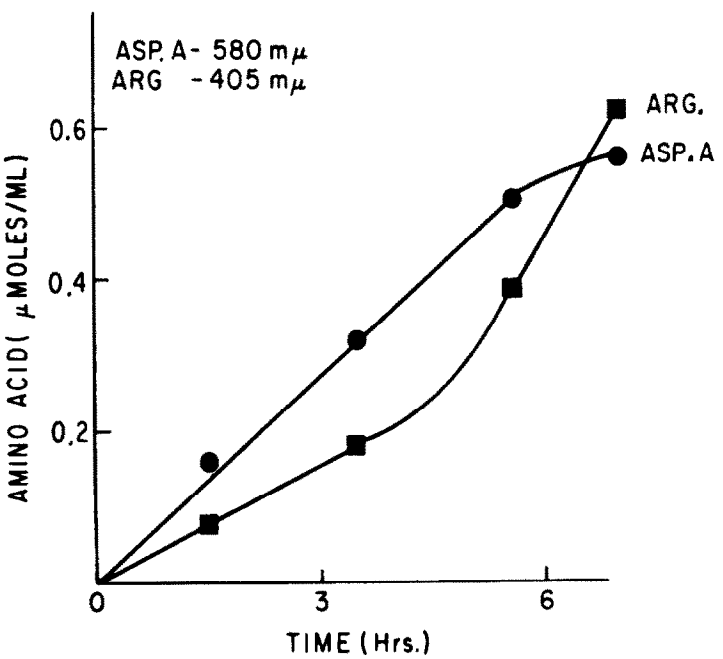

FIG. 2. Time course of hydrolysis of aspartic acid[1] and arginine[2] from angiotensin II.

also constant for the same time period, the cleavage of arginine[2] from angiotensin II was supressed, attaining the level of aspartic acid only after 6 hr. The finding of a larger quantity of arginine than aspartic acid after the 6-hr incubation may be considered within the limits of the error of the technique at a time of nonlinear hydrolysis. The results indicate a rate dependence of arginine[2] release on that of aspartic acid[1] in the presence of human serum with angiotensin II. There is no evidence of such a dependence of arginine[2] release on asparagine[1] release with angiotensin II amide.

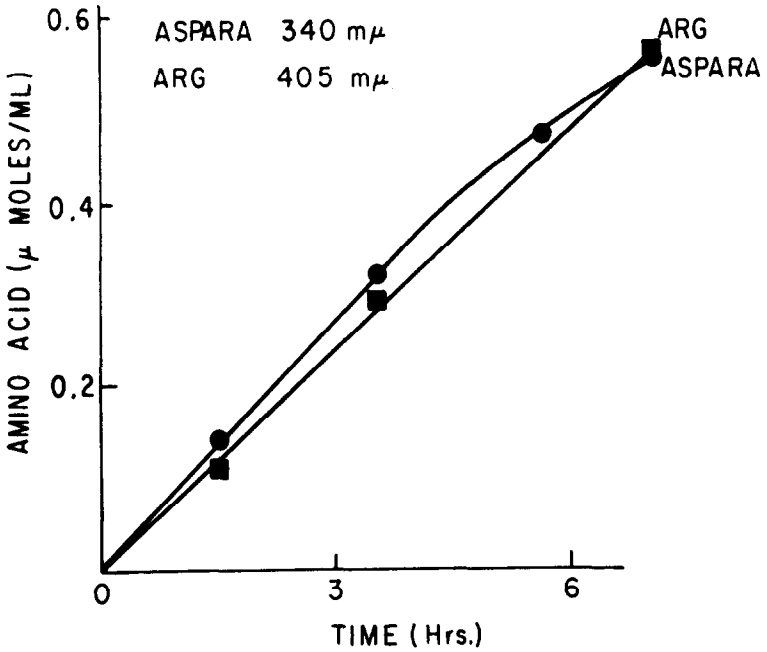

FIG. 3. Time course of release of asparagine[1] and arginine[2] from angiotensin II amide.

