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Purification and Properties of Leucine Aminopeptidase from *Aspegillus japonica*. II[1a-c]

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Leucine aminopeptidase was purified from culture filtrate of *Aspergillus japonica* by calcium acetate treatment, ammonium sulfate fractionation, and column chromatography with diethylaminoethyl (DEAE)-cellulose, Sephadex G-100 and CM-Sephadex C-50. The purified enzyme was homogeneous in disc electrophoretic analysis. Its molecular weight was estimated to be 57000 by Sephadex G-75 gel-filtration.

The enzyme was most active at pH 8.0 towards L-leucylglycylglycine (Leu-Gly-Gly) and L-leucyl β-naphthylamide (Leu-β-NA), and its optimum temperature was 50°. The enzyme was stable in a pH range of 5.5 to 8.5 and below 50°. The purified enzyme was highly activated by Co^{2+} and was strongly inhibited by Fe^{3+}, Hg^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+}, ethylenediaminetetraaceticacid (EDTA), *o*-phenanthroline and N-bromosuccinimide. However, it was not affected by SH-reagents and diisopropyl fluorophosphate (DFP). The enzyme is considered to be leucine aminopeptidase, because it preferentially hydrolyzed di- or tripeptides with N-terminal leucine.

Aminopeptidases are widely distributed in plants, microorganisms and animals, and their properties are varied among the enzyme sources.[3] Leucine aminopeptidase from swine kidney was purified to homogeneous state[4] and the enzyme was a metal enzyme containing Zn which was activated by Mn^{2+} and Mg^{2+}.[5,6] The enzyme has been used for the determination of sequence of peptides, because it hydrolyzed not only peptides with N-terminal leucine but also peptides with the other amino acid as N-terminal amino acid at a slow rate.[7] As regard to aminopeptidases from microorganisms, the enzymes from *Bacillus subtilis*,[8] *B. stearothermophiles*,[9] *Aeromonas proteolytica*[10] and *Aspergilli*[11-14] were purified and characterized. Among aminopeptidases from *Aspergilli*, Lehmann and Uhlig[11] have reported on the purification and properties of leucine aminopeptidases from *Asp. parastica* and *Asp. oryzae*. Nakadai, *et al.*[12,13] has shown that exopeptidases from *Asp. oryzae* or *Asp. sojae* played an important role in digestion of soybean protein to yield free amino acids and also studied on the purification and properties of leucine aminopeptidase I, II, and III from *Asp. oryzae*.[14]

To utilize exopeptidase as digestive enzyme preparation, the authors have searched for exopeptidase from a number of microorganisms and have found that *Asp. japonica* produces

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1) *a*) A part of this report was presented at the 94th annual Meeting of the Pharmaceutical Society of Japan, Sendai, April, 1974; *b*) Part I: M. Sugiura, M. Suzuki, M. Ishikawa, and M. Sasaki, *Chem. Pharm. Bul.* (Tokyo), "accepted"; *c*) This work forms part CXIX of a series entitled "Studies on Enzymes" by M. Sugiura.
2) Location: *1432-1, Horinouchi, Hachioji-shi, Tokyo, 192-03, Japan.*

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a potent aminopeptidase. In the previous paper, we have examined the properties and stability against various pharmaceutical conditions.[1b] In this report, we describe the purification and properties of leucine aminopeptidase from *Asp. japonica*.

Materials and Methods

Crude Enzyme Prepararion——The crude enzyme preparation was a ethanol precipitate of the culture filtrate of *Asp. japonica*.[1b]

Reagents——Diethylaminoethyl (DEAE)-cellulose was a product of Brown, New Hampshire, U.S.A., Sephadex G-50 and G-100, and CM-Sephadex C-50 were obtained from Pharmacia Fine Chemicals, Sweden. L-Leucyl β-naphthylamide (leu-β-NA) was purchased from Wako Pure Chemical Ind., Osaka. Synthetic oligopeptides were from Protein Research Foundation, Osaka, Cyclo Chemical, Calif., U.S.A., and Mann Research Lab., New York, U.S.A. Diisopropyl fluorophosphate (DFP) was a product obtained from Sigma Chemical Co., Mo., U.S.A. Bacterial trypsin inhibitor (Amano Pharm. Co., Nagoya), soybean trypsin inhibitor (Worthington Biochem. Co., New Jersey, U.S.A.), semi-alkaline protease (Amano Pharm. Co., Nagoya), egg albumin (Nutritional Biochem. Co., Ohio, U.S.A.) and human serum albumin (Fr. V, Wako Pure Chemical Ind., Osaka) were used as standards for the determination of the molecular weight of the leucine aminopeptidase. The other chemicals used were of special grade.

