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ISOLATION OF ANGIOTENSIN-CONVERTING ENZYME WITHOUT KININASE ACTIVITY FROM HOG AND GUINEA PIG PLASMA

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SUMMARY

A new angiotensin-converting enzyme activity was found in the blood plasmas of the hog and the guinea pig. This activity was separated from the previously described angiotensin-converting enzyme/kininase by gel filtration on Sephadex G-150. The new enzyme does not possess kininase activity, and does not hydrolyze acetyl-*p*-nitrophenylalanylglycylglycine. It does not need Cl^-, is activated by Co^{2+}, Ca^{2+} and Zn^{2+} and is inhibited by the dipeptides His–Leu and, to a smaller extent, Phe–Arg. The enzyme described here also differs from angiotensin-converting enzyme/kininase in that it is inhibited by 2-mercaptoethanol. Its properties were not altered by 4 M urea, indicating that it is not an associated form of the previously described plasma angiotensin-converting enzyme.

INTRODUCTION

The presence of a halide-activated peptidase that removes the C-terminal dipeptide His–Leu from angiotensin I, yielding angiotensin II, was first demonstrated in horse plasma by Skeggs et al. [1]. This "angiotensin-converting enzyme" was shown to be an α-carboxypeptide dipeptide hydrolase capable of hydrolyzing a variety of peptide dipeptide bonds [2, 3], being activated by divalent cations [3, 4]. The angiotensin-converting activity could not be separated from "kininase II", responsible for the removal of Phe–Arg from the C-terminal end of bradykinin [2]. In this paper we report the separation, from hog and guinea pig plasma, of an angiotensin converting enzyme without kininase activity.

EXPERIMENTAL PROCEDURE

Peptides

The peptides employed in this study were synthesized by the solid phase method [5–7] and purified by counter-current distribution and ion-exchange chromatography until the following criteria for purity were met: (a) the amino acid analysis of acid hydrolyzates yielded a molar ratio within 3% of the theoretical value for each amino acid; (b) only one spot was detected with Pauly, ninhydrin and Sakaguchi

reagents after thin-layer chromatography of a 0.1-μmole sample with three solvent systems and high voltage paper electrophoresis with three different buffers (pH 2.8, 4.9 and 9.9).

Enzyme preparation

Citrate-treated plasma from hog or from guinea pig was submitted to fractionation with $(NH_4)_2SO_4$. The fraction precipitated between 1.4 and 2.8 M was passed through a 105 cm $\times$ 3.2 cm Sephadex G-150 column and eluted with 0.2 M NaCl at a flow rate of 25 ml/h. The eluent was collected in 5-ml fractions which were assayed for converting enzyme activity and for protein concentration (absorbance at 280 nm). The fractions corresponding to each peak were pooled and freeze-dried. The protein content of the different preparations was determined by the method of Lowry et al. [8].

Enzyme assays

All enzyme assays were done in conditions where first-order kinetics applied, and initial rates of reaction were determined.

Angiotensin-converting enzyme activity. For the localization of this activity in eluates of chromatography, 0.1 or 0.2 ml of eluate were added to a $1.25 \cdot 10^{-6}$ M angiotensin I solution of 0.1 M sodium phosphate buffer (pH 8.0) containing 0.2 M NaCl and 10^{-4} M $CoSO_4$, in a final volume of 1 ml. After 5, 10, 15, 20 and 30 min at room temperature aliquots were drawn, in which the reaction was stopped by boiling. After appropriate dilution, the angiotensin II formed was assayed in the isolated rat uterus, by a method that allows estimation of angiotensin II activity in the presence of angiotensin I [9]. The specific activity of the different preparations was determined in the same conditions as described above, but with higher angiotensin I concentration ($7.7 \cdot 10^{-5}$ M). To obtain a more quantitative measurement of the angiotensin II formed, this peptide was separated from the remaining angiotensin I by submitting the incubation mixture to chromatography on a 11 cm $\times$ 0.9 cm carboxymethylcellulose column eluted with 0.1 M ammonium acetate (pH 4.5). This allowed for a good separation of angiotensin I, angiotensin II and His–Leu (Fig. 1). The fractions of eluate corresponding to angiotensin II were pooled and the peptide content was determined both by a quantitative Pauly reaction [10] and by the biological activity in the isolated guinea pig ileum [11].

