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# Physicochemical Properties of Leucine Aminopeptidase from *Aspergillus japonica*[1]

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Physicochemical properties of the purified leucine amino peptidase from *Aspergillus japonica* were studied. The molecular weight of this enzyme was estimated to be approx. 31100 by sedimentation equilibrium, and the sedimentation coefficient ($S_{20,w}$) was determined to be 3.12 S. The extinction coefficient at 278.5 nm, $E^{1\%}_{1cm}$ was 7.72 and the isoelectric point was at around pH 8.0 by gel electrofocusing. The helical content was calculated to be 1.7% from the Moffit-Yang plots. The amino acid analyses indicated that the enzyme was composed of 289 amino acid residues and contained one cystine. The amino-terminal amino acid was lysine, and the partial specific volume calculated from amino acid composition was 0.726.

**Keywords**——leucine aminopeptidase; *Aspergillus japonica*; molecular weight; sedimentation coefficient; extinction coefficient; isoelectric point; optical rotatory dispertion; amino acid composition

Our previous papers in this series[3] dealt with the purification procedure and enzymic properties of leucine aminopeptidase from *Aspergillus japonica*. This enzyme required $Zn^{2+}$ for the full activity and $Co^{2+}$ for the activity. Although there are a number of reports on the physicochemical properties of *Bacillus strearothermophilus*,[4] *Streptomycespeptidofaciens*,[5] *Escherichia coli*,[6] *Aeromonas proteolytica*[7] and animal aminopeptidase such as bovine lens[8] and swine kidney,[9] the physicochemical properties of *Aspergillus* leucine aminopeptidase have not yet been clarified.

This paper describes physicochemical properties of leucine aminopeptidase from *Aspergillus japonica*.

## Materials and Methods

**Purification of Enzyme**——The enzyme used was purified from the ethanol precipitate of culture filtrate of *Aspergillus japonica* by the method described previously.[3c]

**Reagents**——L-Leucyl-*p*-nitroanilide was purchased from Nakarai Kagaku Co., (Kyoto). Dimethylaminonaphthalene sulphonyl-(DNS-) amino acids were purchased from Sigma Chemical Co., (USA). Glucose, L-cysteine, N-acetyl-D,L-tryptophan and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from

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3) *a)* M. Sugiura, M. Suzuki, M. Ishikawa, and M. Sasaki, *Chem. Pharm. Bull.* (Tokyo), **24**, 2026 (1976); *b)* M. Sugiura, M. Suzuki, M. Ishikawa, and M. Sasaki, *Chem. Pharm. Bull.* (Tokyo), **24**, 2286 (1976); *c)* M. Sugiura, M. Ishikawa, and M. Sasaki, *Chem. Pharm. Bull.* (Tokyo), **26**, 3101 (1978).
4) P. Moser, G. Roncari, and H. Zuber, *Int. J. Protein Res.*, **2**, 191 (1970).
5) T. Uwazima, N. Yoshikawa, and O. Terada, *Agric. Biol. Chem.* (Tokyo), **37**, 2727 (1973).
6) V.M. Vogt, *J. Biol. Chem.*, **245**, 4760 (1970).
7) J.M. Prescott, S.H. Wilkes, R.W. Wagner, and K.J. Wilson, *J. Biol. Chem.*, **246**, 1756 (1971).
8) S.W. Melbye and F.H. Carpenter, *J. Biol. Chem.*, **246**, 2459 (1971); F.H. Carpenter and J.M. Vohl, *J. Biol. Chem.*, **248**, 294 (1973).
9) S.R. Himmelhoch, *Arch. Biochem. Biophys.*, **134**, 597 (1969).

Wako Pure Chem. Ind., (Osaka). All other chemicals used in the experiment were commercial product and of reagent grade.

**Enzyme Assay and Protein Determination**——The activity towards L-leucyl-*p*-nitroanilide was measured by the method of Tuppy *et al.*[10] 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. Specific activity was expressed in units per mg of protein. The protein concentration was determined by measuring the absorbance at 278.5 nm.

