Biochimica et Biophysica Acta, 613 (1980) 459—468

BBA 68955

PURIFICATION BY AFFINITY CHROMATOGRAPHY USING AMASTATIN AND PROPERTIES OF AMINOPEPTIDASE A FROM PIG KIDNEY

HIROYASU TOBE, FUKIKO KOJIMA, TAKAAKI AOYAGI and HAMAO UMEZAWA

Institute of Microbial Chemistry, 14-23 Kamiosaki 3-Chome, Shinagawa-ku, Tokyo (Japan)

(Received September 14th, 1979)

Key words: Amastatin; Aminopeptidase A; (Pig kidney)

Summary

1. Amastatin, a specific inhibitor of aminopeptidase A (L-α-aspartyl(L-α-glutamyl)-peptide hydrolase, EC 3.4.11.7), was linked to an agarose matrix and by this affinity chromatography aminopeptidase A of pig kidneys was purified as a single protein shown by acrylamide gel electrophoresis.

2. Aminopeptidase A which was purified 710-fold, hydrolyzed only acidic amino acid β-naphthylamide. The optimum pH and the optimum temperature was 7.5 and 45—50°C, respectively.

3. The molecular weight was approx. 300 000 as determined by Sephadex G-200 gel filtration.

4. The activity of aminopeptidase A was not affected by sulfhydryl agents, S-S dissociating agents and serine proteinase inhibitor, but was inhibited strongly by metal chelating agents, and enhanced by alkaline earth metals.

5. Amastatin inhibited aminopeptidase A in a competitive manner with L-glutamic acid β-naphthylamide, and the K_i value was calculated to be $2.5 \cdot 10^{-7}$ M. The inhibitory effect of amastatin on aminopeptidase A was not reversed by addition of Ca^{2+}.

Introduction

As described in previous reports [1,2], many specific enzyme inhibitors have been isolated from culture filtrates of actinomycetes. Amastatin is a specific inhibitor of aminopeptidase A (L-α-aspartyl(L-α-glutamyl)-peptide hydrolase, EC 3.4.11.7) isolated from culture filtrates of streptomyces ME98-M3 strain

Abbreviation: NA, naphthylamide.

[3]. The structure of amastatin was determined to be [(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl]-L-Val-L-Val-L-Asp [4].

Aminopeptidase A is an exopeptidase which liberates amino terminal glutamyl or aspartyl residues of peptide substrates. This enzyme has been found in various organs and serum of animals, that is, rat kidney and serum [5,13], pig kidney and duodenum [5], rabbit small intestine [15], human serum [6—8,10,14], human red blood cell [11], human parotid gland [8,9] and human kidney [12].

Aminopeptidase A was purified partially from human serum [14] and brush border of rabbit small intestine [15], but it was difficult to separate aminopeptidase A from other aminopeptidases (arylamidases) by Sephadex G-150, DEAE-cellulose and concanavalin A-Sepharose column chromatography. We attempted to isolate aminopeptidase A from pig kidney by using affinity column of an agarose matrix linked with amastatin. The affinity chromatography of aminopeptidase A has not yet been reported. This affinity chromatography of our method was found to be an efficient procedure for purification of aminopeptidase A. In this paper we report purification of aminopeptidase A by using amastatin-affinity chromatography and properties of purified enzyme.

