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PURIFICATION AND PROPERTIES OF *N*-FORMYLMETHIONINE AMINOPEPTIDASE FROM RAT LIVER

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Summary

A specific enzyme for the liberation of N-terminal *N*-formylmethionine from *N*-formylmethionyl peptides was purified 4750-fold from rat liver by successive applications of $(NH_4)_2SO_4$ precipitation, DEAE-cellulose, *N*-formylbestatin-AH-Sepharose 4B and AH-Sepharose 4B chromatography followed by Sepharose CL-6B gel filtration. The molecular weight was determined by gel filtration on Sepharose CL-6B as 290 000 ± 5000. This was suggested to be a tetramer consisting of a subunit which was shown by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis to have a 72 000 ± 2000 molecular weight.

The optimum pH of the enzyme was 7.8. Cd^{2+} and Hg^{2+} were highly toxic to the enzyme. Michaelis constants of *N*-formylmethionyl leucine and *N*-formylmethionine β-naphthylamide were 0.03 and 0.2 mM, respectively.

Introduction

It has been suggested that both *N*-formylmethionine (fMet)-releasing enzyme and methionine aminopeptidase (EC 3.4.11.—) are involved in protein synthesis in rabbit reticulocytes; they were separated by the staining method on starch gel electrophoresis.

Recently, interesting functions of fMet peptides were reported: Shiffmann et al. [1] found that fMet peptides could be chemoattractants for leukocytes;

Abbreviations: Tos-Arg-OMe, *p*-tosyl-L-arginine methyl ester; Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; Bz-Arg-OEt, *N*-benzoyl-L-arginine ethyl ester; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride.

Showell et al. [2] reported that there might be a specific receptor for chemotactically active fMet peptides on plasma membrane of polymorphonuclear leukocytes and this receptor might play an important role in the immune surveillance mechanism. Aswanikumar et al. [3] demonstrated the presence of a receptor for these chemotactic peptides on rabbit neutrophils. fMet peptides were hydrolyzed by the incubation with neutrophils [1]. These observations suggest that fMet-releasing enzyme may be involved in chemoattractance phenomenon.

We attempted the purification and characterization of fMet-releasing enzyme from rat liver.

Materials and Methods

Commercially obtained L-amino acid β-naphthylamides were formylated as described by Sheehan and Yang [4]. Bestatin, (2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-(S)-leucine, was prepared as described previously [5,6] and *N*-formylbestatin was also prepared. Tos-Arg-OMe was obtained from Protein Research Foundation, Osaka; Ac-Tyr-OEt from Tokyo Kasei Co., Tokyo; Bz-Arg-OEt from Sigma Chemical Co., St. Louis; Met-β-naphthylamide, Trp-β-naphthylamide, Tyr-β-naphthylamide and Ser-Tyr-β-naphthylamide from Bachem Co., CA.; Ser-β-naphthylamide, Val-β-naphthylamide and Phe-β-naphthylamide from Protein Research Foundation, Osaka; Leu-β-naphthylamide from Tokyo Kasei Co.; EDC from Nakarai Chemicals, Kyoto; DE-52 from Whatman, U.K.; AH-Sepharose 4B and Sepharose CL-6B from Pharmacia, Sweden. Ferritin and catalase (EC 1.11.1.6) were purchased from Boehringer Mannheim GmbH, F.R.G. Aminopeptidase B (EC 3.4.11.6) was purified by the method of Hopsu et al. [7]. All other reagents were of the best commercial grade.

Enzyme assay. L-Amino acid β-naphthylamide-hydrolyzing activity was measured by the method reported previously [8] using Tris-HCl buffer (pH 7.8).

N-Protected L-amino acid β-naphthylamide-hydrolyzing activity was routinely measured as follows: N-protected L-amino acid β-naphthylamides were dissolved in methanol containing 5% (w/v) Tween 20, at a concentration of 20 mM, and diluted to indicated concentrations with 0.05 M Tris-HCl buffer (pH 7.8). After the reaction mixture was incubated at 37°C for 3 min, an appropriate amount of the enzyme solution (5—50 μl) was added and incubated for 25 min; during the incubation the enzyme reaction proceeded linearly with the time or the enzyme concentrations; liberated β-naphthylamine was measured by the absorbance at 525 nm as reported [8].

Activity of the purified enzyme was determined by the method described by Hummel et al. [9] in phosphate-buffered saline containing Tos-Arg-OMe (7.6 mM), Bz-Arg-OEt (7.6 mM) or Ac-Tyr-OEt (1.6 mM) at 37°C for 1 h.

