Purification and Properties of Leucine Aminopeptidase II from *Aspergillus oryzae*

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Aminopeptidase II from *Aspergillus oryzae* 460 was purified from the rivanol precipitable fraction. The purified enzyme was homogeneous in polyacrylamide gel disc electrophoresis. Its optimum pH is at pH 8.0 for L-leucyl-glycyl-glycine and L-leucyl- β -naphthylamide. The enzyme was inhibited by metal chelating agents and S-S dissociating agents, but not inhibited by SH reagents. The molecular weight of the enzyme was estimated to be about 61,000 by gel filtration. The enzyme was most active toward oligopeptides of amino terminal leucine, indicating that it was a kind of leucine aminopeptidase. Thus, the enzyme was proposed to be called leucine aminopeptidase II of *Asp. oryzae* 460.

Of the proteolytic enzymes of *Aspergillus oryzae*¹⁾ and *Asp. sojae*²⁾ exopeptidases were found to be main enzymes to liberate free amino acids from soybean protein and some enzymatic properties of purified acid carboxypeptidase I,³⁾ II,⁴⁾ III⁵⁾ and leucine aminopeptidase I⁶⁾ were reported in a series of previous papers.

This paper deals with purification and some properties of leucine aminopeptidase II as a part of the systematic purification of peptidases from *Asp. oryzae* 460 (FERM-P No. 1149).⁷⁾

MATERIALS AND METHODS

Aminopeptidase activity was determined with Leu-Gly-Gly by the same method as reported in the previous papers.^{3,4)} In some cases Leu- β -naphthylamide was used as the substrate and activity was determined by the same method as reported in previous papers.^{6,8)} All the other experimental methods were described in the previous paper.³⁾

RESULTS

Gel filtration on Sephadex G-100

The fractions of aminopeptidase II from DEAE-cellulose chromatography reported previously^{*6)} were concentrated to 3 ml by a

Diaflo UM-1 (Amicon Co.) and applied to a column (2.3 $\times$ 150 cm) of Sephadex G-100 equilibrated with 0.1 M acetate buffer at pH 6.5. Elution was performed with the same buffer and 15 ml each of fractions was collected. Active fractions of aminopeptidase were combined, pooled and concentrated to 3 ml by a Diaflo UM-1.

Gel filtration on Sephadex G-200

The concentrated solution was applied to a column (2.8 $\times$ 118 cm) of Sephadex G-200 equilibrated with 0.1 M acetate buffer at pH 6.5. Elution was performed with the same buffer and 15 ml each of fractions was collected. Active fractions (aminopeptidase II) were collected and desalted by dialysis against distilled water.

Column chromatography on DEAE-Sephadex A-50

The above enzyme preparation of aminopeptidase II after dialysis was adjusted to pH 7.0 and charged on a column (3 $\times$ 30 cm) of DEAE-Sephadex A-50 equilibrated with 0.01 M phosphate buffer at pH 6.0. After being washed with 0.01 M phosphate buffer (pH 7.0), the adsorbed protein was eluted by a linear gradient of NaCl concentration. A reservoir was filled with 2 liters of 0.4 M NaCl in the

* See the previous paper⁶⁾ for abbreviations used in this paper.

FIG. 1. Chromatography of Aminopeptidase II on DEAE-Sephadex A-50.

See the text for experimental details.

○—○: peptidase activity for Leu-Gly-Gly. △—△, peptidase activity for Cbz-Glu-Tyr, units/ml $\times 10^{-2}$; ●—●, O.D. at 280 $m\mu$; x—x, NaCl concentration.

same buffer and a mixing chamber contained 2 liters of the same buffer. Figure 1 illustrates an elution pattern of aminopeptidase II. Fractions 172 to 200 were collected, adjusted to pH 5.5 and tentatively named acid carboxypeptidase IV. Fractions 214 to 236 were combined and used for the preparation of purified aminopeptidase II.