Experimental procedure

Materials

DEAE-Sepharose CL-6B, AH-Sepharose 4B and Sephadex G-200 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. *t*-Butyl *S*-4,6-dimethylpyrimidin-2-ylthiolcarbonate (Boc-S) [16] was purchased from Kokusan Chemical Co., Tokyo, Japan. 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride was purchased from Protein Reseach Foundation, Osaka, Japan. Fast Garnet GBC salt (*o*-aminoazotoluenediazonium salt) and phenyl sulfonyl fluoride were purchsed from Sigma Chemical Co., Saint Louis, MI, U.S.A. All substrates, that is, L-alanine β-naphthylamide(L-Ala NA), L-arginine β-napthylamide (L-Arg NA), α-L-aspartic acid β-naphthylamide(L-Asp NA), L-glutamine β-naphthylamide (L-Gln NA), α-L-glutamic acid β-naphthylamide (L-Glu NA), γ-L-glutamic acid β-naphthylamide (γ-L-Glu NA), L-pyroglutamic acid β-naphthylamide (L-pyroGlu NA), L-glycine β-naphthylamide(L-Gly NA), L-leucine β-naphthylamide (L-Leu NA), L-lysine β-naphthylamide (L-Lys NA), L-methionine β-naphthylamide (L-Met NA), L-phenylalanine β-naphthylamide (L-Phe NA), L-tryptophan β-naphthylamide (L-Try NA), L-tyrosine β-naphthylamide(L-Tyr NA), glycyl-L-proline β-naphthylamide (Gly-L-Pro NA) and L-lysyl-L-ananyl β-naphthylamide(L-Lys-L-Ala NA), were purchased from Bachem Feinchemikalien AG, Switzerland. All divalent cation chloride compounds, that is, $CaCl_2$, $SrCl_2$, $BaCl_2$, $MnCl_2$, $MgCl_2$, $FeCl_2$, $CoCl_2$, $ZnCl_2$, $NiCl_2$, $CuCl_2$, $HgCl_2$ and $CdCl_2$, were purchased from Wako Pure Chemical Industries Ltd., Tokyo, Japan. Iodoacetic acid, iodoacetamide, *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, dithiothreitol, 2-mercaptoethanol, HCl · *o*-phenanthroline and EDTA were purchased from Wako Pure Chemical Industries Ltd., Tokyo, Japan. Trypsin (bovine pancreas, (α- and β-trypsin, EC 3.4.21.4)) and α-chymotrypsin type II (bovine pancreas, (chymotrypsin A and B, EC 3.4.21.1) were purchased from Worthington Biochemical Corporation and

Sigma, respectively. Leucine aminopeptidase (leucine aminopeptidase ('LAP'), EC 3.4.11.1) of pig kidney and calibration proteins to determine the molecular weights of proteins were purchased from Boehringer Mannheim GmbH · Biochemica, West Germany.

Methods

Homogenation was done with Ultra Turrax made by Tunke and Kungel KG, F.R.G. Ultracentrifuge was carried out with Hitachi 55P. Absorption at 280, 405 and 525 nm was measured with Hitachi Model 100-41 spectrophotometer.

Preparation of N-t-butyloxyl amastatin. To a solution of amastatin (100 mg) in H_2O (1.5 ml) were added triethylamine (0.042 ml) and a solution of Boc-S (55.2 mg) in dioxane (1.5 ml) and the mixture was reacted under stirring for 18 h at room temperature. After completion of the reaction H_2O (30 ml) was added to the reaction mixture, and the unreacted carbonate was extracted twice with 30 ml of ethyl acetate. Subsequently, the aqueous layer was adjusted to pH 2.0 by addition of 1 N HCl, and was extracted three times with 30 ml of ethyl acetate. Thereafter the ethyl acetate layer was washed three times with 60 ml of 5% HCl cooled at 0°C, and twice with 60 ml of a saturated aqueous NaCl solution and dried over anhydrous Na_2SO_4, and the ethyl acetate was evaporated in vacuo, yielding crystalline *N-t*-butyloxyl amastatin (34 mg, 30% yield), m.p. 120—122°C, $[\alpha]_D^{21}$ −33.0° (c = 1, methanol).
Analysis: Found: C, 53.89; H, 7.72; N, 9.06; O, 27.30.
Calcd. for $C_{26}H_{46}N_4O_{10}$: C, 54.33; H, 8.07; N, 9.75; O, 27.84%
NMR ($C^2H_3O^2H$); δ_{TMS} 1.42, (9H, s, $(CH_3)_3CO$—).

Preparation of amastatin-AH Sepharose 4B. About 2.7 ml of AH-Sepharose 4B washed with 0.5 M NaCl and water was mixed with 30 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 15.5 mg of *N-t*-butyloxyl amastatin in 5 ml of dioxan/H_2O (1 : 5, v/v). The mixture was left at room temperature for 24 h and during that period pH was continuously monitored and maintained at 5.4. After the reaction, the gel was packed in a small column (1.1 × 2.5 cm), and washed successively with each 10 ml of dioxan/H_2O (1 : 5, v/v) and H_2O. One-fourth of *N-t*-butyloxyl amastatin-AH Sepharose 4B thus prepared, about 0.5 ml, was suspended in 4 ml of 0.5 N HCl-methanol and incubated for 1 h at 37°C to remove the protective group. Thereafter, the suspension was poured into a column (1.1 × 0.5 cm) and washed with 50 ml of water. 1 ml of the wet gel was found to contain 10 μmol of covalently bound amastatin, which was determined by amino acid analysis of an acid hydrolyzate of the gel.