K_m values were determined from Lineweaver–Burk plots of the initial velocities of hydrolysis obtained for five substrate concentrations in the range between $2 \cdot 10^{-5}$ and $2 \cdot 10^{-4}$ M. The effect of urea upon the angiotensin-converting activity was studied by pre-incubation of the enzyme preparation in 4 M urea for 20 min at room temperature. This solution was diluted 20-fold in the incubation medium, which contained $7.7 \cdot 10^{-5}$ M angiotensin I, 0.2 M NaCl, 0.1 mM $CoSO_4$ and 0.1 M Tris buffer (pH 7.6).

Dipeptide hydrolase activity. The spectrophotometric method of Stevens et al. [12] was employed, using acetyl-*p*-nitrophenylalanylglycylglycine as a substrate. The incubation mixture contained 50–100 μg enzyme protein and $5 \cdot 10^{-4}$ M substrate in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl, 10^{-4} M $CoSO_4$ and 3% (v/v) methanol. The increase in absorbance at 301 nm was followed with a Beckman Acta V spectrophotometer.

Angiotensinase and kininase activities. These activities were determined by

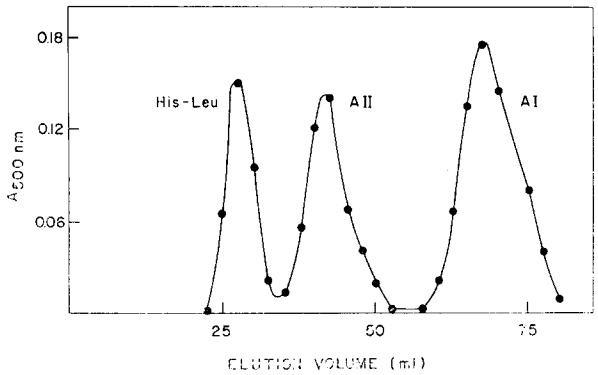

Fig. 1. CM-cellulose chromatography of a mixture of His–Leu, angiotensin II (AII) and angiotensin I (AI) (0.2 μmole of each) on an 11 cm $\times$ 0.9 cm column eluted with 0.1 M ammonium acetate (pH 4.5). Fractions of 2.5 ml were collected and Pauly's reaction was performed with 1-ml aliquots.

using either angiotensin II or bradykinin as substrates at 10^{-4} M concentration. The incubation was performed in 0.1 M sodium phosphate buffer (pH 8.0) containing 0.2 M NaCl and 10^{-4} M $CoSO_4$. At appropriate intervals, aliquots were drawn, in which the reaction was stopped by boiling and the peptides were assayed in the isolated guinea pig ileum.

RESULTS

Two peaks of angiotensin I-converting activity were identified after gel filtration on Sephadex G-150 of preparations from hog and from guinea pig plasma (Fig. 2). When cobalt was omitted from the medium in the enzyme assays of the eluates, only Peak II was detected.

Fraction I had an apparent molecular weight above 400 000 and possessed angiotensin-converting activity, but no kininase activity (Fig. 3). Fraction II, with an apparent molecular weight of 150 000, had both activities present (Fig. 3), and corresponded to the converting enzyme/kininase previously described. Neither of the two fractions had detectable activity upon acetyl-*p*-nitrophenylalanylglycylglycine, and both were devoid of angiotensinase activity. The specificities of Fractions I and II towards angiotensin I and bradykinin were not altered significantly by pre-incubation with 4 M urea.

Table I shows the specific activities at the different stages of the enzyme preparation. Both fractions obtained from Sephadex chromatography had optimum activity at pH 8.0, but they differed in their ion requirements. Fraction I did not require Cl^- for its activity and was activated by Co^{2+}, Ca^{2+} or Zn^{2+}. Fraction II required Cl^- for its activity and, although no activation was observed on addition of Co^{2+}, Ca^{2+} and Zn^{2+} these cations were necessary for the activity of Fraction II that had previously been treated with 1 mM EDTA and dialized.