**Sodium Dodecyl Sulfate (SDS) Electrophoresis**——SDS electrophoresis was carried out as described by Weber and Osborn[11] using 10% polyacrylamide gel containing 0.1% SDS. Electrophoresis was performed at a constant current of 8 mA per tube for 5 hr at room temperature.

**Ultracentrifugal Analyses**——Ultracentrifugal analysis was performed with a Hitachi 282 Analytical Ultracentrifuge equipped with a light absorption scanner. The enzyme sample was dissolved in 0.01 M sodium phosphate buffer (pH 7.8) containing 0.1 M NaCl. Determination of sedimentation velocity was carried out in a 12 mm aluminium double sector cell in an RA-72 rotor at 12.5°. The enzyme solution containing 1.09 mg protein/ml was centrifuged at 60000 rpm. The sedimentation coefficient was calculated and corrected for temperature (12.5°, 20°) and solution (buffer, water). Determination of molecular weight by the sedimentation equilibrium method of Schachman and Edelstein[12] was made in a 12 mm Yphantis cell in a RA-72 TC rotor at 12.5°. The rotor speed was 16000 rpm.

**Isoelectric Point**——Gel electrofocusing was performed by the method of Wrigley[13] using 1% of carrier ampholites which gave a pH gradient from pH 3.5—10 and 5% of polyacrylamide gel. Focusing was carried out 200 V for 3 or 7 hr. After electrophoresis, the course of pH gradient and the enzyme distribution in gels were determined by cutting into 5 mm sections. The sections were soaked in 1 ml of distilled water for several hours.

**Optical Rotatory Dispertion (ORD)**——The ORD measurement was performed in an automatic recording Nihon Bunko J-20 Spectropolarimeter. The samples used were dissolved in 10 mM phosphate buffer (pH 7.8) containing 100 mM NaCl.

**Amino Acid Analyses**——Hydrolysis was performed in a tube containing approx. 2 mg of protein and 1 ml of 6 N constant boiling HCl. The tube was then evacuated, sealed and kept at 110° for 24 or 48 hr. The hydrolysate were evaporated to dryness under reduced pressure and redissolved in deionized water. This procedure was repeated three times. The amino acid analyses were performed with a Nihon Denshi JLC-6AH amino acid auto-analyzer. Tryptophan was determined by the method of Dalby and Tsai,[14] and the determination of sulfhydryl groups was performed by the method of Ellman.[15] The partial specific volume was calculated based on the amino acid composition of the enzyme according to the method of Chen and Edsall.[16]

**Amino-terminal Analysis**——The N-terminal amino acid residue was determined with approx. 0.5 nmol of protein by the improved method of Kimura[17] using DNS-technique according to Gray and Hartley.[18] The DNS-amino acid was identified by polyacrylamide layer (5 × 5 cm) chromatography according to the method of Woods and Wang.[19] Development was carried out with water and 28% $NH_4OH$ (100: 1) as the first solvent, and formic acid, *n*-butylalcohol and heptane (1: 10: 10) as the second solvent.

**Metal Analyses**——Zinc analyses of the enzyme were performed according to the method described in our previous paper.[3c] Metal contents of calcium, cadmium, cobalt and magnecium were determined by the method of Fuwa *et al.*[20]

## Results

### Molecular Weight

The molecular weight of leucine aminopeptidase was determined by the method of SDS electrophoresis and sedimentation equilibrium. The results of SDS electrophoresis are shown

10) H. Tuppy, U. Wiesbauer, and E. Wintersberger, *Hoppe-Seyer's Z. Physiol. Chem.*, **329**, 278 (1962).
11) K. Weber and M. Osborn, *J. Biol. Chem*, **244**, 4406 (1969).
12) H.K. Schachman and S.J. Edelstein, *Biochemistry*, **5**, 2681 (1966).
13) C.W. Wrigley, "Methods in Enzymology," Vol. XXII, ed. by W.B. Jakoby, Academic Press Inc., New York, 1971, pp. 559—564.
14) A. Dalby and Chia-Y. Tsai, *Anal. Biochem.*, **63**, 283 (1975).
15) G.L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
16) E.T. Cohn and J.T. Edsall, "Proteins, Amino Acids and Peptides," ed. by Reinhold, New York, 1941, pp. 374—376.
17) S. Kimura, *Bunseki Kagaku* (Japanese), **23**, 563 (1974).
18) C.J. Bruton and B.S. Hartley, *J. Mol. Biol.*, **52**, 165 (1970).
19) K.R. Woods and K.T. Wang, *Biochem. Biophys. Acta*, **133**, 369 (1967).
20) K. Fuwa, P. Pulida, R. Mckay, and B.L. Vallee, *Anal. Chem.*, **36**, 2407 (1964).

