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Some Properties of Leucine Aminopeptidase from *Aspergillus japonica* as a Metalloenzyme[1)]

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Some properties of purified leucine aminopeptidase [E.C. 3.4.1.1] from *Aspergillus japonica* as a metalloenzyme were investigated. Leucine aminopeptidase was inhibited by various chelating agents. The addition of Zn^{2+} to the ethylenediaminetetraacetic acid (EDTA)-treated leucine aminopeptidase restored the activity to nearly the original level, but Co^{2+} was partially effective.

Kinetic studies using L-leucyl-β-naphthylamide as substrate were carried out with native leucine aminopeptidase, Zn^{2+} or Co^{2+}-reactivated leucine aminopeptidase after dialysis against EDTA. Michaelis constant (K_m) and activation energy (E_{act}) of native leucine aminopeptidase were in good agreement with those of Zn^{2+} reactivated leucine aminopeptidase (native leucine aminopeptidase K_m: 2.5×10^{-4}M, E_{act}: 9.2×10^3 cal/mol, Zn^{2+}-reactivated leucine aminopeptidase K_m: 2.5×10^{-4}M, E_{act}: 9.8×10^3 cal/mol).

In the presence of L-leucyl-β-naphthylamide, the inhibition of leucine aminopeptidase by various chelating agents and a Cd^{2+} decreased to an extent of 23—46%.

Metal analysis indicated that the purified leucine aminopeptidase containing 1 g-atom of zinc per mol of leucine aminopeptidase, and both the activity and the zinc content were lowered when native leucine aminopeptidase was treated with EDTA or *o*-phenanthroline.

Keywords——leucine aminopeptidase; *Aspergillus japonica*; metalloenzyme; kinetics; metal analysis; chelating agents; zinc

It is well known that many aminopeptidase are inactivated by the addition of chelating agents. Leucine aminopeptidase from animal organs such as the swine kidney and bovine lens is reported to be a zinc metalloenzyme having molecular weight of about 3×10^5, and is activated by the addition of Mn^{2+}, Mg^{2+}, or Co^{2+}.[3)] On the other hand, that of microorganisms such as *Streptomyces peptidofaciens*,[4)] *Aeromonas proteolytica*,[5)] *Bacillus stearothermophilus*,[6)] and yeast[7)] were found to be activated by Ca^{2+} or Co^{2+}. These enzymes were metalloenzyme containing calcium, zinc, or cobalt. Molecular weights falls in a range of 1.9×10^4—6.0×10^5.[4—6)]

There are few reports concerning the metalloenzymic properties of leucine aminopeptidase from *Aspergillus* and the metal analysis of this enzyme has not yet been performed.

The present authours have reported purification and enzymic properties of leucine aminopeptidase from *Aspergillus japonica* and pharmaceutical application of this enzyme.[8,9)] In a

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previous paper,[8] we reported that leucine aminopeptidase from *Aspergillus japonica* was activated by Co^{2+} and inhibited by various chelating agents. These results suggested that the enzyme was a metalloenzyme.

This paper deals with the effect of inhibitors on the leucine aminopeptidase activity, determination of kinetic parameters (K_m and E_{act}), and metal analysis of the enzyme. The obtained results have demonstrated that leucine aminopeptidase from *Aspergillus japonica* is a zinc metalloenzyme.

Materials and Methods

Enzyme and Reagents——Purification of leucine aminopeptidase from *Aspergillus japonica* was performed by the method described in a previous paper[8] with modification. That is, heat treatment of the crude enzyme was followed by ammonium sulfate precipitation and N,N'-dimethyl formamide precipitation. The obtained precipitate was dissolved in 10 mM acetate buffer (pH 5.5) and subjected to Sephadex G-25 gel chromatography and then SP-Sephadex C-50 ion exchange chromatography. L-Leucyl-β-naphthylamide was purchased from Daiichi Kagaku Co. (Tokyo). Chelating agents used were of reagent grade: EDTA from Daiichi Kagaku Co. (Tokyo), 8-hydroxyquinoline-5-sulfonic acid from Nakarai Kagaku Co. (Kyoto), *o*-phenanthroline and iminodiacetic acid from Tokyo Kasei Co. (Tokyo), 2,6-pyridine dicarbonic acid, α,α'-dipyridyl, 8-hydroxyquinoline, and 2-mercaptoethanol from Wako Pure Chemical Ind. (Osaka), L-cysteine from Nihon Rikagaku Ind. (Tokyo). All other chemicals used were of special or reagent grade.