Table II shows the effect of some ions on the activity of Fraction I from hog plasma. It is seen that full activity is obtained in the presence of 10^{-4} M Ca^{2+} or Co^{2+} but higher concentrations are inhibitory. Zn^{2+} activated the enzyme at lower concen-

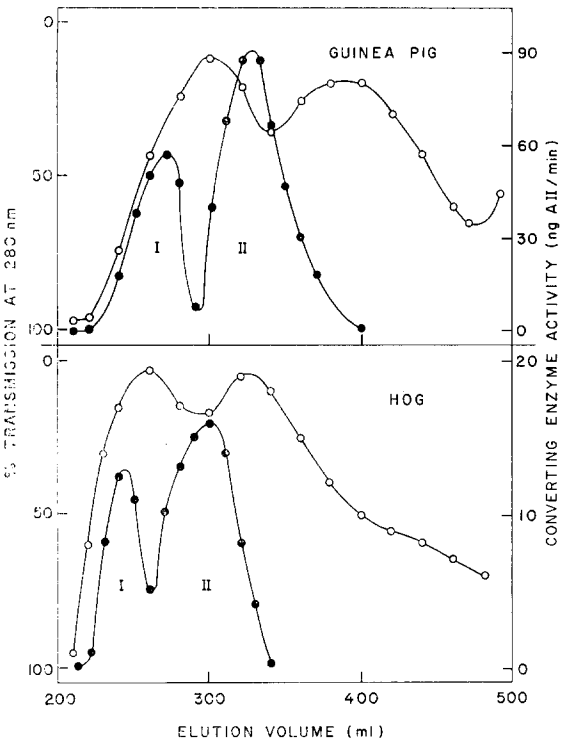

Fig. 2. Gel filtration on Sephadex G-150 of the fractions from hog and guinea pig plasmas precipitated between 1.4 and 2.8 M ammonium sulphate. The 105 cm × 3.2 cm column was equilibrated and eluted with 0.2 M NaCl and 5-ml fractions were collected. Protein (○) was measured by transmittance at 280 nm. Converting enzyme activity (●) was determined by incubation of 0.2-ml aliquots with 1.25 μM angiotensin I in 0.1 M sodium phosphate buffer (pH 8.0) containing 0.2 M NaCl and 0.1 mM $CoSO_4$ in a final volume of 1 ml.

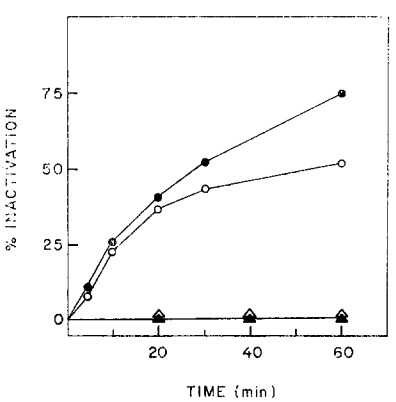

Fig. 3. Inactivation of 0.1 mM bradykinin by Fractions I and II obtained from hog and guinea pig plasma. Incubations were performed in 0.1 M sodium phosphate buffer (pH 8.0) containing 0.2 M NaCl and 0.1 mM $CoSO_4$. Protein concentration, 0.1 mg/ml. Aliquots were assayed for bradykinin activity on the isolated guinea pig ileum. ●, Fraction II from guinea pig; ○, Fraction II from hog; ▲, Fraction I from guinea pig; △, Fraction II from hog.

TABLE I

PURIFICATION OF ANGIOTENSIN-CONVERTING ENZYME FROM HOG AND GUINEA PIG PLASMAS

Enzyme assays were performed in 0.1 M phosphate buffer, pH 8.0, containing 0.2 M NaCl and 10^{-4} M Co^{2+}. Substrate concentration: $7.7 \cdot 10^{-5}$ M.

	Hog		Guinea pig	
	Total protein (mg)	Specific activity (μg/mg per min)	Total protein (mg)	Specific activity (μg/mg per min)
Blood plasma	58 900	0.1	2890	0.1
Ammonium sulphate (1.4–2.8 M)	5 500	0.9	960	2.5
Sephadex G-150				
Fraction I	240	4.3	6.2	6.0
Fraction II	340	4.0	22.9	44

trations than the other cations, but inhibition also occurred at lower concentrations, and full activity was not observed in this case.

EDTA-treated Fraction II from guinea pig plasma was also found to be activated by Co^{2+}, Ca^{2+} or Zn^{2+}, but the effect of ion concentration was not studied in this case.

The K_m values found for the angiotensin-converting activity of Fractions I and II from hog plasma were $8.69 \cdot 10^{-5}$ and $4.52 \cdot 10^{-5}$ M, respectively.