Gel filtration on Sephadex G-200

Aminopeptidase II fraction obtained by DEAE-Sephadex A-50 chromatography was concentrated to 3 ml by a Diaflo UM-1 and applied to a column (2.8×118 cm) of Sephadex G-200 equilibrated with 0.1 M acetate buffer at pH 6.5. Figure 2 shows a typical elution pattern of aminopeptidase II. Pooled fractions of 27 to 38 were frozen and stored at

FIG. 2. Gel Filtration of Aminopeptidase II through Sephadex G-200.

See the text for experimental details.

○—○, peptidase activity for Leu-Gly-Gly; ●—●, O.D. at 280 $m\mu$.

—15°C.

A summary of the purification procedure is shown in Table I.

Determination of molecular weight by gel filtration

TABLE I. PURIFICATION OF LEUCINE AMINOPEPTIDASE II

Procedure	Total volume ml	Total activity Units ^{a)}	Specific activity U/mg protein ^{a)}	Yield %
Crude enzyme solution	10,100	5910	0.181	100
Batchwise treatment with Amberlite IRC-50	10,200	5740	0.490	97.3
Fractionation with ammonium sulfate	235	3280	0.545	55.5
Rivanol precipitation	104	2190	1.44	38.2
DEAE-cellulose chromatography	810	1870	2.72	31.7
Gel filtration on Sephadex G-100	66	974	3.24	16.5
Gel filtration on Sephadex G-200	220	587	3.02	9.94
DEAE-Sephadex A-50 chromatography	330	468	4.70	7.93
Gel filtration on Sephadex G-200	166	262	4.54	4.43

^{a)} Peptidase activity was determined with Leu-Gly-Gly.

TABLE II. ACTION OF LEUCINE AMINOPEPTIDASE II ON VARIOUS SYNTHETIC PEPTIDES

Peptide	Δ O.D.	Peptide	Δ O.D.	Peptide	Δ O.D.	Peptide	Δ O.D.
Gly-Gly	0.040	Glu-Pro	0	Leu-Gly-Glu	1.263	Cbz-Gln-Pro	0
Gly-Leu	0.138	Glu-Tyr	0.470	Leu-Gly-Gln	2.087	Cbz-Gln-Gly	0
Leu-Gly	0.912	Glu-Ser	0.109	Leu-Gly-Asp	1.138	Aoc-Gln-Pro	0
Gly-Phe	0.100	Glu-Anilide	0.020	Leu-Gly-Asn	2.616	Bz-Gly-Lys	0
Gly-Glu	0.066	Try-Glu	0.025	Glu-Ala-Gly	0.745	Cbz-Gly-Gly-Phe	0
Gly-Asp	0.026	Arg-Glu	0.159	Phe-Gln-Gly	0.963	Bz-Arg-NH ₂	0.014
Gly-Gln	0.072	Met-Glu	0.994	γ -Glu-Cys-Gly H	0	Cbz-Pro-Leu-NH ₂	0
Gly-Asn	0.047	Glu-Try	0.704	PyroGlu-Ala	0.015	Cbz-Gly-Ala-NH ₂	0
Pro-Gln	0.305	Glu-Glu	0.136	PyroGlu-Gln-Ala	0.028	Cbz-Gly-Phe-NH ₂	0.014
Tyr-Glu	0.034	Glu-Lys	0.132	Cbz-Ala-Glu	0.009	Cbz-Gly-Leu-NH ₂	0
Tyr-Gln	0.594	Glu-Tyr-Glu	1.122	Cbz-Gly-Glu	0.020	Cbz-Ala-Glu-Dibenzylester	0
His-Glu	0.124	Glu-Try-Glu	0.334	DiCbz-Lys-Glu	0	Leu- β -NA	5.326 ^{a1}
Ala-Glu	0.256	Glu-Val-Phe	0.384	Cbz-Glu-Tyr	0.021	PyroGlu- β -NA	0.007 ^{a1}
Lys-Glu	0.153	Glu-Glu-Glu	0.408	Cbz-Glu-Gly	0	Glu- β -NA	0.216 ^{a1}
Phe-Glu	0.170	Gly-Gly-Gly	0.159	Cbz-Glu-Val	0.008	Poly-Glu (Approx. mol. wt. 72,000)	0.022
Glu-Ala	0.360	D,L-Ala-Gly-Gly	0.600	Cbz-Gly-Phe	0	Poly-Lys (Approx. mol. wt. 2600)	0.380
Glu-Val	0.421	Leu-Gly-Gly	1.346	Cbz-Gly-Try	0	Poly-Pro (Approx. mol. wt. 3000)	0.043
Glu-Gly	0.155	Gly-Gly-Glu	0.266	Cbz-Gly-Leu	0	Ileu-Gln-Asn-Cys-Pro-Leu-Gly-NH ₂ H	3.963
Gln-Gly	0.583	Glu-Gly-Phe	0.173	Cbz-Glu-Pro	0.015		
Glu-Asp	0.156	Glu-Ala-Ala	0.414				

