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STUDIES ON THE ANGIOTENSIN CONVERTING ENZYME WITH DIFFERENT SUBSTRATES

YVONNE PIQUILLOUD, ANJA REINHARZ AND M. ROTH
Laboratoire Central, Hôpital Cantonal Universitaire, Geneva (Switzerland)

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SUMMARY

A method has been developed for the chemical assay of the angiotensin converting enzyme. It is based on the fluorimetric determination of histidyl-leucine, a product of the enzymic reaction. It is shown that not only angiotensin I, but also the artificial substrates Z–Phe–His–Leu and Z–Pro–Phe–His–Leu are hydrolysed by the equine and human enzymes. Z–Phe–His–Leu is hydrolysed about 10 times faster than angiotensin I and Z–Pro–Phe–His–Leu by the human enzyme, and is therefore a convenient substrate for quantitative determinations. Investigations in which this new substrate was utilized showed that human converting enzyme inactivated by dialysis was reactivated by anions in the order $Cl^- > NO_3^- > Br^- > F^- > I^-$. The enzyme activity is highest between pH 7.5 and 8.5.

INTRODUCTION

SKEGGS *et al.*[1] have found in horse plasma, an enzyme capable of hydrolysing the decapeptide angiotensin I, thus releasing the vasopressive octapeptide angiotensin II and the inactive dipeptide histidyl-leucine. This so-called "converting enzyme" occurs in several species. It has so far been determined mainly by biological measurement of angiotensin II produced upon incubation with angiotensin I. THAMPI AND HUGGINS[2] described a radiometric assay using labelled angiotensin I as the substrate, in which the release of radioactive histidyl-leucine serves as an index of enzymic activity. Histidyl-leucine, indeed, may be estimated with good sensitivity by fluorimetry[3,4]. Using the latter technique, we have shown that shortening of the peptide chain of angiotensin I from the amino end does not abolish the susceptibility to the converting enzyme[4]. Thus Z–Pro–Phe–His–Leu and Z–Phe–His–Leu are good substrates. These peptides are easier to synthesize than angiotensin I, and studies with such substrates may contribute to a better understanding of the specificity of the enzyme. The present report gives a detailed account of our studies with such substrates as well as with angiotensin I, and describes a fluorimetric assay of the converting enzyme.

Abbreviation: Z, benzyloxycarbonyl.

MATERIALS AND METHODS

Materials

o-Phthaldialdehyde, Z-phenylalanine *p*-nitrophenylester, histidine methyl ester dihydrochloride and leucine methyl ester hydrochloride were from Fluka (Buchs SG, Switzerland), and histidyl-leucine from ICN (City of Industry, Calif.). The synthesis of Z–Pro–Phe–His–Leu has been described earlier[5]. Synthetic (5-isoleucine)angiotensin I was obtained from Schwarz BioResearch, Orangeburg, N.Y., U.S.A.

Borax–phosphate buffer (pH 8.0 at 20°) was prepared by mixing of 0.05 M borax with the appropriate quantity of 0.1 M KH_2PO_4. The buffers utilized for the pH curves were prepared in the same manner.

The spray reagent utilized to detect histidyl-leucine on chromatograms consisted of *o*-phthaldialdehyde (2 g/l) dissolved in a solution of KOH in methanol (40 g/l).

Synthesis of peptides

The amino acids contained in the starting materials were of the L form. Melting points were determined on a Kofler block and are uncorrected. Thin layer chromatography was performed on silicagel G (Merck). The solvent system for $R_{F,1}$ was methanol–acetone (8:2, v/v) and for $R_{F,2}$ *n*-butanol–acetic acid–water (8:8:2, v/v/v).

Z-phenylalanyl-histidine methyl ester was prepared by allowing Z-phenylalanine *p*-nitrophenyl ester to react with histidine methyl ester according to the procedure of BODANSZKI *et al.*[6]. Yield 62%; m.p. 115–116°. Thin-layer chromatography: $R_{F,2} = 0.6$; ninhydrin-negative, Pauly-positive.