Assay of enzyme activity. The assay system contained the following components in a total volume of 1.0 ml in the test tube (1.5 × 10 cm): 0.25 ml of 2 mM of substrate solution, 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.5), an appropriate amount of enzyme, and water or reagent solution. The reagent solution was used in the case of testing an effect of the reagent on the enzyme activity. After 3 min incubation at 37°C, the reaction was initiated by addition of the enzyme solution. Studies on effect of temperature or metal ions on enzyme activity were carried out using the Ca^{2+}-free enzyme solution after dialysis against H_2O for 24 h. Exactly 30 min later, the reaction was stopped by

adding 1 ml of a solution of Fast Garnet GBC (1 mg/ml) in 1 M acetate buffer (pH 4.2), containing 10% Tween 20. After 20 min at room temperature, absorbance was measured at 525 nm.

One unit of enzyme activity was defined as the amount of enzyme producing 1 nmol of β-naphthylamine per min at 37°C under the assay condition. Specific activity was expressed as units/mg protein.

Determination of protein. Protein concentration was measured by the method of Lowry et al. [17] using bovine serum albumin as the standard. Protein concentration of individual fractions of column chromatography was estimated by measuring the absorbance at 280 nm in a 1.0 cm cell.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of the enzyme preparations at various steps of the purification was carried out by the method of Davis [18]. The stacking (2.5% acrylamide) and running (7.5% acrylamide) gels were polymerized at pH 6.7 and 8.9, respectively, in a Pyrex tube (0.8 × 12 cm). Electrophoresis was performed in a cold room (4°C) at 3 mA per gel at pH 8.3 (5 mM, Tris-Gly buffer). After electrophoresis, the gel was incubated in 2 mM of L-Glu NA for 1 h and stained with Fast Garnet GBC solution described above for 30 min.

Molecular weight estimation. An approximate molecular weight was determined by gel filtration on Sephadex G-200 according to the method of Whitaker [19] and Andrews [20]. Protein concentration was measured by absorbance at 280 nm and the method of Lowry et al. [17]. Catalase fraction was detected by its specific absorbance, the so-called Soret band [21,22], at 405 nm.

Aminopeptidase A purification. Pig kidney was frozen and stored at −20°C untile used. All procedures were carried out 0—4°C.

Step 1. About 26 g of kidney was homogenized in 260 ml of 0.06 M phosphate buffer (pH 7.4) containing 0.25 M sucrose with Ultra Turrax. The homogenate was filtered through silk gauze and the filtrate was centrifuged at 10 000 × *g* for 20 min.

Step 2. The supernatant obtained by Step 1 was centrifuged at 80 000 × *g* for 2 h. The precipitate was suspended in 35 ml of 0.01 M Tris-HCl buffer (pH 7.5) containing 1 mg each of trypsin and α-chymotrypsin. After standing at 37°C for 1 h, the suspension was centrifuged at 80 000 × *g* for 2 h.

Step 3. The supernatant obtained by Step 2 was applied to a 2 × 15 cm column containing DEAE Sepharose CL-6B which had been equilibrated with 0.06 M phosphate buffer (pH 7.5). The adsorbed protein was eluted by 1000 ml of the same buffer and the aminopeptidase A activity appeared in a single peak.

Step 4. 2 ml of the active solution (the fraction No. 3 and 4) was diluted with 8 ml of water and was applied to an amastatin-AH Sepharose 4B column (1.1 × 0.5 cm). After the column was washed with H_2O and 0.1 M Tris-HCl buffer (pH 7.5), aminopeptidase A was eluted with 0.1 M Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$. As shown in Fig. 1, aminopeptidase A appeared in fractions from No. 29 to 31 as a single peak. The volume of each fraction was 2.0 ml. This affinity column could be used repeatedly.