^{a1} Δ O.D. at 535 m μ measured with the reagent for serum leucine aminopeptidase assay (Iatron Co.)²⁷¹

FIG. 3. Plots of Elution Volume, V_e , on a Sephadex G-200 Column against Logarithms of Molecular Weights of Proteins.

See the previous paper³¹ for experimental details. A, trypsin; B, ovalbumin; C, human albumin; D, intestinal alkaline phosphatase; E, human albumin dimer; F, blue dextran; G, aminopeptidase II.

FIG. 4. Polyacrylamide Gel Electrophoresis of Purified Aminopeptidase II from *Aspergillus oryzae* at pH 9.4 for 30 min.

The electrophoresis was operated in the cold (4°C) for 30 min at 4 mA per gel column.²⁸⁾

FIG. 5. Time Course of Hydrolysis of Leu-Gly-Gly by Aminopeptidase II.

Peptidase activity was assayed at various time at 30°C with purified enzyme (3.48 μ g). 0.05 M Tris-HCl buffer (pH 8.0) was used.

Figure 3 shows the plots of elution volume (V_e) through a Sephadex G-200 (pH 5.5) column against logarithms of molecular weights of standard proteins and aminopeptidase II. The molecular weight of aminopeptidase II

determined by this method was calculated to be 61,000.

Disc electrophoresis

To check the purity of the enzyme, the final enzyme preparation was submitted to polyacrylamide gel disc electrophoresis at pH 9.4. A single protein band corresponding to aminopeptidase II was found as shown in Fig. 4.

Action on various peptides

The hydrolytic action of the aminopeptidase II on various peptides was examined by the ninhydrin method. To 0.5 ml of enzyme solution (348 μ g protein/ml) buffered to pH 6.5 with 0.1 M acetate buffer was added 0.5 ml each of 5×10^{-4} M solution for di- and tripeptides and each 10^{-3} M solution for other peptides. After incubation at 30°C for 30 min in the presence of 10^{-3} M CoSO_4 , the amino acid liberated was measured by ninhydrin method. The results are summarized in Table II. The aminopeptidase II preferentially hydrolyzed the oligopeptides that possess leucine at the N-terminal, liberating amino terminal amino acid. Thus, we propose to call this enzyme leucine aminopeptidase II.

Time course of hydrolysis of Leu-Gly-Gly

The time course of hydrolysis of Leu-Gly-Gly by the leucine aminopeptidase II gave straight line to O.D. 0.4 (Fig. 5).