Enzyme Assay and Protein Determination——Activities against L-leucyl-β-naphthylamide and L-leucyl-*p*-nitroanilide were measured by the method of Kato[10] and that of Tuppy *et al.*,[11] respectively. The protein content was determined by measuring the dry weight or by the procedure of Lowry *et al.*,[12] using bovine serum albumin as standard.

Preparation of Chelating Agent-treated Enzyme——Native leucine aminopeptidase (0.5 mg/ml) was dialyzed against 600 ml of 10 mM phosphate buffer (pH 7.0) containing 2 mM EDTA and 100 mM NaCl, or 600 ml of 10 mM phosphate buffer (pH 7.5) containing 1 mM *o*-phenanthroline and 100 mM NaCl at 4° for 18 hr.

Metal Analyses——All glasswares were placed in 2 N HCl and these were rinsed with distilled water before use. Two mg of enzyme protein was dissolved in 4 ml of 0.1 N HCl and the zinc content in it was measured on Perkin-Elmer model 303 spectrophotometer using analytical grade metal salt as standard according to the method of Fuwa *et al.*[13]

TABLE I. Comparison of Effectiveness of Various Chelating Agents as Inhibition of Leucine Aminopeptidase Activity with Order of Their Zinc Stability Constant

Inhibitor	$-\log [I]_{50}$[a]	$\log K_{Zn}$[b]
EDTA	5.73	16.7
o-Phenanthroline	4.23	17.6
2,6-Pyridinedicarboxylic acid	3.87	11.9
8-Hydroxyquinoline	3.23	18.9
α,α'-Dipyridyl	2.40	13.3
Iminodiacetic acid	2.23	12.2

Enzyme was preincubated for 1 hr at 37° in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), with various concentrations of chelating agents. After 100 fold dilution with 0.1 M Tris-HCl buffer (pH 8.0), the enzyme activity was measured using L-leucyl-*p*-nitroanilide as substrate.

a) $[I]_{50}$ is defined as the effective concentration of chelating agents required to give 50% inhibition of enzyme activity.

b) K_{Zn} is defined as $(Zn \times Ln)/(Zn^{2+}) \times (L)^n$; values are from Ueno and Sakaguchi (ed.)[14]; *L*, chelating agent; *n*, number of ligand equivalents.

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Results

The Effect of Chelating Agents on the Activity of Native Leucine Aminopeptidase

It is well known that the inhibition of chelating agents on the activity of metalloenzyme is generally attributed to the binding or removal of an essential metal. It is important to know whether or not the order of inhibition by chelating agents of different types is, in fact, attributed to the stability constant of chelates.

The effective concentration of each chelating agent required to give 50% inhibition of leucine aminopeptidase (I_{50}) was compared with the ability of the chelating agent to form chelate with Zn^{2+}, (log K_{Zn}).[14] As shown in Table I, the order of inhibition was EDTA *o*-phenanthroline 2,6-pyridinedicarboxylic acid iminodiacetic acid α,α'-dipyridyl. There was not a good correlation between the order of inhibition ($-\log[I]_{50}$) and log K_{Zn}.

Restration of the Enzyme Activity on the Addition of Metal Ion to the EDTA-treated Leucine Aminopeptidase

The effect of divalent metal ions on the EDTA-treated leucine aminopeptidase was examined and the result is presented in Table II. An extensive regeneration of the enzyme activity was observed on the addition of Zn^{2+} and Co^{2+} to the reaction mixture.

Table II. Effect of Various Metal Ions on Reactivation of EDTA-treated Leucine Aminopeptidase

Metal ion (1×10^{-4} M)	Relative activity (%)[a]
None	0
$CoCl_2$	84
$NiCl_2$	0
$MnCl_2$	7
$MgCl_2$	0
$CaCl_2$	3
$CdCl_2$	0
$CuCl_2$	0
$HgCl_2$	0
$ZnCl_2$	100
$FeCl_3$	0
NaCl	0

EDTA-treated enzyme was prepared as described in the text. Adequate amount of EDTA-treated enzyme reacted with L-leucyl-β-naphthylamide in the presence of 1×10^{-4} M of metal ion.

a) Activity was expressed as a percentage of zinc ion reactivated leucine aminopeptidase activity.