The ester, when reacted with hydrazine hydrate, gave *Z-phenylalanyl-histidyl-hydrazide* of m.p. 173–174° in 99% yield.

Z-phenylalanyl-histidyl-leucine methyl ester was prepared with a combination of two azide procedures[7–9] as described by MAZUR AND SCHLATTER[10]. The above hydrazide, after reaction with leucine methyl ester hydrochloride, gave the tripeptide ester in 83% yield; m.p. 176–180°. Thin-layer chromatography with methanol–chloroform (1:9, v/v), $R_F = 0.68$.

Z-phenylalanyl-histidyl-leucine was prepared by hydrolysing the above ester in 1 M NaOH for 2 h at room temperature. Yield 72%, m.p. 207–209°. Thin-layer chromatography with *n*-butanol–acetic acid–water (7:1:2, v/v/v), $R_F = 0.75$; ninhydrin-negative, Pauly-positive.

Preparation of the converting enzyme

The enzyme from horse plasma was prepared according to SKEGGS *et al.*[1].

The same procedure was also applied to human plasma, and it yielded preparations that were used in the first experiment. Another purification technique, which is more easily reproducible and yields a material of better purity, was subsequently developed. In this technique, which uses a system described by FLODIN AND KILLANDER[11], 2 ml of serum are applied to a 100 cm × 2.5 cm column containing 17 g of Sephadex G-200 equilibrated with an 0.1 M Tris–HCl buffer (pH 8.0), with 0.2 M NaCl incorporated. The elution is performed with the same buffer and is monitored by continuous recording of protein absorption at 280 nm. The fractions corresponding to the second protein peak contain the converting enzyme. They are pooled, dialysed overnight against deionized water and lyophilized.

Assay of the converting enzyme

Either Z–Pro–Phe–His–Leu or Z–Phe–His–Leu were utilized as the substrate. Z–Phe–His–Leu is more readily hydrolysed by the enzyme, and for this reason it is used in the current assay. The conditions were modified several times during the progress of the investigation, and the procedure finally adopted is as follows: 20 μl of substrate solution (2 mg/ml in methanol) are placed in a test tube and mixed with 3 ml of borax–phosphate buffer (pH 8.0) containing 1% NaCl. The tubes are placed for 3 min or more in a water-bath at 37°, and the reaction is started by the addition of 20 μl of enzyme solution. After 1 h incubation at 37°, the reaction is stopped by placing the tubes for 5 min in a boiling-waterbath. When they have cooled, one adds 0.4 ml 2 M NaOH and 0.1 ml of *o*-phthaldialdehyde solution (0.1% in methanol). After 4 min, 0.2 ml 6 M HCl is added. The fluorescence produced is stable for between 30 and 90 min after the addition of HCl and is read during this period. It will be seen in the RESULTS section that the substrate concentration utilized in this routine assay is lower than optimal. This is because we wanted to spare the small quantities of substrate available.

Apparatus

The fluorescence was measured either with a Farrand spectrofluorimeter (λ_{ex} = 365 nm, λ_{fl} = 500 nm), or with a Photovolt model 540 fluorimeter equipped with the standard mercury lamp and filters B-Hg-1 (primary) and CS 3-71 (secondary).

RESULTS

Identification of histidyl-leucine as a product of enzymic activity

With the aid of thin layer chromatography, histidyl-leucine was identified as a product arising upon incubation of Z–Pro–Phe–His–Leu and Z–Phe–His–Leu with the converting enzyme.