Effect of pH on leucine aminopeptidase II

The effect of pH on leucine aminopeptidase II activity was studied with some peptides in acetate (pH 3.0 to 6.0), tris-HCl (pH 6.5 to 9.0) and phosphate (pH 6.0 to 8.0) buffers. Purified leucine aminopeptidase II was most active at pH 8.0 toward both Leu-Gly-Gly and Leu- β -naphthylamide, at pH 5.0 for α -Glu-Tyr-Glu and at pH 5.5 for Met-Glu (Fig. 6) in the presence of 10^{-3} M CoSO_4 . The ratio of enzyme activity in units for Leu-Gly-Gly (pH 8.0) to Leu- β -naphthylamide (pH 8.0), α -Glu-Tyr-Glu (pH 5.0) and Met-Glu (pH 5.5) was 92.9:43.8:18.4:1. The enzyme was found to be stable in the pH range of 5.5 to 9

FIG. 6. Effect of pH on Activity of Leucine Aminopeptidase II.

●—●, Leu-Gly-Gly; ○—○, α -Glu-Tyr-Glu; $\triangle$ — $\triangle$, Met-Glu (units/ml $\times 10^{-1}$); □—□, Leu- β -NA; ■—■, Leu- β -NA (10^{-3} M CoSO_4 was omitted.) ----, phosphate buffer (pH 6.0 to 8.5); —, acetate buffer (pH 3.0 to 6.0) and Tris-HCl buffer (pH 6.5 to 9.0). Peptidase activity was assayed after 10 min at 30°C with purified enzyme (348 $\mu\text{g/ml}$).

FIG. 7. pH Stability of Leucine Aminopeptidase II.

●—●, Leu-Gly-Gly (pH 8.0); ○—○, Leu- β -NA (pH 8.0). To 25 μl of 0.25 M buffer of various pH values, 100 μl of purified and dialyzed enzyme solution was added, and the mixture was incubated at 37°C for 2 hr. 0.05 M acetate buffer (pH 3.0 to 6.0) and 0.05 M Tris-HCl buffer (pH 6.5 to 9.0) were used.

during preincubation at 37°C for 2 hr. Preincubation at 37°C at pH 7.5 to 9.0 for 2 hr resulted in roughly 10 to 20% increase of arylamidase activity measured at 30°C (Fig. 7).

Effect of temperature on leucine aminopeptidase II

FIG. 8. Effect of Temperature on Activity of Leucine Aminopeptidase II.

●—●, Leu-Gly-Gly (pH 8.0); ○—○, Leu- β -NA (pH 8.0).

The incubation mixtures contained 0.05 M Tris-HCl buffer (pH 8.0), 2.5×10^{-4} M Leu-Gly-Gly or 0.02% Leu- β -naphthylamide and the purified enzyme. Enzyme activity was assayed after 10 min at various temperatures.

FIG. 9. Heat Stability of Leucine Aminopeptidase II.

●—●, control; ○—○, in the presence of 10^{-2} M $\text{Ca}(\text{CH}_3\text{COO})_2$; □—□, in the presence of 10^{-2} M CoSO_4 . —, Leu-Gly-Gly (pH 8.0); ----, Leu- β -NA (pH 8.0). The incubation mixtures consisting of purified enzyme and 100 μl of 0.05 M acetate buffer at pH 6.5 were incubated at various temperatures for 10 min.

The activity of purified leucine aminopeptidase II at various temperature was determined with Leu-Gly-Gly and Leu- β -naphthylamide for 10 min incubation. The enzyme was found to be most active at 50°C (Fig. 8). Heat stability tests showed that the enzyme

was stable up to 60°C, but lost its activity almost completely at 70°C for 10 min. Calcium ions showed some protective effect on the enzyme activity toward Leu-Gly-Gly from heat denaturation. Thermo-activation was studied at various temperatures for 10 min. Heating leucine aminopeptidase II at 40, 50 and 60°C for 10 min in the presence of 10^{-2} M CoSO_4 caused the increase of arylamidase activity. Thermo-activation was not observed for peptidase activity with Leu-Gly-Gly (Fig. 9).

TABLE III. EFFECT OF VARIOUS METAL SALTS ON LEUCINE AMINOPEPTIDASE II

Reaction mixtures were 1 ml of 0.05 M Tris-HCl buffer containing of the purified enzyme, substrate and varying concentrations of metal salts at pH 8.0. After 10 min reaction at 30°C, the amino acid or naphthylamine liberated was measured by the method described in the text.