Enzyme Assay——a) Activity towards Leu-β-NA: The activity was measured by the method as described in the previous paper.[1b]

b) Activity towards Synthetic Peptide: To 4 ml of the enzyme solution (5 μg/ml) bufferized to pH 8.0 with 0.1 M veronal buffer containing 1 mM $CoCl_2$, was added 1 ml of 1 mM L-leucylglycylglycine (Leu-Gly-Gly) or the other synthetic peptides. After incubation at 37° for 30 min, the amino acid liberated was measured by the ninhydrin methods.[15] One unit of enzyme activity was defined as the enzyme quantity which liberates 1 μmole of β-naphthylamine or amino acid from the substrate per minute under above conditions.

Determination of Protein——Protein concentration was determined by measuring the absorbance at 280 nm or by the method according to Lowry[16] using bovine serum albumin as standard.

Disc Electrophoresis——Disc electrophoresis was performed by the procedure of Reisfeld.[17] Protein was stained with Amino Schwarz 10B and activity was detected with coupling β-naphthylamine liberated from Leu-β-NA to Fast Garnet GBC (Sigma Chemical Co. Mo., U.S.A.).[18] Sugar was stained with fuchsin-bisulfite after periodic acid oxidation (PAS).[19]

Thin-Layer Chromatography (TLC)——Amino acids and peptides produced from synthetic peptides in reaction mixture was 2,4-dinitrophenylated by the method of Sanger.[20] The dinitrophenylates was identified by TLC on Kieselgel G(E. Merck AG. Germany) of 0.5 mm thickness. 20 μl aliquots of the dinitrophenylates ethylacetate solution was spotted on the thin layer and developed at 25° for 3 hr by ascending method using a mixture of toluene, pyridine, 2-chloroethanol and 0.8 N ammonia water (10:3:6:6, v/v) as solvent.

Results

Purification of Leucine Aminopeptidase

Two g of crude enzyme powder were dissolved in 40 ml of 0.01M phosphate buffer (pH 8.0) and then the solution was centrifuged at 10000 rpm for 10 min at 0°. To the clear supernatant, was added 0.8 g of calcium acetate and after stirring for 1 hr at 0°, the resultant precipitate was removed by centrifugation at 10000 rpm for 10 min at 0°. To the supernatant, was added 25 g of ammonium sulfate and stirred overnight at 4°. The precipitate formed was collected by centrifugation and dissolved in a small volume of 0.01M phosphate buffer (pH 8.0). The solution was desalted with gel-filtration on Sephadex G-50 column (3×70 cm). The desalted enzyme solution was applied to DEAE-cellulose column (3×28 cm) equilibrated with 0.01M phosphate buffer (pH 8.0) and eluted with the same buffer. The enzyme was unadsorbed to the column, but the pigment was adsorbed and was not eluted with the buffer. The active fractions were concentrated with an ultra-filtration and was subjected to gel-filtration

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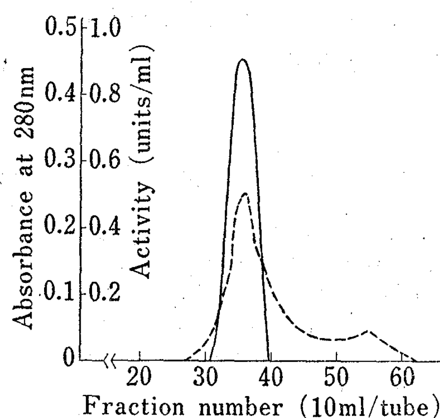

Fig. 1. Gel Filtration of Leucine Aminopeptidase on Sephadex G-100

Active fractions from DEAE-cellulose column chromatography was applied to a column (3 × 70 cm) of Sephadex G-100 equilibrated with 0.01 M phosphate buffer (pH 8.0) and eluted with the same buffer at 4°.
----: absorbance at 280 nm; ——, activity

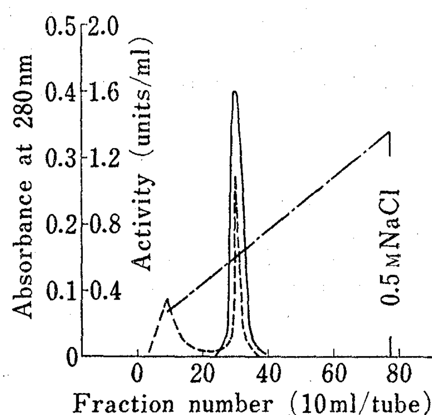

Fig. 2. Chromatography of Leucine Aminopeptidase on CM-Sephadex C-50

Gel filtrated enzyme was adsorbed on a column (3 × 15 cm) of CM-Sephadex C-50 equilibrated with 0.01M acetate buffer (pH 5.0) containing 0.1M NaCl and the elution was performed with a linear gradient of NaCl (0—0.5M) at 4°.
------: absorbance at 280 nm
——: activity
-·-·-·-: concentration of NaCl with linear gradient elution