Enzyme concentration and substrate concentration

The rate of cleavage of aspartic acid[1] from angiotensin II and asparagine[1] from angiotensin II amide was proportional to the concentration of the dialyzed and lyophilized human serum. The rate of cleavage of these amino acids at a constant serum concentration was independent of substrate concentration for both angiotensin II and angiotensin II amide from 1·6 mμM to 3·2 mμM up to 4-hr incubation.

Affector studies

The effect of calcium and nickel salts and ethylenediamine tetraacetate (EDTA) on the enzymic release of aspartic acid[1] from angiotensin II and asparagine[1] from angiotensin II amide are summarized in Table 2. EDTA was preincubated with serum

TABLE 2. AFFECTOR CHARACTERISTICS OF N-TERMINAL AMINO ACID RELEASE FROM ANGIOTENSIN II AND ANGIOTENSIN II AMIDE BY HUMAN SERUM CONCENTRATE

Affector	Asp. acid from angio. II (%)	Aspara. from angio. II amide (%)
None	100 (1·17)	100 (1·61)
Calcium (12·0 mM)	161 (1·88)	83 (1·34)
Nickel (2·4 mM)	0 (0·00)	53 (0·85)
EDTA (12.0 mM)	13 (0·15)	0 (0·00)

Values in per cent of activity of untreated serum. Values in parentheses, μmoles of amino acid released/min/liter of serum at 37°. Incubation time 4 hr. Calcium and nickel salts were added to the serum with the substrate. EDTA was preincubated with serum for 1 hr prior to substrate addition.

for 1 hr at 25° prior to addition of substrate. The metal salts were incorporated directly into the substrate-incubating solution. There is a striking distinction in the effect of cations on aspartic acid[1] release from angiotensin II and asparagine[1] release from angiotensin II amide. No significant difference in the inhibition by EDTA of the hydrolysis of the N-terminal amino acids from both octapeptides is apparent, however. From these data it would appear evident that the hydrolysis of aspartic acids from angiotensin II is calcium activated, but strongly inhibited by nickel and EDTA. On the other hand, the release of asparagine[1] from angiotensin II amide is slightly inhibited by calcium, markedly inhibited by nickel, and completely inhibited by EDTA.

Angiotensins as competitive inhibitors

The inhibitory properties of angiotensin II were investigated by the use of the aminopeptidase A substrates, α-L-aspartyl β-naphthylamide, and α-L-glutamyl β-naphthylamide. Inhibition of a competitive nature was observed (Figs. 4, 5). How-

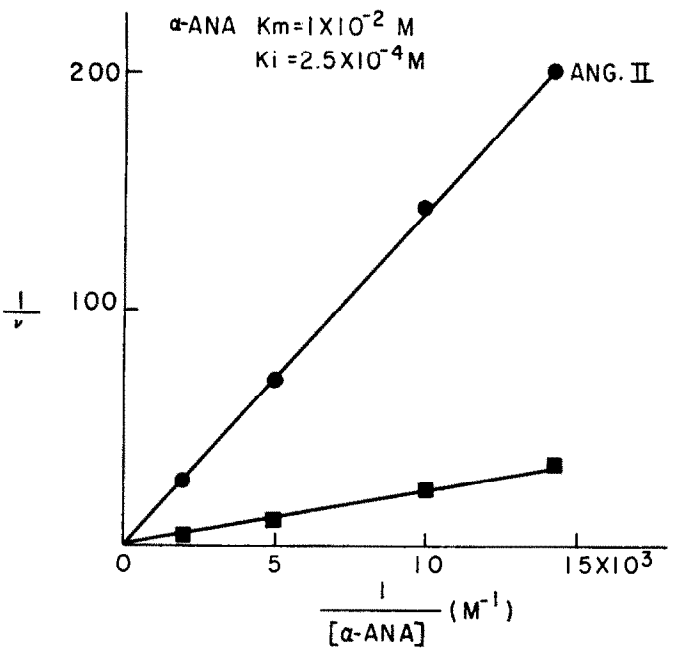

FIG. 4. Inhibition of hydrolysis of α-L-aspartyl β-naphthylamide by angiotensin II. Concentration of angiotensin II, 1 μM.

ever, different K_I values for angiotensin II were obtained with the two naphthylamide substrates. No inhibition by angiotensin II was detected when L-leucyl β-naphthylamide was used as substrate. When angiotensin II amide was tested as an inhibitor, competitive inhibition of the hydrolysis of L-leucyl β-naphthylamide occurred (Fig. 6), but no inhibition of the hydrolysis of either α-L-aspartyl or α-L-glutamyl β-naphthylamide was observed.