Metal salts added, 10^{-3} M	Relative activity (%)	
	Leu-Gly-Gly	Leu- β -NA
None	100	100
CoSO_4	115.0	172.5
MnCl_2	108.0	113.7
NiSO_4	86.2	85.5
ZnSO_4	97.0	70.8
MgSO_4	113.2	111.2
CaCl_2	118.6	111.1
$\text{Pb}(\text{CH}_3\text{COO})_2$	0	6.8
CuSO_4	0	5.7
HgCl_2^*	42.6	19.0

* 4×10^{-3} M

Effect of metal ions on the enzyme activity

As shown in Table III, remarkable inhibition of the enzyme action was shown by Pb^{2+} , Cu^{2+} and Hg^{2+} ions. Co^{2+} , Mn^{2+} , Mg^{2+} and Ca^{2+} ions activated the enzyme.

Effect of various reagents on the enzyme activity

Table IV shows the effect of various reagents on the enzyme activity which are known to inactivate proteases of various origins. The enzyme was inhibited by metal chelating agents such as EDTA, α, α' -dipyridyl and *o*-phenanthroline and S-S dissociating agents like β -mercaptoethanol and sodium thioglycolate. Specific sulfhydryl reagents like *p*-chloromercuribenzoate and moniodoacetate did not show any inhibitory effect on the peptidase activity.

Hydrolysis of some peptides

Two-tenth ml of enzyme solution (69.6 μg protein) in 0.1 M acetate buffer (pH 6.5) was added to 1 mg of Leu-Gly-Glu (kindly supplied by Dr. S. Sakakibara), Pro-Leu-Gly-Gln, Gly-Pro-Leu-Gly-Gln and angiotensin I (supplied by Protein Research Foundation, Osaka, Japan). After incubation for appropriate period at 30°C, 10 μl aliquots of the reaction mixture were spotted on a thin layer of Kieselgel G (E. Merk AG, Darmstadt) and developed at 25°C for 3 hr by the ascending method using a mixture of *n*-butanol, acetic

TABLE IV. EFFECT OF VARIOUS REAGENTS ON THE LEUCINE AMINOPEPTIDASE II

Mixtures of the purified enzyme and substances indicated were incubated in 0.05 M acetate buffer (pH 6.5) for 20 min at 30°C. After this period, activities were assayed and compared with a control.

Inhibitor	Concentration M	Remaining activity (%)	
		Leu-Gly-Gly	Leu- β -NA
None		100	100
EDTA	4×10^{-3}	51.8	57.8
α, α' -Dipyridyl	4×10^{-3}	0.3	3.4
<i>o</i> -Phenanthroline	4×10^{-3}	0	0
<i>p</i> -Chloromercuribenzoate	10^{-3}	114.1	94.0
Monoiodoacetate	4×10^{-3}	125.7	103.2
β -Mercaptoethanol	4×10^{-3}	0	0
Sodium thioglycolate	4×10^{-3}	0	0.3
Sodium hexametaphosphate	4×10^{-3}	82.7	92.0
L-Cysteine	4×10^{-3}		93.6
DFP	10^{-2}	75.5	28.6
DFP	10^{-3}	78.5	9.4

FIG. 10. Actions of Leucine Aminopeptidase II on Various Peptides.

The enzymatic hydrolysis of the peptides was made in 0.1 M acetate buffer (pH 6.5) for 24 hr at 30°C. The numerical figures indicate the relative quantity of liberated amino acids. Arrow marks indicate the peptide bonds split by the enzyme.

acid and water (4:1:1) as the solvent. The thin layer was then dried and detection of separated components was carried out by spraying of 0.25% solution of ninhydrin dissolved in ethanol. After 24 hr incubation, amino acids in the reaction mixtures were determined with a Hitachi model 034 liquid chromatograph by the two column system (Hitachi custom ion-exchange resin 2611 and 2612 columns of 0.6×10 cm and 0.9×50 cm) according to the procedure of Moore *et al.*⁹⁾ Based on the results summarized in Fig. 10 the enzyme was found to hydrolyze the peptide liberating amino terminal amino acid and was confirmed to be aminopeptidase.

