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Dipeptidase 로서의 Histidylleucine 가수분해 효소

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Histidylleucine Hydrolyizng Enzyme: A Dipeptidase From Hog Lung

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요 약. 돼지의 폐(lung)로 부터 histidylleucine dipeptide 를 가수분해 시키는 효소를 추출 정제 하였으며, 정제된 효소의 비활성도는 1.6 μmoles/min/mg protein(약 50배 정제)이었다. 이 새로운 dipeptidase 의 분자량은 sucrose density gradient 원심침강법에 의하여 측정한 결과 약 80,000 daltons 이었으며 효소의 기질 물질로서 요구되는 조건은 방향족 잔기를 갖는 dipeptide 를 필요로 하나 histidylleucine 을 기질로 사용하였을 경우 K_m 값은 $2\times10^{-4}M$ 이었다. 본문에서는 이효소의 일반적 특성과 그 밖의 생리학적 및 생화학적 기능에 관하여 검토하였다.

ABSTRACT. An enzyme capable of hydrolyzing histidylleucine was purified 50 fold from hog lung. The final preparation hydrolyzed 1.6 μmoles of histidylleucime per minute per mg of protein. The K_m of the enzyme for the enzyme was found to be $2\times10^{-4}M$. The enzyme was required a number of free dipeptides for the substrate specificity, and was inhibited by EDTA and 1, 10-phenanthroline. The molecular weight of the enzyme was estimated to be 80,000 daltons from sucrose density gradient sedimentation analysis. The corrected $s_{20,w}$ value was 5.3 S.

INTRODUCTION

It is now evident that the conversion of angiotensin I to angiotensin II occurs much more rapidly in the passage of blood through the lung than in blood itself[1~4]. The half-time is on the order of 15 seconds in lung and about 3 minutes in blood[5,6]. If the mechanism of conversion is the same in lung as in blood[7,8], i.e. if the lung contains the same kind of converting enzyme that specifically splits off histidylleucine from angiotensin I, it would be of interest to see if lung contains an enzyme which is capable of splitting histidylleucine.

The possible existence of such an enzyme is also of interest in regard to the question of the nature of the conversion that occurs in lung. Converting enzyme has not actually been demonstrated in lung tissue and there is some speculation[4,5] that the actual reaction may involve

a kind of carboxypeptidase that sequentially removes leucine and then histidine from angiotensin I. This suggestion is based upon the appearane of ^{3}H-leucine as the principal metabolic product arising from 5-Ileu, 10-Leu(H^3) angiotensin I (decapeptide) accounted for in two ways: (1) aspecific converting enzyme cleaves histidyl-leucine from angiotensin I and histidyllecucine is in turn rapidly hydrolyzed by one or more other enzymes. (2) a carboxypeptidase acts directly on angiotensin I to release free leucine and produce a nonapeptide. The nonapeptide might itself be active. Alternatively, the nonapeptide might be further hydrolyzed to produce angiotensin II and histidine.

Actually we have known for some time in connection with other related work that lung does contain a relatively large histidylleucine hydrolyzing activity and we report our findings in this paper along with some properities of the enzyme.

EXPERIMENTAL PROCEDURES

Enzyme Preparation. Frozen hog lung was thawed and then homogenized in a Waring blender with 5 volumes of 0.05 *M* sodium phosphate buffer, pH7.0 at 5°. The homogenate was filtered through cheesecloth and the cellular debris was then removed by centrifugation at 13,200×g for 30 minutes. The centrifugate was filtered through glass wool prior to ammonium sulfate fractionation. The protein which precipitated between 1.8 *M* and 2.2 *M* ammonium sulfate was collected, dissolved in a minimum volume of 0.05 *M* sodium phosphate buffer, pH 7.0 and then dialyzed against the same buffer at 5° until the sulfate concentration was negligible. This crude ammonium sulfate fraction was concentrated to 25 mg/m*l* with an Amicon ultrafiltration system employing a P-10 filter. The concentrated preparation was then passed on a 2.2 cm×30 cm bed of DEAE-cellulose column, Whatman DE-52, seies III, with a NaCl gradient from zero to 0.5 *M* in 0.05*M* sodium phosphate buffer, pH 7.0 (*Fig.* 1).

The active enzyme fractions were combined, dialyzed against 0.01*M* sodium phosphate buffer, pH 7.0 and lyophilized. The specific activity of this final partially purified enzyme was obtained as 1.6 μmoles per minute per mg protein when it was assayed using the synthetie substrate, His-^{14}C-Leu(UL).