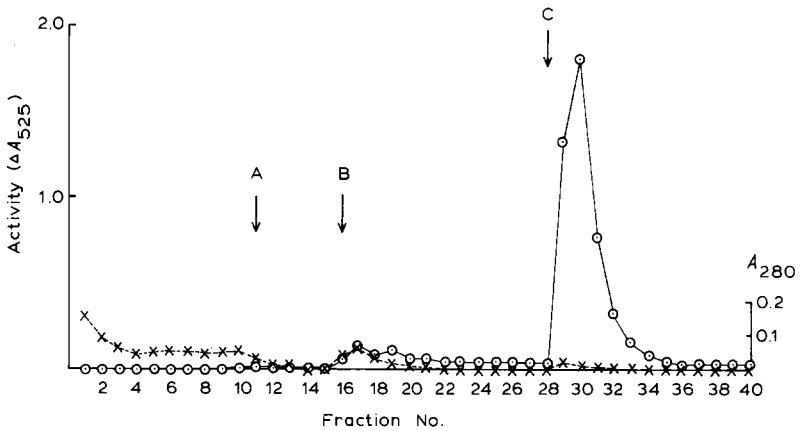

Fig. 1. Affinity chromatography of aminopeptidase A from pig kidney. The elution was performed with the following solutions, A, H_2O; B, 0.1 M Tris-HCl buffer (pH 7.5); C, 0.1 M Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$. Each fraction was 2 ml, and the enzyme activity against L-Glu NA with 50-μl aliquots of each fraction is demonstrated. ⊙———⊙, ΔA_{525}; X- - - - -X, A_{280}.

Results

Table I summarized the results of the purification procedure. The final recovery was 3.9% and the final purification was 710-fold.

Estimation of molecular weight of aminopeptidase A. The molecular size of the aminopeptidase A was estimated by gel filtration on Sephadex G-200. The calibration curve obtained with standard proteins indicated the molecular weight of 300 000.

Effect of pH on enzyme activity. The effect of pH on the rate of hydrolysis of L-Glu NA was studied. The pH-activity profiles of aminopeptidase A indicated pH optimum value of 7.5.

Effect of temperature on enzyme activity. The reaction mixture was allowed to react at various temperatures for 30 min. The purified enzyme was found to be thermostable up to 55°C. When the enzyme was incubated with 1 mM of $CaCl_2$, the enzyme activity was still to be restored at 65°C, but lost its activity

TABLE I

PURIFICATION OF AMINOPEPTIDASE A FROM PIG KIDNEY

	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification
Homogenate	270	2 100	67 900	32.3	100	1
Step 1 (Supernatant, 10 000 × *g* for 20 min)	265	1 480	41 700	28.2	61.4	0.9
Step 2 (Trypsin and Chymotrypsin treatment)	31	52.7	10 000	190	14.9	5.9
Step 3 (DEAE-Sepharose CL-6B)	18	2.25	1 960	870	2.9	27.0
Step 4 (Affinity column)	18	0.105	2 400	22 860	3.9	710

at 65°C when the enzyme was incubated without $CaCl_2$.

Effect of metal ions and reagents on enzyme activity. The effect of various divalent cations on the activity of aminopeptidase A was investigated (Table II). Ca^{2+}, Ba^{2+} and Sr^{2+} markedly activated the hydrolysis of L-Glu NA approx. 3-fold, but Zn^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+} and Cd^{2+} almost completely inhibited the activity at the concentration of 1 mM. The maximal activation of the enzyme was obtained with Ca^{2+} at 1—2 mM. Table III shows that the enzyme was not affected by sulfhydryl-reagents, S-S dissociating agents and serine proteinase inhibitor, but was inhibited completely by EDTA and *o*-phenanthroline at the concentration of 1 mM.

Substrate specificity. The relative rates of hydrolysis of varirou amino acid β-naphthylamides by the purified enzyme are examined. L-Glu NA was most rapidly hydrolyzed, while neutral and basic amino acid β-naphthylamides were not hydrolyzed. γ-L-Glu NA, L-pyroGlu NA, L-Gln NA and Gly-L-Pro NA were not also hydrolyzed.

Inhibition of aminopeptidase A by amastatin. Amastatin strongly inhibited aminopeptidase A at a fairly low concentration. Kinetic studies on amastatin were carried out using the purified and Ca^{2+}-free enzyme after dialysis against H_2O for 24 h. Amastatin inhibited aminopeptidase A in a competitive manner with L-Glu NA as substrate. In the absence of Ca^{2+}, K_i and K_m values were calculated to be $2.5 \cdot 10^{-7}$ M by Dixon plot [23] and $8 \cdot 10^{-4}$ M by Lineweaver-Burk plot [24], respectively. In the presence of $2.5 \cdot 10^{-4}$ M of Ca^{2+}, K_i and K_m values were $1 \cdot 10^{-6}$ M and $5 \cdot 10^{-4}$ M, respectively.