TABLE I. Purification of Leucine Aminopeptidase from *Aspergillus japonica*

Step of purification	Activity (units)	Protein (mg)	Specific activity (units/mg)	Yield of activitiy (%)
Crude enzyme	274	864	0.31	100
$Ca(OAc)_2$ treatment	244	750	0.32	89
Sephadex G-50 after $(NH_4)_2SO_4$ fractionation	142	150	0.96	52
DEAE-cellulose	70	17	4.08	26
Sephadex G-100	44	9.3	4.08	16
CM-Sephadex C-50	29	5.2	5.42	11

Peptidase activity was determined with L-leucyl β-naphthylamide.

on Sephadex G-100 column equilibrated with 0.01M phosphate buffer (pH 8.0). The result is shown in Fig. 1. The active fractions were concentrated and adsorbed to CM-Sephadex C-50 column equilibrated with 0.01M acetate buffer (pH 5.0) containing 0.1M NaCl and eluted with linear gradient of NaCl (0.1—0.5M). In Fig. 2, the enzyme was eluted with 0.15M NaCl. The active fractions were dialyzed against water for 20 hr and lyophylized. The results of purification are summarized in Table I. Leucine aminopeptidase was purified 17.5 fold and the yield of activity of the purified enzyme was 11%.

Homogeneity

The homogeneity of the purified enzyme was examined with disc electrophoresis. A homogeneous single band for protein or activity is shown in Fig. 3, where the mobility of protein band coincided with that of the activity. To know whether the enzyme is a glycoprotein or not, the gel was subjected to PAS stain, but the enzyme protein was not stained suggesting that the enzyme does not contain sugar.

Molecular Weight

The molecular weight of the purified enzyme was determined by gel-filtration on Sephadex G-75. Relation between Kav, calculated by the formula defined by Gelotte,[21] and logarithm

21) B. Gelotte, *J. Chromatog.*, 3, 330 (1960).

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of molecular weight is presented in Fig. 4. The molecular weight of the enzyme was estimated to be 57000.

Substrate Specificity

The hydrolytic action of the leucine aminopeptidase on various peptides was examined by the ninhydrin method (enzyme assay(b)). The result is shown in Table II. The enzyme

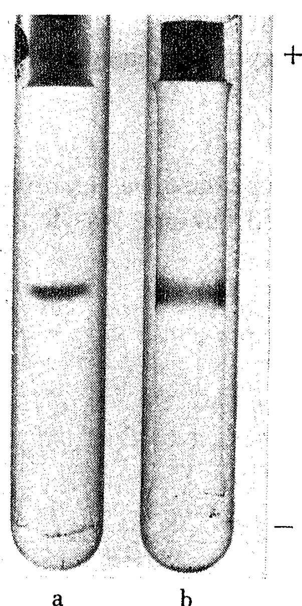

Fig. 3. Disc Electrophoretic Pattern of Purified Leucine Aminopeptidase

Conditions: pH 4.0, 3 mA/tube, 100 min. 4°
a : stained for protein
b : stained for activity

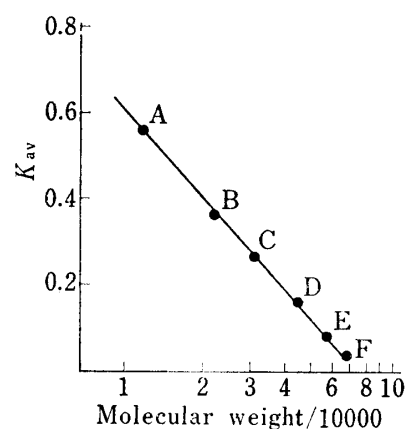

Fig. 4. Plots of Kav against Logarithms of Molecular Weight for Proteins on a Sephadex G-75 Column (2×90 cm)

Each protein sample (20 mg) dissolved in 2 ml of Michaelis veronal buffer (pH 8.0) containing 1 mM $CoCl_2$, was applied to the column and eluted with the same buffer at the flow rate of 15 ml per hr and 3 ml each of fractions was collected. The void volume for the given column was determined from the elution volume of blue dextran. A, bacterial trypsin inhibitor; B, soybean trypsin inhibitor; C, semi-alkaline proteinase; D, egg albumin; E, leucine aminopeptidase; F, bovine serum albumin.