Radioactive Assay for Histidylleucine Cleavage. The ability of enzyme preparations to split histidylleucine was measured by following the release of ^{14}C-leucine(UL) from histidyl-^{14}C-leucine(UL). The standard assay mixture contained 20μl of 0.1 *M* sodium phosphate buffer, pH7; 20μl of 1×10^{-2} *M* unlabeled histidylleucine; 5μl of ($0.75\times10^{-3}M$) histidyl-^{14}C-leucine(UL) of specific activity 0.45 millicuries/millimole; and 5 μl of enzyme solution(0.1 mg/ml). The incubations were carried out at 37° for 30~60 minutes after which the reactionmixtures were immedia-

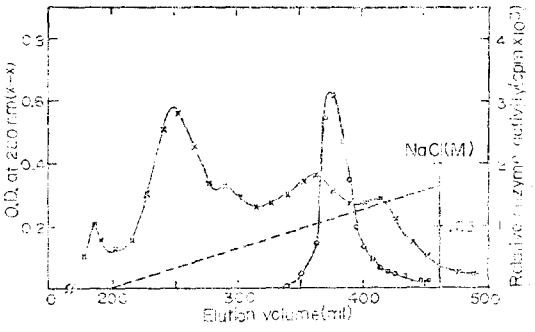

Fig. 1. Purification of hog lung dipeptidase by ion-exchange chromatography. The crude enzyme preparation was passed on 2.2 cm×30 cm bed of DEAE-cellulose colum with a NaCl gradient in 0.01*M* phosphate buffer, pH 7.0. The flow rate was 30 m*l*/hr. The enzyme activity was mesaured by radioactivity assay using His-^{14}C-Leucine (UL).

tely spotted on Whatman 3MM paper. High vol-

tage paper electrophoresis was performed at 2,500 volts for 45 minutes in a pyridine-acetic acid-water buffer (1:10:289 v/v), pH 3.6. The extent of histidylleucine hydrolysis was determined from the amount of radiocativity present as leucine and histidylleucine. Leucine and histidylleucine migrated approximately 5 and 15 cm, respectively. The radioactivity present in each spot was measured by counting 1×2 cm sections of the dried electrophoretogram for 10 minutes or a pre-set error of 5% in 25 m*l* scintillation vials with 10 m*l* of scintillation mixture (7 g of PPO and 0.45 g of POPOP/liter). A Beckman, LS-250 Liquid Scintillation System was used. Portein concentrations were determined by the method of Lowry[9].

Enzymic Hydrolysis of Peptides. The peptide substrate at 2×10^{-3} *M* concentrations was incubated in 50 μl of 0.01 *M* phosphate buffer, pH 6.4, containing 10 μl of enzyme preparation. After incubation at 37° for 1 hr the reaction mixture was then diluted to 1.0 m*l* with sample dilution buffer, pH 2.2, for application to the amino acid analyzer. The JEOLCO amino acid analyzer, model JLG-5AH was used for analysis of the enzymic products (free amino acids)[10]

Sucrose Density Gradient Centrifugation. Sedimentation experiments were carried out as described by Martin and Ames[11]. One-tenth mililiter of test enzyme or standard known enzyme markers, catalase, cytochrome c and alkaline phosphatase, was applied to a 5% to 20% sucrose gradient in 0.1*M* Tris, pH 7.65, and centrifugation was carried out at 38,000 rpm for 18 hours at 5° using a SW 50 L rotor in a Model L2 65 B Beckman Spinco Preparative Ultracentrifuge. The sedimentation velocity of the enzymes were measured by the enzyme activities except for cytochrome c which was measured by absorption. Five to ten drop fractions were collected from each tube after centrifugation, and an aliquot of each fraction was analyzed for the enzyme activity as described in the above. Sedimentation coefficients corrected to $s_{20,w}$ values obtained were compared to the reported values and the approximate relationship of sedimentation constant and molecular weight, $(s_1/s_2)^{3/2} = (\text{mol wt}_1/\text{mol. wt}_2)$ was used to determine the relative molecular weight of the histidylleucine cleaving enzyme as compared to catalase[6].