Protein determination. Protein content was measured by the method of Lowry et al. [10] using crystalline bovine serum albumin as a standard.

Preparation of affinity adsorbent. EDC (400 mg, 2.09 mmol) was added to a solution of *N*-formylbestatin (40 mg, 0.12 mmol), which was dissolved as its sodium salt in 5 ml water by adding 0.06 ml of 1 M sodium hydroxide. The solution was stirred for 30 min at room temperature keeping the pH at 5.0 with

1 M sodium hydroxide and, thereafter, washed AH-Sepharose 4B (24 ml) was added and the suspension was stirred for 48 h at room temperature. At the end of this reaction, the *N*-formylbestatin-linked AH-Sepharose 4B was isolated by filtration and washed with 500 ml water and 500 ml 0.5 M NaCl, successively. The remaining amino groups of *N*-formylbestatin-linked AH-Sepharose 4B were masked with an excess amount of acetic anhydride (1 ml), keeping the pH of the suspension at 9.0 with 0.5 M sodium bicarbonate for 30 min at room temperature. The *N*-formylbestatin-linked AH-Sepharose 4B thus obtained was washed with 500 ml water. It was negative to the trinitrobenzene sulfonate test [11]. The *N*-formylbestatin content of the adsorbent was estimated to be 5 μmol/ml by determination of the leucine content after acid hydrolysis (6 M HCl, 16 h). The leucine content of the hydrolysate was determined by TLC (Silica gel F_{254}, E. Merck, F.R.G. Solvent: *n*-butanol/acetic acid/water (4 : 1 : 1, v/v) using Chromoscan 200, Joyce-Loebl Ltd., U.K.).

Polyacrylamide gel electrophoresis. Analytical polyacrylamide gel electrophoresis was carried out following the procedure of Davis [12]. The discontinuous gels contained 7.5% acrylamide (5 mm × 8 cm) stacked at pH 6.7 and run at pH 8.9. Electrophoresis was performed on 20 μg protein at 3 mA per tube, 4°C for 2 h. Gels were fixed with 7% acetic acid and stained with 0.025% Coomassie brilliant blue for 45 min and destained in 7% acetic acid using a Pharmacia Gel Destainer GD-4 at 24 V for 45 min.

SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn [13] with a slight modification. Gels contained 10% acrylamide and 0.27% *N,N'*-methylene-bisacrylamide, and were run in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.2% mercaptoethanol and 0.1% SDS. Samples were treated for 15 min at 50°C with 0.2% SDS in the above buffer and applied with the running buffer containing 50% glycerol and 0.005% bromophenol blue. Electrophoresis was performed at 3 mA per tube at 15°C using an LKB 2103 power supply and Gel Electrophoresis Apparatus GE-4 (Pharmacia). The gel was stained with 0.25% Coomassie brilliant blue in a mixture of 454 ml of 50% methanol and 46 ml acetic acid for 2 h and destained with 7% acetic acid using Gel Destainer GD-4, at 24 V for 90 min. For the location of bands, the absorbance was measured at 590 nm using Chromoscan 200.

Results

Cleavage of fMet-β-naphthylamide by several mammalian organ homogenates

fMet-β-naphthylamide was hydrolyzed after incubation with homogenates of rat liver, kidney and spleen or bovine liver and kidney as shown in Table I. Met-β-naphthylamide-hydrolyzing activities were determined at the same time for comparison. Each organ (4 g) was homogenized in 15 ml of 0.1 M Tris-HCl buffer (pH 7.8) with a Teflon homogenizer and filled up to 24 ml. The homogenate (25 μl) thus obtained was tested for the enzyme activity under the standard assay conditions (20 min at 37°C). The results indicated that fMet-β-naphthylamide-hydrolyzing activity did not correlate with Met-β-naphthylamide-hydrolyzing activity. In addition, bestatin which is a specific inhibitor of aminopeptidase B and leucine aminopeptidase [8] did not show any inhibition

TABLE I

fMet-β-NAPHTHYLAMIDE AND Met-β-NAPHTHYLAMIDE HYDROLYZING ACTIVITY OF RAT AND BOVINE ORGANS

Enzyme activity was expressed in nmol β-naphthylamide hydrolyzed/mg organ per h. NNap, naphthylamide.