TABLE II

EFFECT OF IONS ON ANGIOTENSIN-CONVERTING ACTIVITY OF FRACTION I FROM HOG PLASMA

EDTA-treated and dialyzed Fraction I was assayed in 0.1 M sodium phosphate buffer (pH 8.0). Substrate concentration: $7.7 \cdot 10^{-5}$ M. Activity is expressed as percent of the activity in the presence of 0.2 M NaCl and 0.1 mM Co^{2+}.

NaCl concentration (M)	Other ions added	Concentration (mM)	Activity (%)
0	None		2
0.2	None		2
0.2	Co^{2+}	0.05	29
0.2		0.10	100
0		0.10	90
0.2		0.50	71
0.2		1.00	41
0.2	Ca^{2+}	0.05	17
0.2		0.10	100
0.2		0.50	71
0.2		1.00	29
0.2	Zn^{2+}	0.01	42
0.2		0.03	75
0.2		0.10	50

TABLE III

EFFECT OF ANGIOTENSIN-CONVERTING ENZYME INHIBITORS UPON FRACTIONS I AND II OF GUINEA PIG PLASMA

The reactions were performed in 0.1 M phosphate, pH 8.0, containing 0.2 M NaCl and 10^{-4} M Co^{2+}. Substrate concentration: $7.7 \cdot 10^{-5}$ M. Protein concentration: 0.1 mg/ml. Inhibition is expressed in percentage of the activity of a control reaction. Each value is the average of three determinations and the average deviations are shown inside parentheses.

Inhibitor	Concentration (mM)	Inhibition (%)	
		Fraction I	Fraction II
2-Mercaptoethanol	0.1	0	0
	1.0	52 (3)	5 (1)
His–Leu	1.0	0	0
	2.0	14 (1)	19 (2)
Phe–Arg	1.0	4 (1)	39 (1)
	2.0	16 (1)	60 (2)
Gly–Gly	1.0	0	0
	2.0	0	0

The inhibitory effect of the dipeptides His–Leu and Phe–Arg, known to be inhibitors of converting enzyme kininase activities [2], were studied for both fractions from the hog and the guinea pig plasma, and the results are presented in Table III. This table also shows that Fraction I was inhibited by 2-mercaptoethanol, in contrast to Fraction II.

DISCUSSION

Fraction II obtained by us from gel filtration of hog and guinea pig plasma is identical with the previously described angiotensin-converting enzyme because of: (a) its specificity towards angiotensin I and bradykinin [2]; (b) the characteristics of its dependence on Cl^- and the divalent cations [13]; (c) its inhibition by Phe–Arg and His–Leu and resistance to inhibition by 2-mercaptoethanol [2]; (d) its K_m of $4.5 \cdot 10^{-5}$ M, very similar to the value $4.2 \cdot 10^{-5}$ M reported for the hog plasma converting enzyme/kininase [14].

We have shown that blood plasma of the hog or the guinea pig contains, in addition to the previously described activity, an angiotensin-converting enzyme (Fraction I) that does not hydrolyze bradykinin. This activity could not be detected in the absence of added Co^{2+} or other divalent cations, and this may be the reason why it has not been previously reported.

This new converting enzyme activity does not appear to be due to association of the enzyme of 150 000 molecular weight, since the above described difference in specificity was maintained when Fractions I and II were treated with 4 M urea. In contrast, two high molecular weight converting enzymes found in hog lung were shown to be associated species, being dissociated by 4 M urea [15].

The new converting enzyme activity is also distinguished from the previously

described one by its independence of Cl^- concentration, its inhibition by 2-mercaptoethanol, and by a greater resistance to inhibition by Phe–Arg.

Both Fractions I and II did not hydrolyze acetyl-*p*-nitrophenylalanylglycylglycine. This is in contrast with the observation that carbobenzoxy-nitrophenylalanylglycylglycine is a good substrate for converting enzyme from calf lung [12]. However, it agrees with the observation that the enzyme from hog plasma does not attack Dns–Gly–Gly–Gly, while the same substrate is hydrolyzed by converting enzyme from hog lung [16].

Angus et al. [14] have found that hog plasma contains a peptide dipeptidyl hydrolase of higher molecular weight than the angiotensin-converting enzyme. This enzyme, however, was not able to convert angiotensin I into angiotensin II and thus is different from the one described here. This is the first observation of an angiotensin-converting enzyme in the absence of kininase activity.

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