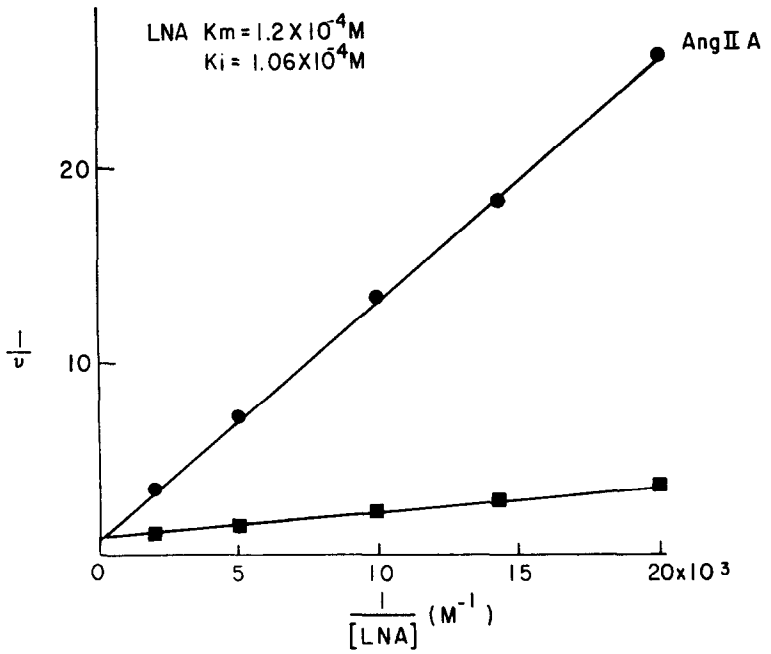

FIG. 5. Inhibition of hydrolysis of α-L-glutamyl β-naphthylamide by angiotensin II. Concentration of angiotensin II, 1 μM.

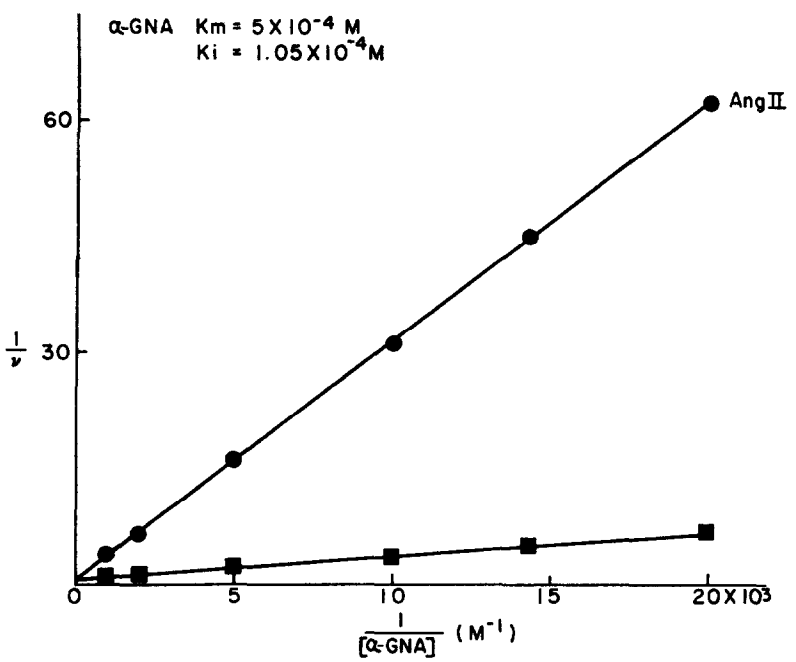

FIG. 6. Inhibition of hydrolysis of L-leucyl β-naphthylamide by angiotensin II amide. Concentration of angiotensin II amide, 1 μM.