Organ	Enzyme activity		
	fMet-β-NNap	Met-β-NNap	Ratio *
Rat			
Liver	0.60	2.8	0.21
Kidney	0.46	3.4	0.14
Spleen	0.49	1.1	0.45
Bovine			
Liver	0.92	2.0	0.46
Kidney	0.62	4.9	0.13

* Each value for fMet-β-naphthylamide was divided by that for Met-β-naphthylamide.

at 0.1 mM for the hydrolysis of fMet-β-naphthylamide, while Met-β-naphthylamine-hydrolyzing activity was completely inhibited by this inhibitor. The results indicate that the enzyme capable of hydrolyzing the amide bond between fMet and β-naphthylamine is different from deformylase and methionine aminopeptidase.

Purification of fMet-releasing enzyme from rat liver

Rat liver was chosen as the source of fMet-releasing enzyme which hydrolyzes fMet-β-naphthylamide to fMet and β-naphthylamine. The following procedure resulted in 750-fold purification with a 17% yield. The results of a typical purification are given in Table II. All steps were carried out at 0—4°C.

Step 1. Preparation of the crude extract. Male albino rats of the Wister strain, weighing 150—200 g were used. Rat liver (100 g) was minced with scissors and homogenized in 1 l of 0.1 M Tris-HCl buffer (pH 7.8) with a Teflon homogenizer. After debris was removed by filtration through four layers of cheesecloth, the homogenate was centrifuged at 15 000 × *g* for 30 min. More than 90% of the enzyme activity was found in the supernatant.

Step 2. $(NH_4)_2SO_4$ precipitation. $(NH_4)_2SO_4$ powder (280 g) was added

TABLE II

PURIFICATION OF fMet AMINOPEPTIDASE FROM RAT LIVER

Specific activity was expressed in μmol β-naphthylamine hydrolyzed/mg protein per h.

Isolation step	Volume (ml)	Protein (mg)	Specific activity	Yield (%)	Purification
1. 15 000 × *g* supernatant	820	9760	0.21	100	1
2. $(NH_4)_2SO_4$ (45% saturation)	200	3570	0.48	82	2.3
3. DEAE-cellulose	378	570	2.2	59	10.4
4. Affinity chromatography	55	11	86	45	408
5. AH-Sepharose 4B	60	2.7	218	28	1040
6. Sepharose CL-6B	20	0.35	997	17	4750

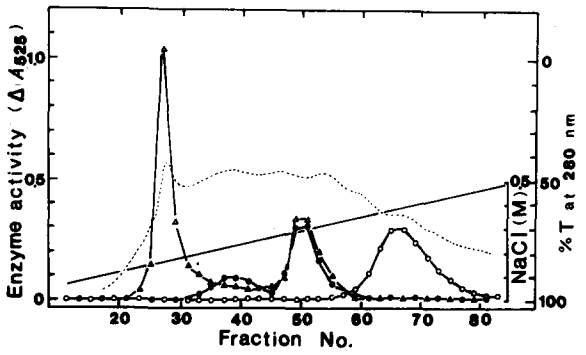

Fig. 1. Chromatographic profile of 45% $(NH_4)_2SO_4$ precipitate on DEAE-cellulose (DE-52). Specific details of the chromatographic procedure are described in the text. Enzyme activities were measured for 15 μl of each fraction incubated for 20 min at 37°C using the assay method described. The substrates used were fMet-β-naphthylamide (○———○), Met-β-naphthaylamide (●———●) and Arg-β-naphthylamide (△———△).

slowly with stirring to the supernatant described above, up to 45% saturation. After the addition, the suspension was allowed to stand for 2 h and was centrifuged at 3000 × *g* for 30 min. The supernatant was discarded and the precipitate was dissolved in 150 ml of 0.01 M Tris-HCl buffer (pH 7.8). The solution was dialyzed against 10 l of the same buffer for 16 h.

Step 3. DEAE-cellulose chromatography. The material from Step 2 was chromatographed on a column of DE-52 (4 × 20 cm) previously washed with 4 column vols. of 0.01 M Tris-HCl buffer (pH 7.8). The column was eluted with a linear gradient of NaCl (up to 0.5 M) in the same buffer, total vol. 1.2 l. Fractions of 15 g were collected at a flow rate of 100 ml per h. As shown in Fig. 1, separation of fMet-aminopeptidase from Met-aminopeptidase was achieved in this step.