TABLE II. Action of Leucine Aminopeptidase on Various Synthetic Substrate

Peptide	OD 570 nm	Peptide	OD 570 nm
Gly-Gly	0.01	Leu-NH_2	1.15
Gly-Leu	0.01	N-Ac-Leu	0
Gly-Phe	0.01	Leu-Gly	0.22
Gly-Pro	0.01	Leu-Leu	0.35
Gly-Val	0.01	Leu-Gly-Gly	0.70
Gly-Asp	0.01	Gly-Gly-Gly	0.01
Gly-Glu	0.02	Ala-Gly-Gly	0.01
Ala-Glu	0.03	Lys-Gly-Pro	0
Lys-Glu	0.01	Tyr-Gly-Gly	0.02
Phe-Glu	0.01	Val-Gly-Gly	0.01
Glu-Gly	0.01	Cbz-Leu-Gly	0
Glu-Asp	0.01	Cbz-Glu-Tyr	0
Glu-Pro	0.01	Cbz-Glu-Gly	0
Glu-Tyr	0.01	Cbz-Gly-Leu	0
Tyr-Glu	0	Cbz-Glu-Pro	0
Arg-Glu	0.02	Bz-Arg-NH_2	0.01
Met-Glu	0.02	BAEE	0
Val-Leu	0.01	TAME	0
His-Leu	0.01		
Met-Leu	0.02		

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preferentially hydrolyzed oligopeptides with N-terminal leucine, liberating the leucine. The enzyme showed neither activity towards peptides in which N-terminal amino group was blocked, nor esterase activity.

Hydrolysis of Leu-Gly-Gly

To 20 ml of enzyme solution (0.1 mg/ml) dissolved in 0.1M veronal buffer (pH 8.0) containing 1 mM $CoCl_2$, was added 5 ml of 10 mM Leu–Gly–Gly and incubated at 37°. The reaction mixture was 2,4-dinitrophenylated and identified by TLC. As shown in Fig. 5, The enzyme hydrolyzed the tripeptide, only liberating amino terminal leucine. From the results of substrate specificity and hydrolysis of Leu–Gly–Gly, the enzyme is considered to be a leucine aminopeptidase.

Optimum pH, Temperature and Stability

The effect of pH on the activity of the leucine aminopeptidase was examined with Leu–β-NA and Leu–Gly–Gly. As shown in Fig. 6a, the purified enzyme was found to be most

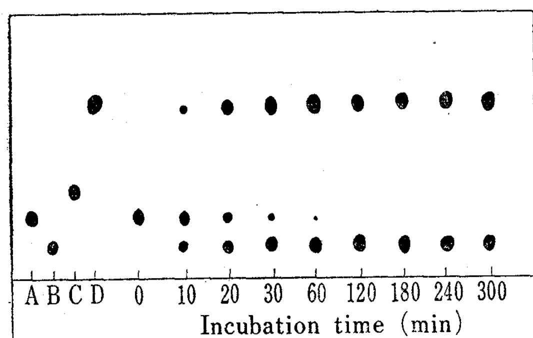

Fig. 5. Thin-Layer Chromatography of Dinitrophenylate of Leucyl glycylglycine Hyarolyzate

A, leucylglycylglycine; B, glycylglycine; C, glycine; D, leucine

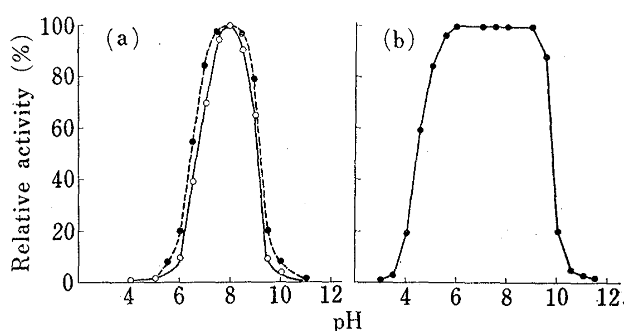

Fig. 6. Effect of pH on Activity (a) and Stability (b) of Leucine Aminopeptidase

(a) the reaction was carried out at various pH (Britton-Robinson buffer) in a usual manner.
(b) the enzyme was incubated at various pH (Britton-Robinson buffer) and 37° for 15 min and then the solution was diluted 10-fold with 0.2M Tris-HCl buffer (pH 8.0) and the remaining activity was determined by the usual method.
(a) —○—: leucyl β-naphthylamide
---●---: leucylglycylglycine

TABLE III. Effect of Various Metal Ions and Reagents on Activity of Leucine Aminopeptidase from *Aspergillus japonica*