A preparation of the human enzyme (2 ml containing approx. 17 mg/ml protein), obtained by partial purification on a column of Sephadex G-200, was mixed with 2 ml borax–phosphate buffer (pH 7.0) containing 1% NaCl. The reaction was started by the addition of 0.2 ml of a methanolic solution of Z–Phe–His–Leu (2 mg/ml). After 2 h incubation at 37°, the reaction was stopped by the addition of 4 ml ethanol, the samples were centrifuged and the supernatant was evaporated to dryness. The residue, taken up in 3 ml of methanol, was centrifuged, and the supernatant concentrated by evaporation to a small volume which was applied to a thin layer plate of silica gel G. After chromatography with the system *n*-butanol–acetic acid–water (12:3:5, v/v/v), the plate was sprayed with the *o*-phthaldialdehyde reagent. Histidyl-leucine (R_F = 0.25) appeared as a strongly fluorescing blue-green spot.

Unhydrolysed substrate (R_F = 0.68) gave a blue fluorescing spot. In contrast, only trace amounts of leucine (R_F = 0.43, weak orange-yellow fluorescence) and histidine (R_F = 0.10, pale yellow fluorescence) could be detected.

Similar results were obtained when Z–Pro–Phe–His–Leu (R_F = 0.57) was used as the substrate, either with the same enzyme preparation, or with a preparation of human enzyme obtained by the procedure of SKEGGS *et al.*[1].

The excitation and fluorescence spectra of the final solution of the standard assay presented under MATERIALS AND METHODS were the same as those of a solution

TABLE I

CONVERTING ENZYME ACTIVITY IN THE THREE FIRST PROTEIN PEAKS ELUTED FROM A COLUMN OF SEPHADEX G-200 LOADED WITH HUMAN SERUM

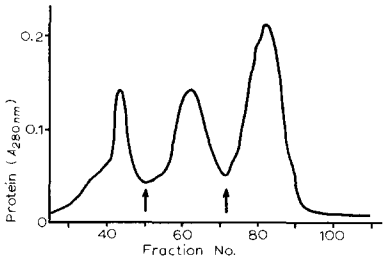
Substrate: Z–Phe–His–Leu. Conditions as under MATERIALS AND METHODS.

Serum number	*Total activity (mU/ml)*			*Relative activity (mU/g protein)*		
	Peak 1	*Peak 2*	*Peak 3*	*Peak 1*	*Peak 2*	*Peak 3*
1	0.08	1.54	0.04	5	100	2
2	0.07	0.90	0.03	6	43	3
3	0.06	1.84	0.05	5	94	3
4	0.02	1.33	0.04	1	89	3

in which pure histidyl-leucine had been subjected to the reaction with *o*-phthaldialdehyde.

Fractionation of human serum by gel filtration

The typical protein elution pattern from a Sephadex G-200 column loaded with human serum is shown in Fig. 1. Three peaks appeared successively. The fractions corresponding to each peak were pooled, dialyzed against water and lyophilized, and the enzymic release of histidyl-leucine from Z–Phe–His–Leu was assayed for each pool. Results are shown in Table I. Most of the activity was located in peak 2. The procedure has been repeated several times for preparative purposes, and the material obtained from peak 2 was utilized in the studies on the properties of the converting enzyme reported below.



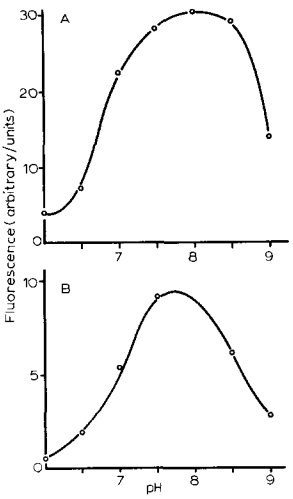
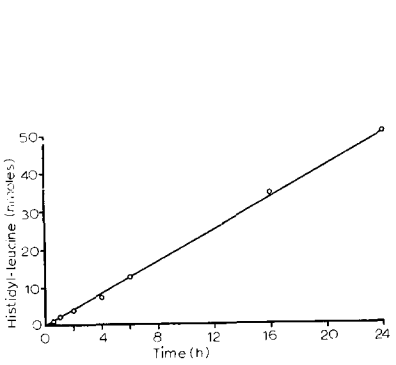


Fig. 1. Gel filtration of human serum on Sephadex G-200. Two ml serum were applied to a 100 cm × 2.5 cm column equilibrated at 4° with a 0.1 M Tris–HCl buffer (pH 8.0) with 0.2 M NaCl incorporated. Partially purified preparations of the converting enzyme were obtained by pooling the fractions comprised between the two arrows (corresponding to the second peak).