Step 4. Affinity chromatography. *N*-Formylbestatin inhibited fMet aminopeptidase with an IC_{50} value at 1 mM and was not hydrolyzed by prolonged incubation with this enzyme. Therefore, it was chosen as an affinity ligand. The affinity adsorbent, *N*-formylbestatin-AH-Sepharose 4B prepared as described in Materials and Methods, was poured into a column (2.5 × 20 cm) to yield a 20 ml packed volume, and the column was equilibrated with 0.01 M Tris-HCl buffer (pH 7.8). The enzyme solution from Step 3 was diluted with 3 vols. of 0.05 M Tris-HCl buffer (pH 7.8) and passed through the column at a flow rate of 50 ml per h. Thereafter, the column was washed with 200 ml of 0.05 M Tris-HCl buffer (pH 7.8) and the enzyme was eluted with a linear gradient of NaCl (up to 0.4 M) in 0.05 M Tris-HCl buffer (pH 7.8, total vol. 400 ml) at a flow rate of 50 ml per h. Fractions of 10 g each were collected. The fractions with peak activity were combined. About 40-fold purification of the enzyme was achieved by this affinity chromatography with 76% yield.

Step 5. AH-Sepharose 4B chromatography. The enzyme preparation from Step 4 was diluted 3-fold with 0.05 M Tris-HCl buffer (pH 7.8) and chromatographed on a column of AH-Sepharose 4B (2.5 × 5 cm) equilibrated with the same buffer, with a linear gradient of NaCl (0.2—0.8 M) in 0.05 M Tris-HCl buffer (pH 7.8, total vol. 400 ml) at a flow rate of 50 ml per h. Fractions of

10 g each were collected. The enzyme activity was concentrated in the fractions 20 to 25.

Step 6. Gel filtration on Sepharose CL-6B. The active fractions from Step 5 were concentrated using an Amicon PM-10 membrane and chromatographed on a column of Sepharose CL-6B (2.4 × 82 cm), using 0.05 M Tris-HCl buffer (pH 7.8) at a flow rate of 20 ml per h. Fractions of 5 g each were collected. Each fraction with high enzyme activity was tested for its purity by analytical polyacrylamide gel electrophoresis, using Tris-glycine system with 7.5% (5 mm × 8 cm). The enzyme (20 μg) was purified as a single band by this gel filtration. 1 μg of the isolated enzyme hydrolyzed fMet-β-naphthylamide at a rate of 16.3 nmol per min under standard assay conditions.

Molecular weight determination of the enzyme and its subunit

The molecular weight of the isolated enzyme was determined by gel filtration method using a column of Sepharose CL-6B (2.4 × 82 cm) under the same condition used in purification Step 6. Ferritin, catalase and aminopeptidase B were used as calibration proteins. The molecular weight of the enzyme was shown to be 290 000 ± 5000.

SDS-polyacrylamide gel electrophoresis indicated only one subunit having a molecular weight of 72 000 ± 2000.

Optimum pH and Cl^- activation

Optimum pH for the enzyme was determined using sodium phosphate buffer (pH 5.5—8.0), Tris-HCl buffer (pH 6.8—9.3) and glycine-NaOH buffer (pH 8.7—10.1) under standard assay conditions. The results are shown in Fig. 2. Among the buffer systems used, Tris-HCl buffer was shown to be preferable for the enzyme having optimum pH about 7.8. Using sodium phosphate buffer (pH 8.0) at a concentration of 0.05 M, the effect of Cl^- was examined as shown in Fig. 3. The results showed that the enzyme was activated by Cl^-.

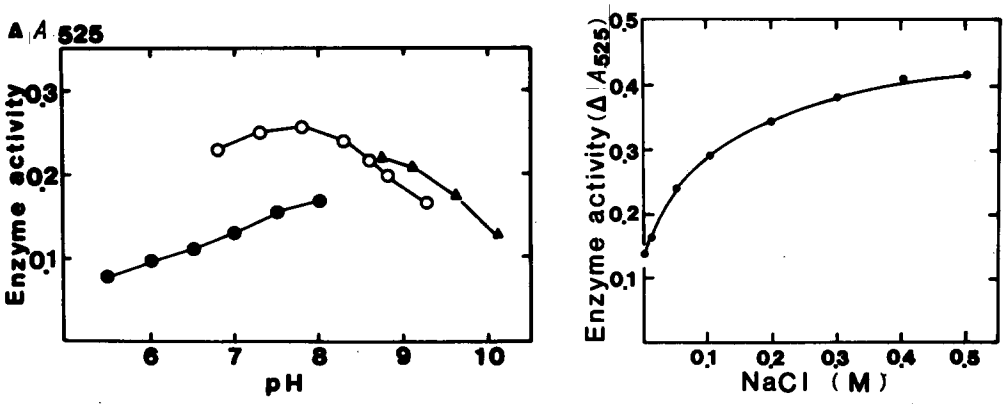

Fig. 2. Activity of fMet-aminopeptidase toward fMet-β-naphthylamide substrate buffered as described in the text at pH values shown. The experiments were performed at 37°C for a period of 20 min. The buffer systems used were sodium phosphate buffer (●——●), Tris-HCl buffer (○——○) and glycine-NaOH buffer (▲——▲).

