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PURIFICATION AND PROPERTIES OF HUMAN LIVER PEPTIDASE

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Summary

The peptidase from human liver was purified using L-Leu-L-Leu as a substrate, in adapted purification techniques including treatment with *n*-butanol, acetone precipitation, ammonium sulfate fractionation, DEAE-cellulose chromatography, Sephadex G-150 gel filtration and CM-cellulose chromatography. The purified enzyme exhibited homogeneity in disc electrophoresis. The molecular weight of the enzyme was estimated to be 130 000 by Sephadex G-200 gel filtration. The isoelectric point of the enzyme was found to be pH 5.6. The enzyme was activated by Mn^{2+} and inhibited by *o*-phenanthroline. L-Leu-L-Leu and L-Phe-L-Phe were hydrolyzed effectively by the peptidase. By electrophoresis on Cellogel, the electrophoretic mobility of purified enzyme was same as that of the peptidase in serum of patients with hepatic disease.

Introduction

Recently a number of reports have appeared on peptidases in serum [1], and human [2,3] and mammalian tissues [4—6]. Several peptidases were isolated from those tissues [7—10]. A determination of leucine aminopeptidase activity in serum using L-leucyl-β-naphthylamide as a substrate is used generally for diagnosis of hepatic disease [11,12] and assessment of placental function for evaluating the status of pregnancy [13,14]. However, previous investigators reported that L-leucyl-β-naphthylamide used as a substrate of leucine aminopeptidase might be hydrolyzed by a variety of peptidases in serum [15,16].

In a previous paper, we demonstrated that the method for determination of peptidase activity in serum coupling with L-amino acid oxidase obtained from

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venom of *Agkistrodon caliginosus* could be applied in clinical diagnosis of hepatic diseases [17]. The activity of peptidase, where L-Leu-L-Leu served as a substrate, was markedly elevated in the serum of patients with acute hepatitis and hepatoma. From the result of experiments with rats, this peptidase seems to leak out from the liver [17]. In this paper, we described the purification of peptidase from human liver, and discussed its properties.

Materials and Methods

DEAE-cellulose and CM-cellulose were obtained from Brown Co. (U.S.A.). The following peptides were used in this study: L-Leu-L-Leu, purchased from Fluka AG (Switzerland); L-Phe-L-Tyr and L-Leu-Gly-Gly from Miles-Yeda Ltd. (U.S.A.); Gly-L-Leu, L-Phe-L-Phe, hippuryl-L-Phe, L-Leu-NH_2, L-Phe-L-Pro, L-Leu-β-naphthylamide and L-Cys-di-β-naphthylamide from Sigma Chem. Co. (U.S.A.). Peroxidase (Sigma Type I, 94 units/mg) was obtained from Sigma Chem. Co. (U.S.A.).

The L-amino acid oxidase used was a peptidase-free preparation purified from venom of *Agkistrodon caliginosus* in the same manner as described previously [17].

The crude enzyme was prepared in the following procedure. 1 kg of frozen human liver was homogenized with 3 l of water and 1 l of *n*-butanol (liver/water/*n*-butanol, 1 : 3 : 1) and was centrifuged at 10 000 × *g* for 10 min. The supernatant was treated with 60% acetone at 0°C to precipitate the protein. Distilled water was added to the precipitate and the soluble fraction was used as the crude enzyme.

Peptidase activity was measured in the same manner as described previously [17]. The incubation mixture contained 4 μmol of L-Leu-L-Leu in 1 ml of 50 mM Tris · HCl buffer (pH 8.0), 20 μl of L-amino acid oxidase (20 units/ml), 2 ml of color reagent in which 8 mg of 4-aminotipyrine, 20 μl of *N*,*N*-dimethylaniline and 300 units of peroxidase were dissolved in 100 ml of 50 mM Tris · HCl buffer (pH 8.0), and 0.1 ml of the enzyme solution. The reaction was carried out at 37°C for 20 min and was stopped by addition of 1 ml of 0.1 M acetic acid. Absorbance (a purple color was produced) was measured at 550 nm. A unit of peptidase activity is defined as 1 μmol of L-Leu-L-Leu hydrolyzed per min at 37°C.