When amounts of Ca^{2+} were successively increased in the reaction mixture of aminopeptidase A and amastatin, this inhibitory effect of amastatin was not reversed, while that of *o*-phenanthroline on aminopeptidase A was reversed by this procedure (Fig. 2).

Inhibition of other arylamidases of pig kidney by amastatin. Amastatin, at the concentration of $1 \cdot 10^{-5}$ M, inhibited not only acidic amino acid β-naphthylamidase but also neutral amino acid β-naphthylamidases. It inhibited leucine aminopeptidase in a competitive manner with L-leucine NA as substrate, and

TABLE II

EFFECT OF DIVALENT CATIONS ON AMINOPEPTIDASE A ACTIVITY

Divalent cation (1 mM)	Activity
—	100
Ca^{2+}	328
Sr^{2+}	395
Ba^{2+}	281
Mn^{2+}	155
Mg^{2+}	120
Fe^{2+}	68
Co^{2+}	68
Zn^{2+}	14
Ni^{2+}	11
Cu^{2+}	2
Hg^{2+}	1
Cd^{2+}	0

TABLE III

EFFECT TO AMINOPEPTIDASE A ACTIVITY BY VARIOUS REAGENTS

Reagent	Concentration (mM)	Activity
None		100
EDTA	1	0
	0.1	7
o-Phenanthroline	1	0
	0.1	33
Iodoacetic acid	1	125
Iodoacetamide	1	118
p-Chloromercuribenzoate acid	1	104
N-Ethylmaleimide	1	97
Dithiothreitol	0.01 *	107
2-Mercaptoethanol	0.01 *	102
Phenyl sulfonyl fluoride	1	102

* These concentrations were chosen because S-S dissociating agents inhibited color reaction between Fast Garnet GBC salt and β-naphthylamine in the concentration of S-S dissociating agents higher than 0.01 mM.

the K_i and K_m values were calculated to be $1.6 \cdot 10^{-6}$ M and $3.7 \cdot 10^{-3}$ M, respectively. This leucine aminopeptidase had no activity to hydrolyze L-Glu NA and L-Arg NA under our assay condition. Amastatin did not inhibit dipeptidyl aminopeptidases.

Comparison of aminopeptidase A of pig kidney, pig serum and human serum. Studies on polyacrylamide gel disc electrophoresis of aminopeptidase A of pig kidney, pig serum and human serum showed that aminopeptidase A from

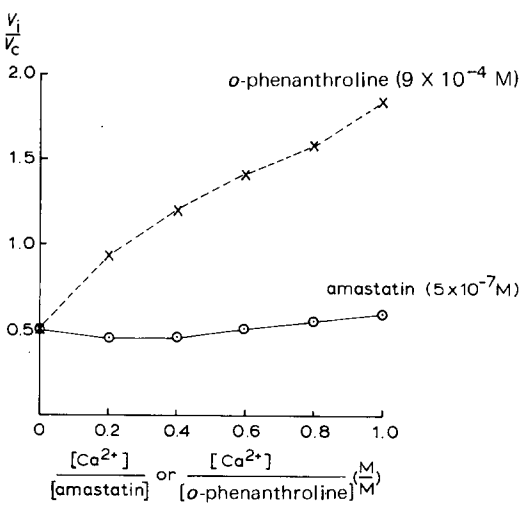

Fig. 2. Reversal test of the inhibition of aminopeptidase A by amastatin or *o*-phenanthroline on the addition of Ca^{2+}. Each cuvette contained 1 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.5 mM L-Glu NA, and $5 \cdot 10^{-7}$ M amastatin or $9 \cdot 10^{-4}$ M *o*-phenanthroline. V_i is the initial rate of reaction observed in the presence of inhibitor and Ca^{2+}. V_c is the initial rate observed in a simultaneous control assay from which the inhibitor and Ca^{2+} were omitted. ⊙———⊙, Amastatin ($5 \cdot 10^{-7}$ M) in the reaction mixture; X- - - - -X, *o*-phenanthroline ($9 \cdot 10^{-4}$ M) in the reaction mixture.