in Fig. 1. The molecular weight was estimated to be $3.4 \times 10^4$ based on the relative mobility. In sedimentation equilibrium experiments, the molecular weights in different protein concentration were calculated to be 29930 (0.43 mg/ml), 32200 (0.50 mg/ml) and 31020 (0.74 mg/ml). We used the average molecular weight of 31100 in the subsequent experiments. In Fig. 2 are shown the representative data on molecular weight when the protein concentration was 0.43 mg/ml. The molecular weight was determined based on the partial specific volume of 0.73 which was calculated from amino acid composition.

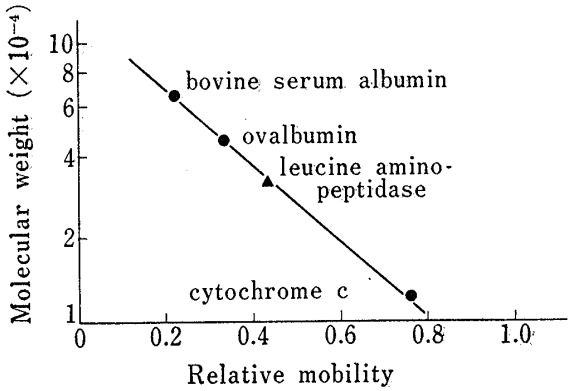


Fig. 1. Estimation of Molecular Weight of Leucine Aminopeptidase by SDS Polyacrylamide Gel Electrophoresis

Electrohhoresis was carried out at pH 7.2 and 8 mA/tube for 5 hr, and the relative mobility was expressed taking that of bromphenol blue as 1.0.

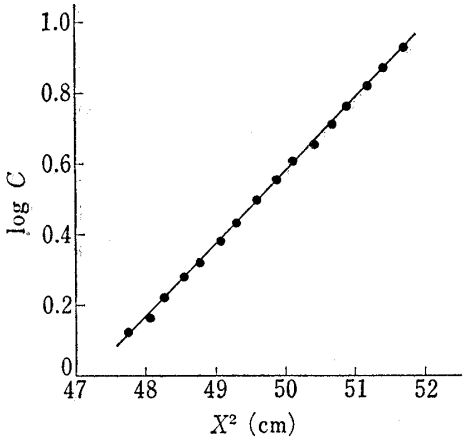


Fig. 2. Representative Sedimentation Equilibrium Data for Leucine Aminopeptidase from *Aspergillus japonica*

The experiment was performed with 0.43 mg of protein/ml in 10 mM phosphate buffer (pH 7.8) containining 100 mM NaCl. The rotor speed was 16000 rpm.

## Sedimentation Coefficient

The sedimentation coefficient ($S_{20,w}$) was calculated from the slope obtained by plotting the log of distance of the boundary from the axis of rotation against time. Fig. 3 shows the sedimentation pattern of leucine aminopeptidase.

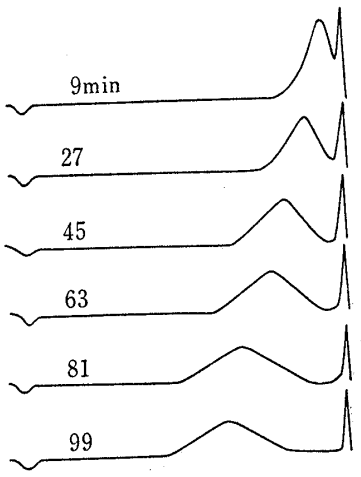


Fig. 3. Representation of Sedimentation Pattern of Leucine Aminopeptidase from *Aspergillus japonica*

The patterns were taken at indicated time after reaching full speed (60000 rpm) and sedimentation is from top to bottom.