Stability of EDTA-treated Leucine Aminopeptidase

Figure 1 shows the effect of preincubation time on the inhibition of leucine aminopeptidase activity by EDTA. Under the experimental conditions employed, 40 min was suffi cient to give the maximal inhibition. The addition of Zn^{2+} to the reaction system resulted in the reactivation of the enzyme activity, but the extent of reactivation decreased with an increase in preincubation time.

The Effect of Metal Ion Concentration Reactivation of EDTA-treated Leucine Aminopeptidase

The effect of Co^{2+} or Zn^{2+} concentration on the reactivation of EDTA-treated leucine aminopeptidase is shown in Fig. 2. In the reaction system, the maximal reactivation of

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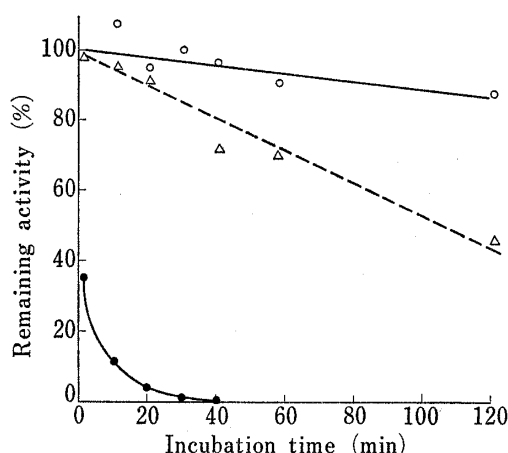
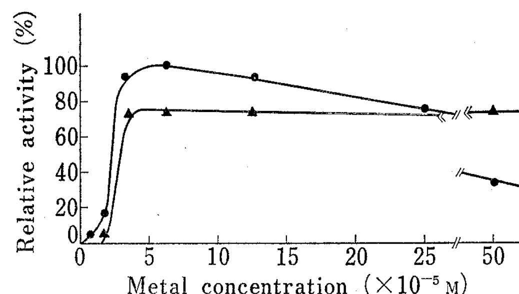


Fig. 1. Inactivation of Leucine Aminopeptidase by EDTA and Reactivation by Zinc Ion

The enzyme was incubated with 1 ml of 0.1 M phosphate buffer (pH 7.5) containing 5 mM EDTA. After incubation at 37°, the incubation mixture was diluted 100 fold with 0.1 M Tris-HCl buffer (pH 8.0), and the enzyme activity was assayed in the presence or absence of zinc ion using L-leucyl-*p*-nitroanilide as substrate.
○: control, ●: remaining activity,
△: activity when 1×10^{-4} M of zinc ion was added to the reaction system.



Eig. 2. Effect of Zinc or Cobalt Ion Concentration on the Reactivation of EDTA-treated Leucine Aminopeptidase

EDTA-tretaed enzyme was prepared as described in the text, and was further dialyzed in the absence of EDTA. One ml of the treated enzyme solution was added to the reaction system containing various concentration of metal ion, and enzyme activity was measured using L-leucyl-β-naphthylamide as substrate.
●: Co^{2+}-reactivated enzyme.
▲: Zn^{2+}-reactivated enzyme.

leucine aminopeptidase occured at about 5×10^{-5} M of Co^{2+} or Zn^{2+}. With increasing the concentration of Zn^{2+}, however, the relative activity decreased gradually. At the concentration of 5×10^{-4} M the relative activity of leucine aminopeptidase was only 45%. On the other hand, an increase in Co^{2+} concentration did not result in a decrease in the relative activity up to the concentration of 5×10^{-4} M.