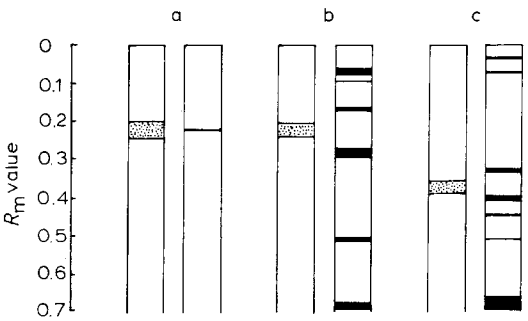

Fig. 3. Polyacrylamide-gel disc electrophoresis of aminopeptidase A of pig kidney, pig serum and human serum. a, b and c are purified pig kidney aminopeptidase A, pig serum and human serum. a is 1.0 μg, b, 100 μl and c, 100 μl. The gels on the left of the pair were stained for enzyme activity to hydrolyze L-Glu NA. The gels on the right were stained for protein with 0.25% Coomassie brilliant blue and were destained in 7% acetic acid. R_m value was calculated from the ratio: mobility of aminopeptidase A to that of bromophenol blue.

pig kidney and serum had a similar mobility (R_m value 0.22) but that human serum aminopeptidase A had a large mobility (R_m 0.37) as shown in Fig. 3. The approximate molecular weight of human serum aminopeptidase A determined by Sephadex G-200 gel filtration, in the same manner as the purified pig kidney, was 210 000. From these results, it may be that pig kidney aminopeptidase A is different from human serum aminopeptidase A. IC_{50} value, concentration of amastatin to inhibit 50% of the enzyme activity, was $4.5 \cdot 10^{-7}$ M against 0.04 μg of the purified pig kidney aminopeptidase A, $4.0 \cdot 10^{-6}$ M against aminopeptidase A in 100 μl of pig serum and $1.4 \cdot 10^{-7}$ M against aminopeptidase A in 100 μl of human serum.

Discussion

Amastatin was effective in binding the aminopeptidase A and other aminopeptidases and separating them from dipeptidyl aminopeptidases. Amastatin-affinity column adsorbed aminopeptidase A, leucine aminopeptidase and other arylamidases, all of which were inhibited by amastatin. But the column did not adsorb dipeptidyl aminopeptidases, because they were not inhibited by amastatin. Among aminopeptidases adsorbed by the column, only aminopeptidase A was eluted from the column with 10 mM $CaCl_2$. This should be due to the effect of Ca^{2+} to reduce the binding affinity of aminopeptidase A to amastatin on the column. This idea was supported by kinetic studies which showed that the presence of $2.5 \cdot 10^{-4}$ M of Ca^{2+} increased the K_i value by 24-fold. The other aminopeptidases were not eluted from the column with 10 mM $CaCl_2$ because they were not sensitive to Ca^{2+}.

From the study of the substrate specificity, it was found that aminopeptidase A was four times more active on L-Glu NA than on L-Asp NA. A free α-amino group and γ-carboxyl group were required in a substrate for enzyme activity, because L-pyroGlu NA, γ-L-Glu NA and L-Gln NA were not hydrolyzed.

Table III suggests that free sulfhydryl groups and a reactive serine residue are

not involved in the enzyme activity. The enzyme activity was inhibited completely by some chelating agents such as EDTA and *o*-phenanthroline, and reactivated by the addition of Ca^{2+} as shown in Fig. 2. This supported that pig kidney aminopeptidase A is a metalloenzyme. Recently, human liver alanine aminopeptidase, which is activated by Co^{2+} but not by Ca^{2+}, was described to be a zinc metalloenzyme containing one atom of zinc per molecule of enzyme [25].

In the present article we showed that amastatin is a suitable ligand in the affinity column for purification of aminopeptidase A. The purified preparation of aminopeptidase A obtained from amastatin-Sepharose column was employed for the comparison of properties among related enzymes.

Optimal pH, the activation by alkaline earth metals, the substrate specificity and sensitivity to amastatin of pig kidney aminopeptidase A was similar to those of human serum aminopeptidase A [14]. But it was concluded that pig kidney aminopeptidase A showed different behavior from human serum aminopeptidase A in acrylamide gel disc electrophoresis and had a different molecular weight estimated by gel filtration. Cheung and Cushman [26] reported that the aspartate aminopeptidase from dog kidney was neither stimulated by Ca^{2+} nor inhibited by EDTA, but was activated by preincubation with $MnCl_2$. It hydrolyzed L-Asp NA three times more rapidly than L-Glu NA. These properties of aspartate aminopeptidase of dog kidney are different from those of pig kidney and human serum aminopeptidase A. Pig serum aminopeptidase A coincided with pig kidney aminopeptidase A in acrylamide gel disc electrophoresis and the sensitivity to amastatin.