Protein was determined by the method of Lowry et al. [18] using bovine serum albumin as a standard, and its absorbance was measured at 280 nm.

Disc electrophoresis in polyacrylamide gel (7.5%, pH 9.4) was carried out according to the procedure of Davis [19]. After electrophoresis, the gel was placed in 12.5% trichloroacetic acid to fix the protein, and the protein was stained with 1% Amido Black 10B. Staining for enzyme activity was also carried out, using a formazan system of the following composition: 10 mg of L-Leu-L-Leu, 10 mg of 3-(*p*-iodophenyl)-2-(*p*-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), 0.2 mg of *N*-methylphenazine methosulfate (PMS) and 2 units of L-amino acid oxidase in 10 ml of 10 mM Tris · HCl buffer (pH 8.0). The gels were placed in this staining solution and kept in the dark at the room temperature for 1 h.

SDS disc electrophoresis in polyacrylamide gel (5%) was performed by the method of Weber et al. [20].

Protease activity was determined by the method of Matsubara et al. [21] using casein as a substrate.

Results

Purification of peptidase from human liver

The crude enzyme was fractionated with ammonium sulfate. The precipitate between 40—50% of $(NH_4)_2SO_4$ saturation was collected and dialyzed against 5 l of 10 mM Tris · HCl buffer (pH 8.0) for 20 h at 4°C.

The enzyme was applied to a DEAE-celluose column (2 × 26 cm) equilibrated with 10 mM Tris · HCl buffer (pH 8.0). After the column had been washed with 300 ml of the same buffer, the peptidase was eluted with a linear concentration gradient from 0 to 0.3 M NaCl in 10 mM Tris · HCl buffer (pH 8.0), shown in Fig. 1.

The active fraction was concentrated by a membrane filter (Diafilter MC-2 Type, membrane; G-10T, Nihonshinkugijutsu Co. Ltd., Japan) and dissolved in 10 mM Tris · HCl buffer containing 0.1 M NaCl (pH 8.0), and applied to a Sephadex G-150 column (3.5 × 65 cm) equilibrated with the same buffer. The elution pattern is shown in Fig. 2. The active fraction was collected and the buffer was exchanged for 10 mM phosphate buffer (pH 6.0).

The purified fraction of the Sephadex G-150 gel filtration was passed through CM-cellulose column (2 × 16 cm) equilibrated with 10 mM phosphate buffer (pH 6.0), and the effluent was collected and used as a purified peptidase preparation. The above-mentioned purifying procedure is summarized in Table I. The final preparation was purified about 1100-fold based on crude enzyme with a recovery of 17.5%.

To prove homogeneity of the purified enzyme, the preparation was sub-

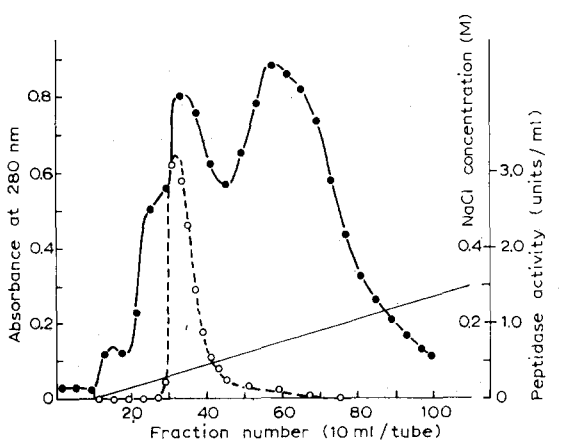


Fig. 1. Purification of human liver peptidase by DEAE-cellulose chromatography. The crude enzyme was applied to a DEAE-cellulose column (2 × 26 cm) equilibrated with 10 mM Tris · HCl buffer (pH 8.0). The column was washed with the same buffer, and then eluted with a linear concentration gradient of 0 to 0.3 M NaCl in 10 mM Tris · HCl buffer (pH 8.0) (500 ml + 500 ml). •———•, protein (A_{280}); - - - -, peptidase activity; ——— NaCl concentration.