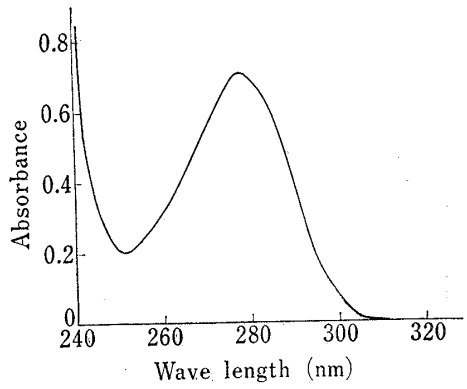


Fig. 4. Ultraviolet Absorption of Leucine Aminopeptidase from *Aspergillus japonica*

The protein concentration was 0.85 mg/ml. The experiment was performed in 10 mM Tris-HCl buffer (pH 8.0).

### Extinction Coefficient

As shown in Fig. 4, the absorption spectrum of leucine aminopeptidase in 10 mM Tris-HCl buffer (pH 8.0) gave a maximum at 278.5 nm and the ratio of absorption at 278.5 to 260 nm was 2.05. The extinction coefficient of the enzyme at 278.5 nm, $E^{1\%}_{1cm}$ was calculated to be 7.72.

### Isoelectric Point

The results of gel electrofocusing are given in Fig. 5. One active peak at around pH 8.0 was observed when 0.4 mM of $Zn^{2+}$ was added to the reaction system.

### Optical Rotatory Dispertion

The measurement of neat ultra-violet optical rotatory dispersion curve was carried out with 0.195 mg/ml of the enzyme solution, and the results are shown in Fig. 6. The ORD spectrum of leucine aminopeptidase showed a minimum at 233 nm and the reduced mean residure rotation [m'] at 233 nm was calculated by the equation, $H=[[m']_{233}-(-22000)]/-12800$. The helical content was calculated to be 0.2%. By using 2.45 mg/ml of the enzyme

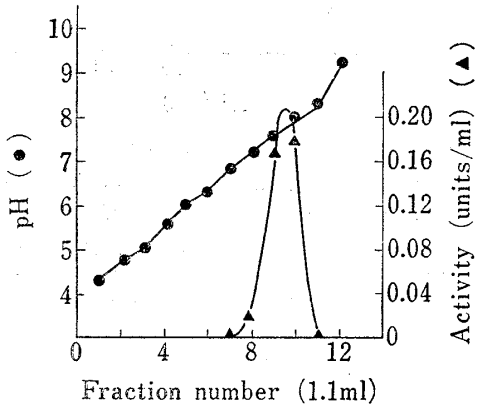


Fig. 5. Determination of Isoelectric Point by Gel Electrophofocusing of Leucine Aminopeptidase from *Aspergillus japonica*

The focusing was performed for 3 hr with addition of 23 μg of leucine aminopeptidase (2.3 units) per tube.

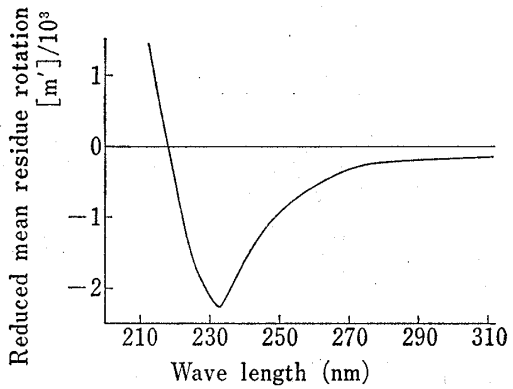


Fig. 6. Optical Rotatory Dispertion Curve of Leucine Aminopeptidase from *Aspergillus japonica*

The experiment was performed with 0.195 mg of protein/ml in 10 mM phosphate buffer (pH 7.8) containing 100 mM NaCl.