The Effects of Metal Ions on the Michaelis Constant and Activation Energy

The Michaelis constant (K_m) and activation energy (E_{act}) of the native leucine aminopeptidase, Zn^{2+} or Co^{2+}-reactivated leucine aminopeptidase after dialysis against EDTA for

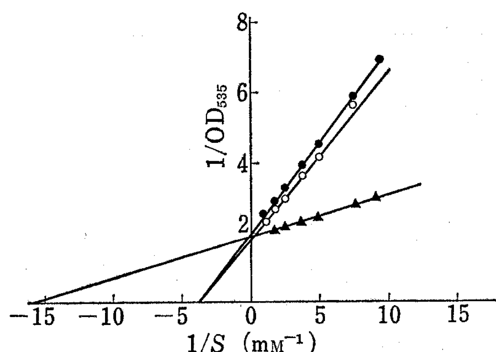


Fig. 3. Lineweaver-Burk Plots of the Activity of Native- and EDTA-treated Leucine Aminopeptidase in the Presence of Co^{2+} or Zn^{2+}

EDTA-treated leucine aminopeptidase(LAP) was prepared as described in the text, and was further dialyzed in the absence of EDTA.
▲: EDTA-treated LAP containing 37 μM of Co^{2+}.
○: EDTA-treated LAP containing 37 μM of Zn^{2+}.
●: native LAP.

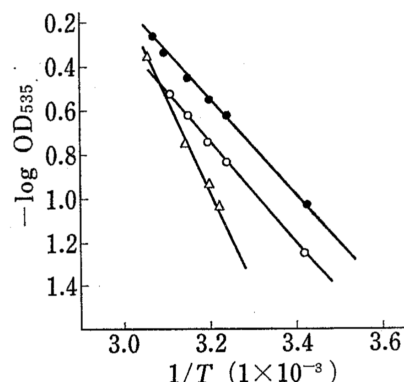


Fig. 4. Arrhenius Plots for the Activity of Native- and EDTA-treated Leucine Aminopeptidase in the Presence of Co^{2+} or Zn^{2+}

EDTA-treated leucine aminopeptidase(LAP) was prepared as described in the text, and was further dialyzed in the absence of EDTA.
△: EDTA-treated LAP containing 50 μM of Co^{2+}.
○: EDTA-treated LAP containing 50 μM of Zn^{2+}.
●: native LAP.

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L-leucyl-β-naphthylamide were calculated by the methods of Lineweaver-Burk plots and Arrhenius Plots. As shown in Fig. 3, K_m values of native and Zn^{2+}-reactivated leucine aminopeptidase were the same (2.5×10^{-4} M), whereas the K_m of Co^{2+}-reactivated leucine aminopeptidase was 6.4×10^{-5} M.

As shown in Fig. 4, the E_{act} of native and Zn^{2+}-reactivated leucine aminopeptidase were 9.2×10^3 cal/mol and 9.8×10^3 cal/mol, respectively, while the E_{act} of Co^{2+}-reactivated leucine aminopeptidase was 1.56×10^4 cal/mol. These results indicate that the K_m value and the E_{act} of native leucine aminopeptidase are similar to those of Zn^{2+}-reactivated leucine aminopeptidase.

The Effects of Substrate on the Inhibition of the Leucine Aminopeptidase Activity against the Inhibitors

Inhibition of leucine aminopeptidase activity by chelating agents and other inhibitors were examined in the presence and absence of leucyl-β-naphthylamide. The result is shown in Table III. Without adding substrate the inhibition of the leucine aminopeptidase activity by EDTA was 91%. The inhibition became 45% with the addition of substrate. *o*-Phenanthroline inhibited the leucine aminopeptidase activity to an extent of 40% without substrate. This compound did not show any inhibition when the substrate was added. Differences in the inhibition between the presence and absence of substrate were about 20% for 8-hydroxyquinoline-5-sulfonic acid, $CdCl_2$, and L-cysteine. The inhibition by 2-mercaptoethanol was practically the same in the presence or absence of L-leucyl-β-naphthylamide.

Table III. The Protection of Substrate (L-Leucyl-β-naphthylamide) against Inhibition of Leucine Aminopeptidase Activity by Chelating Agents and Inhibitors

Inhibitor	Concentration (mM)	pH	Inhibition (%)		Difference
			None	Substrate added	
None		8.5	0	0	0
EDTA	5.0	7.5	91	45	46
o-Phenanthroline	0.2	8.5	40	0	40
8-Hydroxyquinoline-5-sulfonic acid	0.5	8.5	65	41	24
$CdCl_2$	5.0	7.5	66	42	24
L-Cysteine	10	8.5	54	31	23
2-Mercaptoethanol	20	8.5	94	92	2

In the presence and absence of L-leucyl-β-naphthylamide, the inhibitor and the enzyme were preincubated for 1 hr at 0°. After 100 fold dilution, the enzyme activities were assayed using L-leucyl-*p*-nitroanilide.