Kenny, et al. [27—29] reported that aminopeptidase A is one of microvillar enzymes which were solubilized by detergent or papain. The molecular weights of the detergent forms of microvillar enzymes are greater than those of the proteinase forms. The two forms also differ in electrophoretic mobility. We will attempt to solubilize aminopeptidase A by not only trypsin-chymotrypsin mixture but also papain or detergent, and to compare the proteinase form enzymologically with the detergent form.

Acknowledgements

This work was partially supported by a contract from the Division of Cancer Treatment, the National Cancer Institute, N01-CM-57009 and the Ministry of Education, Science and Culture.

References

1 Umezawa, H. (1972) Enzyme Inhibitors of Microbial Origin, Univ. of Tokyo Press, Tokyo
2 Aoyagi, T. and Umezawa, H. (1975) Proteases and Biological Control (Reich, E., Rifkin, D.B. and Shaw, E., eds.), Vol. 2, pp. 429, Cold Spring Harbor Conferences on Cell Proliferation.
3 Aoyagi, T., Tobe, H., Kojima, F., Hamada, M., Takeuchi, T. and Umezawa, H. (1978). J. Antibiot. 31, 636—638
4 Tobe, H., Morishima, H., Naganawa, H., Takita, T., Aoyagi, T. and Umezawa, H. (1979) Agric. Biol. Chem. 43, 591—596
5 Glenner, G.G., McMillan, P.J. and Folk, J.E. (1962) Nature 194, 867
6 Nagatsu, I., Gillespie, L., Folk, J.E. and Glenner, G.G. (1965) Biochem. Pharmacol. 14, 721—728
7 Nagatsu, I., Gillespie, L., George, J.M., Folk, J.E. and Glenner, G.G. (1965) Biochem. Pharmacol. 14, 853—861

8 Nagatsu, I., Nagatsu, T. and Yamamoto, T. (1968) Experientia 24, 347—348
9 Oya, H., Nagatsu, I., Harada, M. and Nagatsu, T. (1970) Experientia 26, 252—253
10 Nagatsu, I., Nagatsu, T. and Glenner, G.G. (1968) Enzymologia 34, 73—76
11 Klairallah, P.A., Bumpus, F.M., Page, I.H. and Smeby, R.R. (1963) Science 140, 672—674
12 Francis, J.B. and Mayr, N.S. (1969) Arch. Biochem. Biophys. 131, 74—82
13 Hess, R. (1965) Biochim. Biophys. Acta 99, 316—324
14 Nagatsu, I., Nagatsu, T., Yamamoto, T., Glenner, G.G. and Mehl, J.W. (1970) Biochim. Biophys. Acta 198, 255—270
15 Andria, G., Marzi, A. and Auricchio, S. (1976) Biochim. Biophys. Acta 419, 42—50
16 Nagasawa, T., Kuroiwa, K., Narita, K. and Isawa, Y. (1973) Bull. Chem. Soc. Jpn. 46, 1269—1272
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
19 Whitaker, J.R. (1963) Anal. Chem. 35, 1950—1953
20 Andrews, P. (1964) Biochem. J. 96, 595—606
21 Samejima, T. and Yang, J.T. (1963) J. Biol. Chem. 238, 3256—3261
22 Hiraga, M., Anan, F.K. and Abe, K. (1964) J. Biochem. 56, 416—423
23 Dixon, M. (1953) Biochem. J. 55, 170—171
24 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658—666
25 Garner, C.W. and Behal, F.J. (1974) Biochemistry 13, 3227—3232
26 Cheung, H.S. and Cushman, D.W. (1971) Biochim. Biophys. Acta 242, 190—193
27 George, S.G. and Kenny, A.J. (1973) Biochem. J. 134, 43—57
28 Andrew, G.B., Lucinda, M.L. and Kenny, A.J. (1979) Biochem. J. 179, 397—405
29 Kenny, A.J. and Booth, A.G. (1978) Essays Biochem. 14, 1—44