Effect of NaCl on the enzyme activity

As shown in Fig. 11, NaCl at high concentration (18%) reduced the enzyme activity about 90% for Leu-Gly-Gly, about 75% for α-Glu-Try-Glu, about 70% for α-Glu-Try and about 25% for Met-Glu, respectively.

DISCUSSION

The aminopeptidase II from *Asp. oryzae* 460 showed different behavior on optimum pH (Fig. 6), thermo-activation (Fig. 9) and effect of NaCl (Fig. 11) for various substrates.

FIG. 11. Effect of NaCl on Leucine Aminopeptidase II.

●-●, Leu-Gly-Gly in 0.05 M phosphate buffer at pH 8.0; ○-○, Leu-β-NA in 0.05 M Tris-HCl buffer at pH 8.0; △-△, α-Glu-Try-Glu in 0.05 M acetate buffer at pH 5.0; ▲-▲, Met-Glu in 0.05 M acetate buffer at pH 5.0; ■-■, α-Glu-Try in 0.05 M acetate buffer at pH 5.0. The incubation mixtures consisting of 50 μl of purified enzyme (17.4 μg), 0.7 ml of varying concentration of NaCl and 0.25 ml of 10^{-3} M substrate were incubated at 30°C.

Such phenomena were frequently observed in other enzymes. For example, aminopeptidase of *Bac. stearothermophilus*¹⁰⁾ and brain arylamidase¹¹⁾ exhibited two different pH optima depending on substrates. Dissimilar patterns of inhibitory effects on the former enzyme by 8 M urea and of stimulatory or inhibitory effects on the latter enzyme by a variety of metal ions were also found for different substrates. Similar results for leucine aminopeptidase I from *Asp. oryzae* 460 was reported in the previous paper.⁶⁾

Thermo-activation in the presence of Co^{2+} ions is also characteristic of *Asp. oryzae* leucine aminopeptidase II. Similar phenomenon was reported for the thermophilic aminopeptidase from *Bac. stearothermophilus*¹²⁾ and the leucine aminopeptidase I from *Asp. oryzae*.⁶⁾

Mammalian tissues contain aminopeptidases or arylamidases with a great variety of substrate specificity. Enzymes have been isolated that preferentially hydrolyze peptide bonds involving N-terminal acidic amino acids (glutamate or aspartate),^{13,14)} basic amino acids

(lysine or arginine),^{15,16)} alanine,^{17,18,19)} methionine,^{20,21)} glycine,¹¹⁾ cystine,²²⁾ β -aspartate,²³⁾ or pyrrolidone carboxylate.²⁴⁾ Since the aminopeptidase II from *Asp. oryzae* preferentially hydrolyzed the oligopeptides that possess leucine as the N-terminal amino acid, the present aminopeptidase was named leucine aminopeptidase II.

The leucine aminopeptidase II from *Asp. oryzae* was not inhibited by SH reagents as the aminopeptidase and dipeptidase from *Asp. oryzae*.²⁵⁾ But the aminopeptidase B from rat liver,¹⁵⁾ arylamidases in regenerating wound tissue of a rat²¹⁾ and the brain arylamidase from the soluble fraction of bovine brain¹¹⁾ were inhibited by SH reagents. This enzyme was inhibited by S-S dissociating agents like the aminopeptidase B from rat liver.¹⁵⁾

Leucine aminopeptidase II was shown to be similar in molecular weight, optimum pH and substrates hydrolyzed to aminopeptidase from *Asp. oryzae*²⁶⁾ reported by Lehmann and Uhlig. Leucine aminopeptidase II was shown to be dissimilar in molecular weight, substrate specificity, optimum temperature and heat stability to leucine aminopeptidase I⁶⁾ from *Asp. oryzae* 460.

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