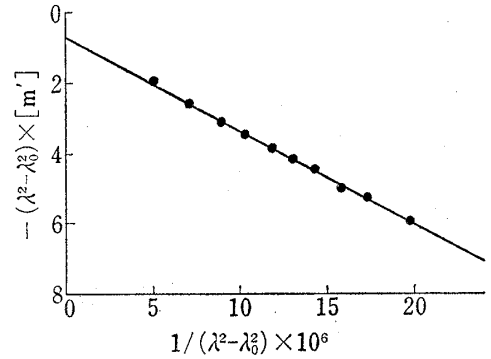


Fig. 7. Moffit-Yang Plots for Leucine Aminopeptidase from *Aspergillus japonica*

The concentration of leucine aminopeptidase was 0.245% in 10 mM phosphate buffer (pH 7.8) containing 100 mM NaCl.

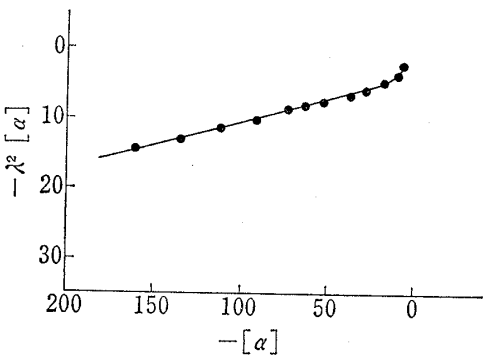


Fig. 8. Drude Plots for Leucine Aminopeptidase from *Aspergillus jajonica*

The sample used was the same as described in Fig. 7.

solution the parameters $a_0$ and $b_0$ were calculated by the Moffit-Yang equation[21] and $\lambda_c$ was by the one-term Drude equation.[22] From the linear curve of Moffit-Yang plots shown in Fig. 7, the $a_0$ and $b_0$ were determined to be $-15°$ and $-11°$, respectively. Using the $b_0$ value of completely helical molecule as $-630°$, the helical content was calculated to be 1.7%. These results suggest that leucine aminopeptidase was low helical structure. The parameter $\lambda_c$ was obtained from the slopes of Drude plots, and calculated to be 266 nm. The result is shown in Fig. 8.

## Amino Acid Composition and N-Terminal Amino Acid

The amino acid composition of leucine aminopeptidase is shown in Table I. The number of residue per mol of the enzyme was 289, and the N-terminal residue was identified as lysine. Sulfhydryl groups were not present and the sugar content determined by the phenol–$H_2SO_4$ method[23] was negligible. The amino acid composition of leucine aminopeptidase from *Aspergillus japonica* was higher for lysine, threonine, and serine and lower for tryptophan, and methionine as compared to aminopeptidase from *Aeromonas proteolytica*.[7]

Table I. Amino Acid Composition of Leucine Aminopeptidase (LAP) from *Aspergillus japonica* and Aminopeptidase (AP) from *Aeromonas proteolytica*

| Amino acid | Amino acid[a] (g/100 g protein) | Number of residue (mol/molecular weight) | |
|---|---|---|---|
| | | LAP[b] | AP |
| Phenylalanine | 6.09 | 13 | 9 |
| Tyrosine | 6.23 | 12 | 12 |
| Leucine | 6.84[c] | 19 | 16 |
| Isoleucine | 4.34 | 12 | 12 |
| Methionine | 0.83[c] | 2 | 6 |
| Valine | 5.68[c] | 18 | 15 |
| Half-cystine | 0.65[d] | 2 | 2 |
| Alanine | 5.43 | 24 | 29 |
| Glycine | 4.00 | 22 | 19 |
| Proline | 4.02 | 13 | 13 |
| Glutamic acid | 14.4 | 35 | 28 |
| Serine | 9.42[d] | 34 | 26 |
| Threonine | 7.07[d] | 22 | 31 |
| Aspartic acid | 8.77 | 24 | 30 |
| Arginine | 2.98 | 6 | 5 |
| Histidine | 3.93 | 9 | 6 |
| Lysine | 8.16 | 20 | 8 |
| Tryptophan | 1.18[e] | 2 | 5 |
| Total | | 289 | 272 |

*a*) Expect where noted, each value is the mean of 24- and 48-hr hydrolysates.
*b*) Based on a molecular weight of $3.11 \times 10^4$.
*c*) Maximum values adopted.
*d*) Extrapolated to zero time of hydrolysis.
*e*) Determined by the method of Dalby and Tsai.[15]

## Metal Analyses

Metal analyses of the enzyme are shown in Table II. In a previous paper we reported that leucine aminopeptidase from *Aspergillus japonica* contained 1 g-atom of zinc per mol of enzyme.[3c] Other metals such as calcium, cadmium, cobalt and magnecium was not detected.