Metal salt	Remaining activity (%)	Reagent	Remaining activity (%)
None	100	EDTA	9.1
NaCl	89	*o*-phenanthroline	3.5
$CaCl_2$	109	α,α-dipyridyl	4.5
$MgCl_2$	105	monoiodoacetic acid	88
$ZnSO_4$	72	*p*-chloromercuribezoate	85
$Pb(OAc)_2$	23	diisopropyl fluorophosphate	89
$CdCl_2$	74	N-bromosuccinimide	5.7
$CuSO_4$	38	ascorbic acid	78
$NiCl_2$	38	H_2O_2	88
$MnSO_4$	57	iodine	81
$CoCl_2$	200		
$FeCl_3$	17	cysteine	89
$HgCl_2$	32	2-mercaptoethanol	92
$AgNO_3$	81	thioglycolic acid	96

The enzyme was incubated with an equal volume of metal salt or reagent solution (2 mM, pH 8.0) at 37° for 30 min. The mixture was diluted 50—fold with cooled water and the remaining activity was determined under standard conditions.

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active at pH 8.0. pH-Stability of the enzyme was investigated at 37° for 15 min. The result is shown in Fig. 6b. The enzyme was stable in a pH range of 5.5 to 8.5. Optimum temperature of the enzyme was determined at pH 8.0 for 30 min using Leu–β–NA. The enzyme was most active at 50°. The thermal stability of the enzyme was examined at pH 8.0 for 15 min. The enzyme was stable up to 50°, but was completely inactivated at 60°.

Effect of Some Metal Ions and Reagents on the Enzyme

Effect of some metal ions and reagents on the activity of the enzyme is shown in Table III. The enzyme was activated 2-fold by Co^{2+}, and also slightly activated by Ca^{2+} and Mg^{2+}, but was strongly inhibited by Hg^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Fe^{3+}. Among various reagents, the enzyme was strongly inhibited by chelators such as ethylenediaminetetraacetic acid (EDTA), α,α'-dipyridyl and *o*-phenanthroline and N-bromosuccinimide, but was not affected by *p*-chloromercuribenzoate, monoiodoacetic acid and DFP.

Discussion

Previously, the authors have reported the utility of the aminopeptidase from *Asp. japonica* as a digestive enzyme preparation.[1b] To clarify the properties of the enzyme, the purification and characterization of the enzyme was dealt with in this paper. The enzyme was purified to homogeneous state from the culture filtrate of *Asp. japonica*, by calcium acetate treatment, ammonium sulfate fractionation, and column chromatography with DEAE-cellulose, Sephadex G-100 and CM-Sephadex C-50. The molecular weight of the enzyme was estimated to be 57000. This was closely similar to those of leucine aminopeptidases II and III from *Asp. sojae*[12] and *Asp. oryzae*[14] and the enzyme from *Asp. oryzae*,[8] having a mol. wt. of 57000—63000, but differed from mol. wt. of leucine aminopeptidase I from *Asp. sojae*[12] and *Asp. oryzae*[14] and the enzyme from *Asp. parastica*,[11] having a mol. wt. of 26000—32000. On the other hand, the molecular weights of leucine aminopeptidases from swine kidney,[4] *B. stearothermophiles*,[9] bovine lens[22] and *E. coli*[23] were estimated to be 3×10^5—4×10^5.

The optimum pH of the purified enzyme, showing 8.0, was similar to that of leucine aminopeptidases from *Asp. sojae* and *Asp. oryzae*. The enzyme is considered to be a metal enzyme, because the enzyme was activated by Co^{2+} and inactivated by chelators, whereas it was not affected by SH-reagents and DFP. The activation by Co^{2+} was also shown with leucine aminopeptidases from *Asp. oryzae*, *Asp. sojae*, *Asp. parastica* and *B. Stearothermophiles*. However, the enzymes from swine kidney, bovine lens, brain[24] and *Aeromonas proteolytica* were stimulated by Mn^{2+} and these enzymes contain Zn in the molecules.

The purified enzyme preferably hydrolyzed peptides with N-terminal leucine, and hydrolyzed Leu–Gly–Gly, liberating only N-terminal leucine. The results suggest that the enzyme has relatively high specificity towards peptides containing leucine as N-terminal amino acid. Leucine aminopeptidases I, II and III from *Asp. oryzae* also showed high specificity towards substrates containing leucine as N-terminal amino acid, but the enzymes showed fairly high activity towards the substrates which were lack of N-terminal leucine. From these results, it is considered that the enzyme from *Asp. japonica* is a leucine aminopeptidase generally similar to the enzymes from *Asp. oryzae*.

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