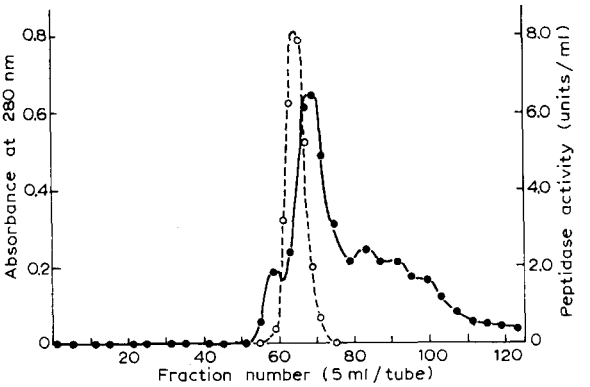


Fig. 2. Purification of human liver peptidase by Sephadex G-150 gel filtration. DEAE-cellulose fraction concentrated to 20 ml was applied to Sephadex G-150 column (3.5 × 65 cm) equilibrated with 10 mM Tris · HCl buffer (pH 8.0) containing 0.1 M NaCl. ●———●, protein (A_{280}); ○- - - - -○, peptidase activity.

mitted to disc electrophoresis (7.5% polyacrylamide gel, pH 9.4). The purified enzyme gave a single band of protein and activity on the gel as shown in Fig. 3. Electrophoresis of the enzyme under denaturing conditions on sodium dodecyl sulfate polyacrylamide gel indicated that the enzyme is a dimer consisting of two subunits of equal molecular weight, 60 000.

Fig. 4. shows the electrophoresis pattern of purified enzyme and serum peptidase on Cellogel (5 × 6 cm). The electrophoretic mobility of purified enzyme was the same as that of serum peptidase; it migrated between α_2- and β-globulin.

Optimum pH and pH stability

The optimum pH of the enzyme was pH 8.2—8.6. Examination of pH-stability of the enzyme was carried out by the following procedure. The enzyme was incubated at various pH values at 37°C for 15 min, and then the activity remaining was determined. The enzyme was stable between pH 8.0 and 10.0, and was extremely unstable below pH 6.0.

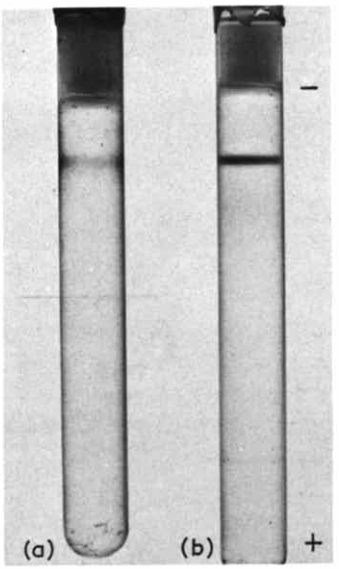
TABLE I

PURIFICATION OF PEPTIDASE FROM HUMAN LIVER

Step of purification	Activity (units) *	Protein (A_{280} nm)	Specific activity **	Yield of activity (%)
Crude enzyme	1990	35400	0.056	100
Precipitation with $(NH_4)_2SO_4$ (0.4—0.5 saturation)	515	4030	0.128	25.9
DEAE-cellulose chromatography	401	289	1.39	20.2
Sephadex G-150 gel filtration	224	33.5	6.67	11.3
CM-cellulose chromatography	346	5.48	63.3	17.5

* Hydrolyzed L-Leu-L-Leu μmol/min.