RESULTS and DISCUSSION

An enzyme capable of cleaving histidylleucine was prepared from hog lung homogenate by ammonium sulfate and further by Whatman DE-52 ion exchange chromatography (*Fig.* 1). The specific activity of the final partially purified preparation was 1.6 μmoles per minute per mg protein which represents a fifty fold purification over the centrifuged and filtered homogenate. The rate of histidylleucine cleavage during standard assay conditions was constant over an incubation period of 60 minutes at 37° and directly

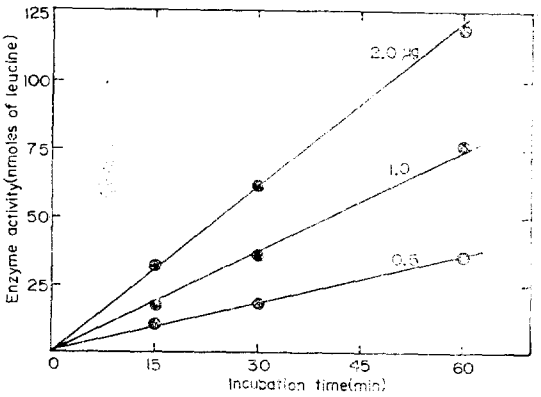

Fig. 2. Time course of histidylleucine hydrolysis by the hog lung enzyme. The incubation mixture contained 200 nmoles of unlabeled histidylleucine, 3.75 nmoles of histidyl-^{14}C-leucine(UL) of specific activity 0.45 millicuries/millimole, 0.5, 1.0, or 2.0 μg of enzyme, and 0.1*M* sodium phosphate, pH 7, in a final volume of 50 μl. All incubations were carried out at 37°.

proportional to the enzyme concentration (*Fig.* 2)

The percent reaction varied from 4 to 60%. However, the enzyme did not exhibit product inhibition when histidine and leucine were present at 2.4×10^{-3} *M* i. e., after 60% reaction. The pH optimum of the enzyme appeared to be about 6.0 (*Fig.* 3). The enzyme was completely inhibited by preincubation with EDTA (6×10^{-4} *M*) or 1, 10-phenanthroline (6×10^{-4} *M*) at 37° for 30 minutes prior to performing the standard assay.

The enzyme preparation showed a broad substrate specificity as a dipeptidase, but free carboxyl and amino groups are absolute requirement for the enzyme activity (*Table* 1). As shown in *Table* 1, dipepties containing *N*-terminal aromatic amino acids were hydrolyzed more easily than other dipeptides. The blocked dipeptides, tripeptides and angiotensins were not hydrolyzed by this enzyme preparation. From this it could be concluded that there was no other peptidases like carboxypeptidase or aminopeptidase as a possible contaminant in the enzyme preparation.

The K_m of the enzyme for histidylleucine was $2\times10^{-4}M$ as calculated from a modified from of the Michaelis-Menten[12] (*Fig.* 4) in which $1/\bar{v}$ was plodtte versus $1/\bar{s}$or $(1/\Delta s)$ ln (S_o/S_t); $\bar{v}$

Table 1. Enzymic hydrolysis of peptides.

Substrate	Products formed (% Hydrolysis)[a]	
His-Leu	Leu(100)	His(95)
Phe-Leu	Leu(85)	Phe(90)
His-Gly	Gly(95)	His(95)
Phe-Gly	Gly(90)	Phe(90)
Phe-Pro	Phe(15)	Pro(?)
Ala-Ala	Ala(35)	
Gly-Leu	Gly(40)	Leu(30)
Ala-Phe-Leu	Ala(5)	Leu(0)
Angiotensin I	None	
Angiotensin II	None	
Z-His-Leu-OMe	None	
Z-His-Leu	None	
His-Leu-OMe	His(3)	Leu-OMe(5)
N-Cbz-Gly-Tyr-Gly	None	

[a]Enzymic hydrolysis products were analyzed by amino acid analyzer, JEOLCO, Model JLG-5AH
[b]*Z*=*N*-benzyloxycarbonyl.

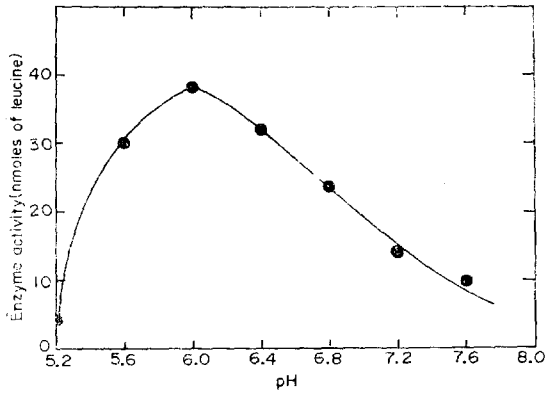

Fig. 3. Depenednce of the histidylleucine cleaving enzyme on pH. The standard radioactive assay was performed with the exception of the buffers. At pH 5.2 and 5.6, 0.1 *M* sodium acetate was used and at pH 6.0, 6.4, 6.8, 7.2, and 7.6, 0.1 *M* sodium phsophate was employed. The incubations were carried out at 37° for 1 hour.