21) M. Moffit and J.T. Yang, *Proc. Natl. Acad. Sci.*, **42**, 596 (1956).
22) J.T. Yang and P. Doty, *J. Am. Chem. Soc.*, **79**, 761 (1957).
23) M. Dubois, K. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

Table II. Metal Content of Leucine Aminopeptidase from *Aspergillus japonica*

| Metal | Content g-atom/mol of enzyme[a)] |
|---|---|
| Ca | 0 |
| Cd | 0 |
| Co | 0 |
| Mg | 0 |
| Zn | 1.0[b)] |

*a*) Based on molecular weight of $3.11\times10^4$.
*b*) Zinc content was reported in a previous paper.[3c)]

## Discussion

Previously we reported the molecular weight of leucine aminopeptidase from *Aspergillus japonica* was $5.7\times10^4$ based on the measurement by gel filtration,[3a)] although the molecular weight determined from SDS electrophoresis and sedimentation equilibrium measurements were $3.4\times10^4$, and $3.11\times10^4$, respectively. This discrepancy in molecular weight may be due to the difference in the principle of molecular weight measurement. The molecular weight of this enzyme was similar to the molecular weights of leucine aminopeptidase from *Aspergillus aryzae*,[24)] *Aspergillus parastica*,[25)] and *Aeromonas proteolytica*,[7)] ranging from $2.6\times10^4$ to $3.2\times10^4$.

The amino acid analysis demonstrated that 289 residues of amino acid contained one mol of leucine aminopeptidase from *Aspergillus japonica*. This was similar to the aminopeptidase from *Aeromonas proteolytica* which contained 272 residues per mole of enzyme.[7)] However, the number of basic amino acids such as arginine, histidine, and lysine, leucine aminopeptidase from *Aspergillus japonica* exceeded from 1 to 12 residues. The isoelectric point of leucine aminopeptidase from *Aspergillus japonica* is at around pH 8.0, suggesting the predominance of basic amino acids over acidic residues in the enzyme. In gel electrofocusing, the low recovery of enzyme activity may be resulted from the chelating reaction and irreversible inactivation of carrier ampholites to the metalloenzyme, since the enzyme residues zinc ion for the optimum activity.

The low helical content in leucine aminopeptidase is similar to other proteolytic anzymes such as trypsin, pepsin, and Aspergillo peptidase.[26)]

Metal analyses have shown that the aminopeptidase from *Streptomyces peptidofaciens*[5)] and *Bacillus stearothermophilus*[27)] contain calcium, magnecium, and cobalt and that leucine aminopeptidase from *Aspergillus japonica*[3c)] contains only 1 g-atom of zinc per mole of enzyme.

The results obtained in the present study confirm that leucine aminopeptidase from *Aspergillus japonica* differs from other microbial and animal aminopeptidases in physicochemical properties such as the kind and content of metals, molecular weight, isolectric point and amino acid composition.

24) T. Nakadai, S. Nasuno, and N. Iguchi, *Argic. Biol. Chem.* (Tokyo), **37**, 757 (1973).
25) K. Lehmann and H. Uhlig, *Hoppe-Seyer's Z. Physiol. Chem.*, **350**, 99 (1969).
26) K. Hamaguchi and H. Takesada, "Seibutsu Kagaku Jikken Ho," Vol. C-4, ed. by I. Uritani, K. Shimura, M. Nakamura, and M. Hunazu, Tokyo Daigaku Shuppan-Kai, Tokyo, 1971, pp. 30—31.
27) G. Roncari, H. Zuber, and A. Wittenbach, *Int. J. Peptide Protein Res.*, **4**, 267 (1972).