Fig. 3. Effect of the addition of sodium chloride to the standard assay system. The assay conditions are described in the text.

Heat stability

Varying the incubation temperature under the standard assay condition, the heat stability of the enzyme was examined. The enzyme was shown to be fairly stable at high temperature up to 50°C.

Inhibition by metal ions

Inhibition by metal ions at a concentration of 0.1 mM was examined under standard assay conditions. The enzyme was not inhibited by Mg^{2+}, Ca^{2+}, Sr^{2+}, Mn^{2+}, Co^{2+} or Ni^{2+}, but markedly inhibited by Cd^{2+} and Hg^{2+}. Moderate inhibition was observed by Fe^{2+}, Cu^{2+} and Zn^{2+}.

Inhibition by enzyme inhibitors

The enzyme was weakly inhibited by *N*-ethylmaleimide and phenylmethanesulfonyl fluoride at a concentration of 3 mM, but not by EDTA. Effects of protease inhibitors of microbial origin were also examined. Leupeptin, antipain, pepstatin, chymostatin, phosphoramidon, elastatinal, bestatin and amastatin at a concentration of 0.1 mM did not show any inhibition.

Substrate specificity

The susceptibility of two series of amino acid β-naphthylamides and *N*-formylamino acid β-naphthylamides to fMet-releasing enzyme was assayed using the method described above. The following β-naphthylamide derivatives of amino acids were not hydrolyzed; Arg-, Asp-, Ser-, Pro-, Leu-, Met-, Phe-, Trp-, Gly-Pro-, Ser-Tyr-, Gly-Pro-Leu- and Gly-His-Lys-β-naphthylamide. Among the β-naphthylamide derivatives of *N*-formylamino acids, i.e., fMet-, fSer-, fVal-, fLeu-, fPhe-, fTyr-, fTrp- and fSer-Met-β-naphthylamide, only fMet-β-naphthylamide was hydrolyzed. The enzyme did not hydrolyze Tos-Arg-OMe, Bz-Arg-OEt or Ac-Tyr-OEt. Therefore it was shown that the enzyme recognized NH_2-blocked methionine residue specifically.

Kinetic properties

The purified fMet-releasing enzyme was confirmed to hydrolyze fMet-Leu at pH 7.8, measuring the amount of liberated leucine by the usual ninhydrin method. Kinetic values were calculated from the results of the duplicate assay and K_m and V were obtained from the Lineweaver-Burk plots. The value for k_{cat} was obtained by dividing V by the concentration of enzyme in μmol per ml of reaction mixture. Apparent K_m of fMet-β-naphthylamide and fMet-Leu were 0.2 and 0.03 mM, respectively. V for fMet-β-naphthylamide was 6.5 nmol/min and k_{cat} for fMet-β-naphthylamide was $13.8 \cdot 10^3$ min^{-1}.

Discussion

We have studied the enzymes on mammalian cell surfaces, which are considered to play an important role in physiological phenomena including immune responses, and their inhibitors from microbial origin [14,15]. For example, bestatin, a specific inhibitor of aminopeptidase B and leucine aminopeptidase, enhanced cell-mediated immune reactions [16]. fMet-releasing enzyme is also located on the cell surface and has been reported to have a role

in chemotaxis. Hence, we were interested in this enzyme and successful with its purification. However, only a few efforts have been made to study the fMet-peptide hydrolysing enzyme itself [17]. This enzyme is known to be contained in rat liver in a high content, and we purified the enzyme, testing the activity to hydrolyze fMet-β-naphthylamide as a substrate, although the relationship of fMet aminopeptidase on the cell surface to that in the cytoplasm remains to be explored. *N*-Formylbestatin was found to act as a specific inhibitor of this enzyme but not a substrate. Therefore, during the course of purification, affinity chromatography of *N*-formylbestatin-AH-Sepharose 4B was effectively used and the enzyme was purified as a single band in analytical polyacrylamide gel electrophoresis. The purified enzyme was very specific to fMet-peptides. Further characterization of the enzyme will be reported in following papers.

We found a strong inhibitor for the enzyme in a culture filtrate of actinomycetes. The purification of this substance is in progress. Such an inhibitor is thought to be useful in analysis of chemotaxis and the relationship of this enzyme and chemoattractant receptor.

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