Fig. 2. Effect of pH on the activity of the human enzyme. Samples partially purified by fractionation of serum on Sephadex G-200 were incubated for 1 h at 37° in phosphate–borate buffer containing 1%NaCl. Histidyl-leucine was measured as under MATERIALS AND METHODS. A. Substrate, Z–Phe–His–Leu (6 μg/ml). B. Substrate, angiotensin I (15.4 μg/ml).



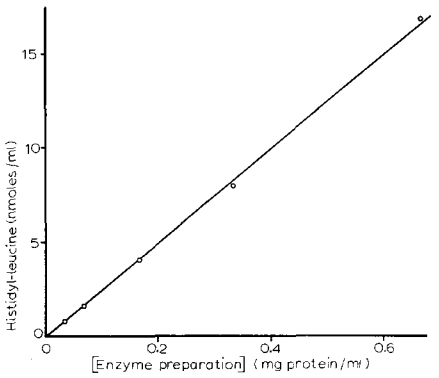


Fig. 3. Activity of the human enzyme as a function of time. The enzyme, partially purified by fractionation of serum on Sephadex G-200, was incubated at 37° in phosphate–borate buffer (pH 8.0) containing 1% NaCl in the presence of 6.7 μg/ml of Z–Phe–His–Leu in a total volume of 3 ml. Histidyl-leucine was measured as under MATERIALS AND METHODS. Blanks without enzyme were treated in the same manner.

Fig. 4. Release of histidyl-leucine plotted against the concentration of enzyme preparation (expressed as mg protein per ml). Incubation time 60 min. Other conditions as for Fig. 3.

Influence of pH

Preparations of human serum enzyme incubated with Z–Phe–His–Leu or Z–Pro–Phe–His–Leu gave the highest rates of release of histidyl-leucine between pH 7.5 and 8.5. When angiotensin I was used as the substrate, the maximum was found to occur at a pH of about 7.5 (Fig. 2).

Activity as a function of time

The amount of histidyl-leucine enzymically released from Z–Phe–His–Leu by the human plasma enzyme purified with Sephadex G-200 increased linearly with time within a 24-h period of incubation (Fig. 3). A time curve obtained with angiotensin I as the substrate is shown in Fig. 6.

Activity as a function of enzyme concentration

This is shown in Fig. 4. Under the conditions employed, the activity was proportional to the quantity of enzyme. The specific activity derived from these data is 4.1 international units per mg protein.

Activity as a function of substrate concentration

The K_m value for the hydrolysis of Z–Phe–His–Leu was determined twice and found to be 61 (Fig. 5) and 40 μM.

Influence of anions

SKEGGS *et al.*[12] showed that the converting enzyme from horse plasma is activated by halogen and nitrate ions. For this reason, NaCl was included in our standard incubation medium. We observed that virtually all the activity of our preparations of horse and human converting enzyme was lost if they were dialyzed against deionized water (until no more Cl^- could be detected in the dialysis bag) and incubated in our

TABLE II

REACTIVATION OF DIALYZED HUMAN CONVERTING ENZYME BY ANIONS

Enzyme from human plasma was purified by gel filtration and dialyzed; it was incubated 30 min with Z–Phe–His–Leu in borax–phosphate buffer (pH 8.0) which included 0.17 M of the following salts: NaF, NaCl, NaBr, KI, $NaNO_3$. Other conditions as under MATERIALS AND METHODS.