** Hydrolyzed L-Leu-L-Leu μmol/min per unit A_{280nm}.



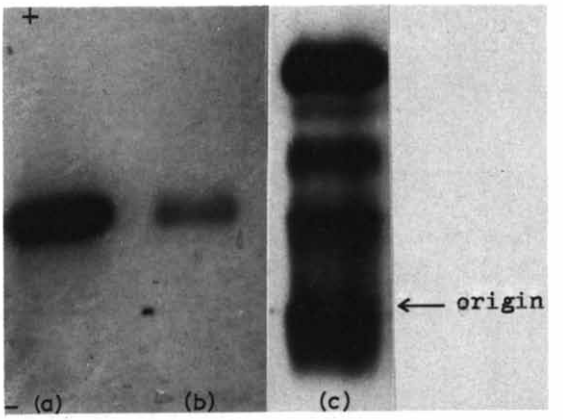


Fig. 3. Disc gel electrophoresis of peptidase purified from human liver. Conditions: pH 9.4, 3 mA/tube, 90 min, 4°C. Gel (b) was stained with Amido Black 10B for protein, gel (a) was stained for activity with L-Leu-L-Leu as substrate.

Fig. 4. Electrophoretic patterns of human liver peptidase and serum peptidase on Cellogel. Conditions: pH 8.6, 0.8 mA/cm, 60 min, 4°C. (a) Peptidase purified from human liver, (b) peptidase in serum of patient with acute hepatitis. (a) and (b) were stained for activity with L-Leu-L-Leu as a substrate, (c) was stained for serum protein with Amido Black 10B.

Thermal stability and optimum temperature

The enzyme was treated in 10 mM Tris · HCl buffer (pH 8.0) for 15 min at the temperature indicated, and the remaining activity was determined. The enzyme activity decreased at 40° C and above, but the enzyme was stable below 40° C. It was found that the optimum temperature of the enzyme was 45° C.

Molecular weight

The molecular weight of the enzyme was determined by Sephadex G-200 gel filtration. The molecular weight of the enzyme was estimated to be 130 000.

Isoelectric point

The isoelectric point of the enzyme was found to be pH 5.6 by isoelectric focusing with 1% ampholyte (pH 3.0—10.0), as shown in Fig. 5. The result indicated that there was no heterogeneity in the enzyme.

K_m value

The K_m value of the enzyme was investigated by the standard assay system and was estimated to be 0.29 mM for L-Leu-L-Leu.

Effect of metal ions and reagents on the peptidase

Table II shows the effects of metal ions and reagents on the enzyme. The purified enzyme was activated by 10^{-3} M Mn^{2+} and inhibited by 10^{-3} M Hg^{2+}.

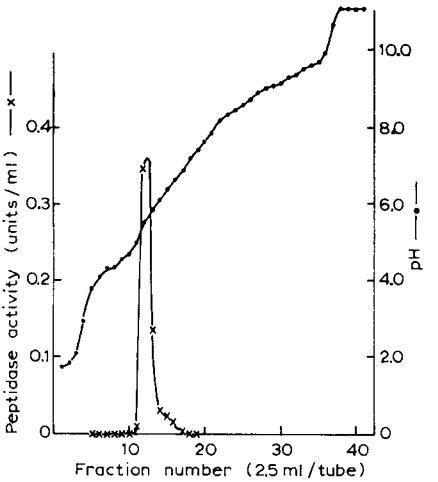


Fig. 5. Isoelectric focusing of human liver peptidase. The purified enzyme (6.12 units) was applied to an isoelectric focusing column (110 ml) with 1% carrier ampholyte (pH 3.0—10.0).

The other metal ions except Mn^{2+} and Hg^{2+} did not affect the enzyme activity. Chelators such as *o*-phenanthroline and cysteine, oxidizers such as *N*-bromosuccinimide and iodine also effectively inhibited the enzyme activity.