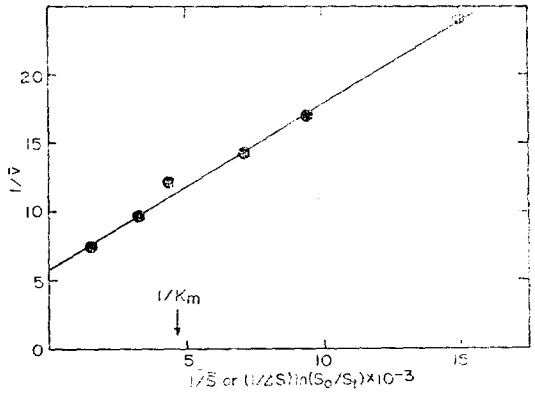

Fig. 4. Modified Lineweaver-Burke plot for the histidylleucine cleaving enzyme. Histidylleucine present at 0.16, 0.22, 0.28, 0.41, 0.53 and 1.3 $\times10^{-3}M$ was incubated with 0.5 μg of enzyme in 50μl of 0.1*M* sodium phosphate buffer, pH 7 at 37° for 30minutes. The average velocity and mean substrate concentration are designated $\bar{v}$ and $\bar{s}$, respectively.

and $\bar{s}$ are the average velocity and the mean substrate concentration, $\bar{s}=\frac{1}{2}\left(S_0+S_t\right)$, respectively, S_0 is the initial substrate concentration, S_t equals the substrate concentration at t minutes, and Δs equals S_0-S_t.

The corrected $s_{20,w}$ values of the histidylleucine cleaving enzyme and known standard marker proteins were calculated (*Table* 2). Based on the assumption that the partial specific volumes and shapes of catalase and the histidylleucine cleaving enzyme are similar, the molecular weight of the cleaving enzyme was estimated to be 80,000 daltons.

Although the lung contains a relatively large amount of dipeptidase which hydrolyzes histidylleucine (20 μmoles/min/g of wet tissue), it is hard to make an accurate comparison with the amount of enzyme in lung that transforms angiotensin I to angiotensin II because this quantity is not given in the literature. However, Huggins, *et al.*[13] report on a purified preparation of this enzyme that has a specific activity of 0.007μmole/min/mg of tissue. If we make the probable assumption that Huggins, *et al*. purified their preparation that hydrolyzes 1.62 μmoles of histidylleucine per min per mg protein, we can conclude that there is at least two hundred times as converting enzyme activity. Under these circumstances it would be difficult to show histidylleucine as a product when angiotensin I is hydrolyzed to angiotensin II. This high histidylleucine splitting activity could easily explain the appearance of ^{3}H-leucine rather than histidyl-^{3}H-leucine in the lung perfusion experiments described in the introduction[4,5] and indicates that we can anticipate some difficulty in demonstrating histidylleucine in homogenates even if it is formed.

Table 2. The calculated $s_{20,w}$ values from the sucrose density Gradient sedimentation experiment.

Protein	$s_{20,w}$ (obtained)	$s_{20,w}$ (literature)	Molecular weoght
Histidylleucine cleaving enzyme	5.3	—	80,000[a]
Catalse (Bovine liver)	11.2	11.3	247,500
Cytochrome c	1.7	1.71	12,500
Alkaline phosphatase (E. coli)	6.2	6.0	~85,000

[a]Molecular weight of histidylleucine cleaving enzyme was calculayed based on bovine liver catalse.

It would seen possible that the histidylleucine splitting enzyme has a physiological function in "removing" histidylleucine, ie., in returning histidine and leucine to their amino acid pools. We have not investigated thorougbly the specificity of the enzyme which will require further purification, but specificity is not a requirement for this function. The K_m value, $2\times10^{-4}M$, is in a suitable range.

Our preparation may contain only one enzyme that hydrolyzes histidylleucine. The evidence for this is that the distribution of enzyme activity in the sucrose gradient experiment was symmetrical and suitably sharp. Also the double reciprocal plot from which the K_m value was calculated was a good straight line.

The enzyme apparently contains an essential metal since two metal chelating agents inactivate the enzyme but chloride ion is not required.

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