Assays for tryptic activity

The smallest peptide fragment containing aspartic acid that might result from serum endopeptidase activity on angiotensin II is α-L-aspartyl-L-arginine. This fragment would most likely occur as the result of the presence of a serum tryptic activity. The R_f of both α-L-aspartyl-L-arginine and β-L-aspartyl-L-arginine was found to be 0·18. No Sakaguchi reaction was noted at this point on chromatography of a 4-hr serum digest of angiotensin II or of β-aspartyl-angiotensin II. Benzoyl-DL-arginine β-naphthylamide, a substrate for trypsin and trypsin-like enzymes,[11, 12] was not hydrolyzed by the serum concentrate during a 4-hr incubation.

DISCUSSION

The enzymes that mediate the breakdown of angiotensin II in tissues and body fluids have been collectively referred to as "angiotensinases." A terminology of this type signifies a limited substrate specificicity for the enzymic activities involved in angiotensin II degradation. From the evidence at present available[1–4] it would appear that angiotensin II is degraded by a variety of aminopeptidases with varying specificities. There is also evidence that a chymotrypsin-like enzymic activity[3] is implicated to a lesser degree in angiotensin II breakdown. None of these activities has been shown to be specific for angiotensin II. In our investigation of angiotensin II degradation by human serum we also considered the existence of a trypsin-like enzyme[13] that might be capable of releasing the dipeptide fragment, α-L-aspartyl-L-arginine, from this octapeptide. Such a dipeptide might undergo further hydrolysis by the action of aminopeptidases or of a basic carboxypeptidase reported to be present in human serum.[14] We were, however, unable to detect such a dipeptide in serum incubated with angiotensin II. We were thus able to confirm the apparent absence in normal human serum of a tryptic activity having the capacity of releasing this dipeptide fragment.[15]

In human serum the release of the N-terminal amino acid, α-L-aspartic acid, from angiotensin II appears to be mediated by an enzymic activity different from that effecting the release of α-L-asparagine from angiotensin II amide. This conclusion is derived from the following data: (a) arginine[2] hydrolysis is rate dependent on aspartyl[1] hydrolysis from angiotensin II, but not on asparaginyl[1] hydrolysis from angiotensin II amide; (b) aspartyl[1] hydrolysis is calcium activated and completely nickel inhibited, while asparaginyl[1] hydrolysis is not activated by calcium and is only partially inhibited by nickel; (c) angiotensin II is a competitive inhibitor of substrates upon which angiotensin II amide has no effect, and vice versa. These results are consonant with those of Regoli *et al.*,[3] who, using various tissue and serum sources, also concluded on the basis of differences in rates of inactivation of angiotensin II and angiotensin II amide, that different enzymes (predominantly aminopeptidases) were involved in the hydrolysis of the two octapeptides. The results with angiotensin II amide also are consistent with the findings of Klaus *et al.*,[1] who noted that, when this compound was used as asubstrate in unlyophilized and either dialyzed or undialyzed human serum, inactivation was not accelerated by calcium, but was completely inhibited by EDTA. Khairallah *et al.*,[16] however, found that with heparinized human plasma, though EDTA completely inhibited its breakdown, calcium activated the hydrolysis of angiotensin II amide. The difference in results of the latter authors may be due to the use of plasma rather than serum as their enzyme source.

The characteristics of the aspartyl[1] hydrolysis from angiotensin II are consistent

with those of several aminopeptidase A preparations that have been partially purified from mammalian sources,[6] i.e. calcium activation, EDTA inhibition, and competitive inhibition of the hydrolysis of α-L-aspartyl and α-L-glutamyl β-naphthylamide. Aminopeptidase A catalyzes the hydrolysis of N-terminal dicarboxylic L-amino acid residues and in the partially purified form acts on α-L-aspartyl and α-L-glutamyl peptides, but has no activity for peptides having such N-terminal residues as asparagine, glutamine, or leucine.[6]

The differences in enzymic characteristics of angiotensin II degradation and those of angiotensin II amide reported here would suggest that clinical investigations of angiotensin II inactivation should employ only the naturally occurring pressor octapeptide.

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