Specificity of the peptidase

Specificity for substrate was investigated and the result is shown in Table III. The amount of hydrolytic activity of this enzyme towards L-Phe-L-Phe was similar to that for L-Leu-L-Leu, but L-Leu-Gly-Gly, L-Phe-L-Tyr and L-Leu-Gly were hydrolyzed slightly by the enzyme. Casein was not hydrolyzed by the enzyme. The enzyme has a high substrate specificity for L-Leu-L-

TABLE II

EFFECT OF VARIOUS METAL ION AND REAGENTS ON ACTIVITY OF HUMAN LIVER PEPTIDASE

The enzyme was incubated with the metal ions and reagnets in 10 mM Tris · HCl buffer (pH 8.0) at 37°C for 30 min. 1 mM metal ions and reagents were used in the enzyme system except for *p*-chloromercuribenzoate, 0.1 mM. After the incubation mixture had been diluted 1000-fold with 10 mM Tris · HCl buffer (pH 8.0), the remaining activity was assayed.

Metal ion	Remaining activity (%)	Reagent	Remaining activity (%)
None	100		
NaCl	105	*o*-Phenanthroline	26
$CaCl_2$	85	EDTA	81
$MgCl_2$	96	Diisopropyl fluorophosphate	83
$ZnSO_4$	100	Monoiodoacetate	104
$CdSO_4$	74	*p*-Chloromercuribenzoate	99
$CuCl_2$	86	*N*-Bromosuccinimide	8
$NiCl_2$	87	Iodine	3
$MnCl_2$	210	Cysteine	26
$CoCl_2$	86	2-Mercaptoethanol	89
$FeCl_3$	81		
$HgCl_2$	52		

TABLE III

SUBSTRATE SPECIFICITY OF HUMAN LIVER PEPTIDASE

The values were calculated as percentage of L-Leu-L-Leu hydrolysis.

Substrate	Concentration of substrate (mM)	Relative activity (%)
L-Leu-L-Leu	1.50	100
L-Phe-L-Phe	0.11	99.4
L-Phe-L-Tyr	1.50	21.1
L-Leu-Gly-Gly	1.50	15.8
L-Leu-Gly	1.50	8.6
L-Leu-β-naphthylamide	0.50	0.2
L-Leu-NH_2	2.00	0
Gly-L-Leu	1.50	0
L-Phe-L-Pro	1.50	0
Hippuryl-L-Phe	1.50	0
L-Cys-di-β-naphthylamide	0.67	0
Casein	1.67 (%)	0

Leu barely hydrolyzed L-Leu-Gly, L-Leu-Gly-Gly, L-Leu-NH_2 and L-Leu-β-naphthylamide, which were used as substrates of leucine aminopeptidase. From this result, it is suggested that the peptidase is different from leucine aminopeptidase.

Discussion

The peptidase which had a high specificity for L-Leu-L-Leu was purified homogeneously from human liver by following procedure; treatment with *n*-butanol, acetone precipitation, ammonium sulfate fractionation, DEAE-cellulose chromatography, Sephadex G-150 gel filtration and CM-cellulose chromatography. It seemed that the treatment with *n*-butanol was necessary for separation of the enzyme protein from lipid material and solubilization of the enzyme. The result agrees with the report of Campbell et al., which describes the purification of peptidase from hog kidney [22].

Although it is recognized generally that peptidases in several mammalian tissues exist as multiple forms [5,22], there is no multiple form in this peptidase from human liver as the result of isoelectric focusing and disc electrophoresis experiment.

A number of peptidase are known to be metalloenzymes containing zinc [8,22,24]. Since this peptidase from human liver was activated by Mn^{2+} and inhibited by chelators such as *o*-phenanthroline, EDTA and cysteine, it is suggested that the peptidase is probably a metalloenzyme. Garner et al. [24] reported that human liver aminopeptidase was activated by Co^{2+}, but this peptidase was not activated by Co^{2+}. This permits the enzymes to be distinguished from each other.

From the elevation of the enzyme activity in serum involved with hepatic disease and the same electrophoretic mobility of the enzyme purified from human liver, and peptidase in serum of patients with hepatic diseaes, it can be suggested that this enzyme may leak from liver tissue to serum.

Considering these results, we should go on working to clarify the relationship between hepatic disease and peptidase.

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