Metal Analyses

The results of metal analyses of native and apo-leucine aminopeptidase are shown in Table IV. The native leucine aminopeptidase contained 1 g-atom of zinc per mol of leucine

Table IV. Metal Analyses of Native- and Chelating Agent-treated Leucine Aminopeptidase (LAP) from *Aspergillus Japonica*

Sample	Zinc content (g-atom/mol LAP)	Specific activity[a] (units/mg protein)
Native LAP	1.0	97.0
EDTA-treated LAP	0.1	0
o-Phenanthroline-treated LAP	0.1	11.6

a) One unit of the enzyme activity was defined as the amount of enzyme which hydrolyzed 1 μmol of L-leucyl-*p*-nitroanilide per min at 37°, pH 8.0.

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aminopeptidase. The zinc content of EDTA or *o*-phenanthroline treated leucine aminopeptidase was less than the native leucine aminopeptidase and leucine aminopeptidase activity was lower in the EDTA or *o*-phenanthroline treated leucine aminopeptidase.

Discussion

It has been reported that leucine aminopeptidase from *Aspergillus* have similar features in the molecular weight and the metal requirement. The leucine aminopeptidase from *Aspergillus oryzae*,[15] *Aspergillus sojae*,[16] and *Aspergillus parastica*[17] having the molecular weight of 2.6×10^3 to 6.3×10^3 was strongly inhibited by chelating agents and was activiated by Co^{2+}. These results indicate that *Aspergillus* leucine aminopeptidase is a metalloenzyme. A more detailed study is, however, needed for the precise characterization of leucine aminopeptidase as a metalloenzyme. We have reported the properties of the metalloenzyme and metal analysis of leucine aminopeptidase from *Aspergillus japonica* in this paper. The relative ability of chelating agents to inhibit leucine aminopeptidase activity (EDTA$>$*o*-phenanthroline$>$2,6-pyridine dicarbonic acid$>$8-hydroxyquinoline$>$iminodiacetic acid$>\alpha,\alpha'$-dipyridyl) differs from those of other zinc metalloenzymes such as alkaline phosphatase,[18] carboxypeptidase,[19] and phosphomannose isomerase.[20] It is reported that there is a good correlation between the effectiveness of various chelating agents to inhibit the activity of the latter three enzymes and their ability to bind zinc ion (log K_{Zn}).

The activity of EDTA-treated leucine aminopeptidase was reactivated to nearly the original level by the addition of of Zn^{2+}. On increasing the concentration of Zn^{2+}, there was gradual decrease in the relative activity. This means that the optimum concentration exists for zinc to give the maximal reactivation of EDTA-treated leucine aminopeptidase.

Using native leucine aminopeptidase, Zn^{2+} and Co^{2+}-reactivated leucine aminopeptidase, the kinetic parameters (K_m and E_{act}) of these enzymes for L-leucyl-β-naphthylamide were compared. The results indicated that the values of the parameters of native leucine aminopeptidase were almost the same as those of Zn^{2+}-reactivated leucine aminopeptidase. On the other hand, the parameters of Co^{2+}-reactivated leucine aminopeptidase largely differed from those of native leucine aminopeptidase. These results suggest that the cobalt metalloenzyme was composed of apo-leucine aminopeptidase.

In the presence of L-leucine-β-naphthylamide, the inhibition of leucine aminopeptidase activity by chelating agents such as EDTA, *o*-phenanthroline, 8-hydroxyquinoline-5-sulfonic acid, and L-cysteine and Cd^{2+} was much less than in the absence of substrate, while the inhibition by 2-mercaptoethanol which has less affinity for metals did not very even in the presence of substrate. These data suggest that the metal ion to form native leucine aminopeptidase exists near the substrate binding site.

From the metal analysis, it became apparent that leucine aminopeptidase from *Aspergillus japonica* contained 1 g-atom of zinc per mole of enzyme, although the zinc content of chelating agent-treated leucine aminopeptidase decreased to 0.1 g-atom.

From these results, it can be concluded that leucine aminopeptidase from *Aspergillus japonica* is a zinc metalloenzyme.

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