	Control	F^-	Cl^-	Br^-	I^-	NO_3^-
Activity (μmoles/min)	0.2	8.0	23.3	11.5	6.5	14.1

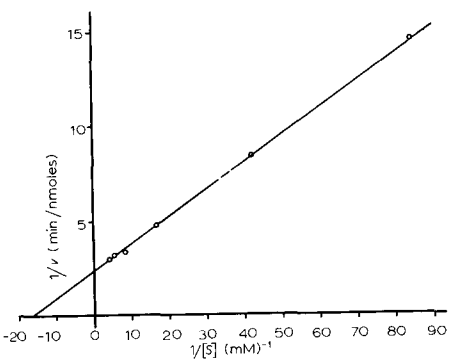
standard assay medium from which NaCl had been omitted. Addition of chloride restored the activity. Table II shows that anions reactivated the dialyzed human enzyme in the order $Cl^- > NO_3^- > Br^- > F^- > I^-$. Reactivation was also observed when Z–Pro–Phe–His–Leu was utilized as the substrate instead of Z–Phe–His–Leu.

Inhibition by EDTA

When 0.1 mM EDTA was incorporated in the standard assay described under MATERIALS AND METHODS, the hydrolysis of Z–Phe–His–Leu by preparations of the human enzyme was inhibited by more than 90%.

Substrate specificity

Under the standard assay conditions described under MATERIALS AND METHODS, Z–Phe–His–Leu was hydrolysed by the human enzyme about ten times faster than Z–Pro–Phe–His–Leu and angiotensin I. Fig. 6 shows the time course of release of histidyl-leucine when the same enzyme preparation was incubated with either Z–Phe–His–Leu or an equimolar amount of angiotensin I.



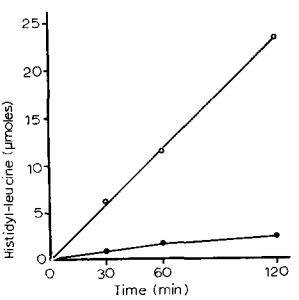


Fig. 5. Enzymic release of histidyl-leucine from Z–Phe–His–Leu expressed by a reciprocal plot as a function of substrate concentration. Human enzyme purified by gel filtration. v, reaction velocity as nmoles/min; $[S]$, substrate concentration as mM. The value for K_m derived from these data is 61 μM.

Fig. 6. Action of human converting enzyme on two different substrates. The enzyme preparation obtained by fractionation of human serum on Sephadex G-200 was incubated in 0.05 M phosphate–borate buffer (pH 7.5) containing 1% NaCl and 60 nmoles/ml substrate. The release of histidyl-leucine was determined as under MATERIALS AND METHODS. Substrates: ○—○, Z–Phe–His–Leu; ●—●, angiotensin I.

DISCUSSION

In these studies, Z–Phe–His–Leu was a much better substrate of human converting enzyme than was a commercial preparation of synthetic angiotensin I. Since it is also easier to synthesize, it is a convenient substrate for the assay of this enzyme. The fluorimetric technique described here is relatively simple, and it has been used successfully in studies on the tissue distribution of the converting enzyme in the rat[13].

The enzymic release of histidyl-leucine from Z–Phe–His–Leu is activated by chloride and inhibited by EDTA, two effects also observed when angiotensin I is the substrate[1,14,15]. This confirms the hypothesis that the hydrolysis of Z–Phe–His–Leu is actually due to the converting enzyme.

The fact that Z–Phe–His–Leu is much more susceptible to hydrolysis by the converting enzyme than are Z–Pro–Phe–His–Leu and angiotensin I might indicate that the proline group hinders enzyme–substrate combination. The term "angiotensin converting enzyme" will have sooner or later to be replaced by a more appropriate one, since this enzyme hydrolysis other substrates as well. The specificity seems to be that of a peptidase capable of catalysing the release of a dipeptide from the carboxyl end of certain polypeptides. Better knowledge of the exact substrate requirements warrants further studies with synthetic substrates. It would be interesting to know whether the sole function of this enzyme in the organism is to convert angiotensin to its active form, or whether it exerts some similar effect on other circulating substrates.

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