Purification and Some Properties of Two Aminopeptidases from Sardines

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Two fish aminopeptidases designated as aminopeptidases I and II were purified by DEAE-cellulose chromatography, gel filtration on Sephadex G-200, and isoelectric focusing. The final preparations of enzymes I and II were judged nearly homogenous by polyacrylamide gel electrophoresis. The molecular weights of enzymes I and II were determined by gel filtration to be 370,000 and 320,000, respectively. The isoelectric points were 4.1 (I) and 4.8 (II). Both enzymes were inhibited by EDTA and activated by Co^{++} . Bestatin could inhibit enzyme I but not enzyme II. Enzymes I and II rapidly hydrolyzed not only synthetic substrates containing alanine or leucine but also di-, tri-, and tetra-alanine. Judged from all of these properties, sardine aminopeptidases resemble human alanine aminopeptidase. Enzyme I retained more than 70% of its original activity in 15% NaCl, suggesting the enzyme participates in hydrolyzing fish proteins and peptides during fish sauce production.

As described in our previously paper,^{1,2)} we isolated and characterized two acid and three alkaline proteases in sardines. Furthermore, we³⁾ observed that an acid protease (II) and an alkaline protease (III) in sardines were stable for three months after the beginning of fish sauce production in the presence of 22% NaCl.

On the other hand, peptides and amino acids in fish sauce markedly increased during its production, suggesting the important role of exo-peptidase(s) or exo-protease(s) in fish sauce production.⁴⁾

Very recently, we found⁴⁾ that aminopeptidase(s) in sardines were stable for three months after the beginning of fish sauce production. However, no detailed studies on aminopeptidases in fish have been done although there has been extensive work on marine bacteria,⁵⁾ microorganisms,⁶⁾ mammalian tissues,^{7,8)} human kidney,⁹⁾ and plant sources.¹⁰⁾

In the present paper, we describe the isolation and some of properties of two aminopeptidases that are present in the internal organs of the sardine.

MATERIALS AND METHODS

Sardine. Sardines (*Sardinops melanosticta*) were caught near Choshi on the Pacific coast of Japan. In all, about 2 hr elapsed from the time the fish were caught until they were completely frozen at -60°C . They were frozen for 3~4 months until their use in our experiments.

Materials. Fast blue B salt (*O*-dianisidine, tetrazotized), L-alanine- β -naphthylamide, L-leucine- β -naphthylamide, L-alanyl-alanine (Ala-Ala), L-leucyl-glycine (Leu-Gly) and L-glycyl-alanyl-alanine (Gly-Ala-Ala) were purchased from Sigma Chemical Co. L-Leucyl-tyrosine (Leu-Tyr), L-leucyl-alanine (Leu-Ala) were obtained from the Protein Research Foundation, Osaka, and Servalyt from Feinbiochemica, Heidelberg. Bestatin was kindly supplied by Dr. Aoyagi. DEAE-cellulose was from Whatman, and Sephadex G-200 from Pharmacia. All other chemicals were of reagent grade and used without further purification.

Protein determination. Protein concentration was determined spectrophotometrically, assuming an absorbance at the concentration of 1 mg/ml at 280 nm to be 1.0.

Enzyme assays. These were two assays of aminopeptidase activity. In the first, activity against leu- β -naphthylamide was measured according to the method of Gomori¹¹⁾; the enzyme was incubated at 37°C for 30 min with 0.25 ml of 0.2 mM leu- β -naphthylamide and 0.5 ml of 0.2 M phosphate buffer (pH 7.0) in a total volume of 1 ml.

The reaction was stopped by addition of 0.5 ml of 40% (w/v) trichloroacetic acid. Diazotization of the liberated β -naphthylamine and color development were performed using the procedure described by Gomori.¹¹⁾ The absorbance of the chromophore produced was measured at 570 nm. In the second assay, the rate of release of *p*-nitroanilide was measured as the change in absorbance at 405 nm.¹²⁾ One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μ mol of β -naphthylamine or *p*-nitroanilide per minute under these conditions.

Determination of molecular weight. The molecular weight of the enzyme was determined by gel filtration on a Sephadex G-200 column (1.6 $\times$ 90 cm).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out in 7.5% gel containing Tris-HCl buffer, pH 8.9, according to the method of Davis.¹³⁾ Proteins were stained with 0.1% Coomassie brilliant blue G-250 in 50% trichloroacetic acid. The enzyme was located by incubation of gel for 15 min at 37°C in 0.06 M acetate buffer, pH 5.5, containing 0.1% Fast blue B salt and 2% leu- β -naphthylamide.

Determination of isoelectric point. Isoelectric focusing was conducted using carrier Servalyts (pH 3~7) according to the method of Vesterberg and Svensson.¹⁴⁾ After the focusing was carried out with a constant potential of 900 V for 24 hr at 4°C, the aminopeptidase activity and pH of the eluate were determined.

Extraction of enzyme. All the following procedures were performed at 4°C. One hundred g of the internal organs of sardines were defatted by being homogenized for 30 sec in 10 volumes of cold acetone with a Polytron and air-

dried after filtration. Thirty-five grams of dry powder thus obtained were extracted with 10 volumes of 0.02 M phosphate buffer, pH 6.0, for 1 hr and the extract was collected by centrifugation at 10,000 $\times$ 60 min. The supernatant was dialyzed overnight against the same buffer and used as a crude enzyme solution.

RESULTS

Purification of enzyme

Aminopeptidases were extracted from internal organs of the sardines, and a crude enzyme solution was obtained as described in MATERIALS AND METHODS. The crude enzyme solution was further purified by chromatography on a first DEAE-cellulose column (Fig. 1), gel filtration on Sephadex G-200 (Fig. 2), rechromatography on the second DEAE-cellulose column (Fig. 3), and an isoelectric focusing column (Fig. 4). After the isoelectric focusing, the aminopeptidase appeared in two different peaks at pH 4.1 and pH 4.8 which were designed as aminopeptidase I and aminopeptidase II, respectively (Fig. 4).

The results of purification are summarized in Table I. The aminopeptidases I and II were purified about 300~600-fold with recovery of 26% from crude extract. The purified aminopeptidase I gave a single band in both stainings of activity and protein on polyacrylamide gel electrophoresis at pH 8.9 (Fig. 5). The purified aminopeptidase II gave a single active

FIG. 1. Chromatography of the Sardine Aminopeptidases on a DEAE-Cellulose Column.

300 ml of the crude enzyme solution was prepared as described in MATERIALS AND METHODS and applied to a DEAE-cellulose column (2 $\times$ 30 cm). After being washed with 0.01 M phosphate buffer, pH 6.0, the column was eluted with a linear gradient from 0.09 M to 0.3 M NaCl in 0.01 M phosphate buffer, pH 6.0 at a flow rate of 20 ml/hr. Each fraction was 5.0 ml. O---O, absorbance at 280 nm; ●—●, activity with leu- β -naphthylamide; ▲—▲, NaCl concentration.

FIG. 2. Gel Filtration of the Sardine Aminopeptidases on a Sephadex G-200 Column.

The enzyme fraction obtained from the first DEAE-cellulose column was concentrated to a small volume by ultrafiltration using a Diaflo UM 10 membrane, and the concentrated solution was applied to a Sephadex G-200 column (4.5×100 cm) previously equilibrated with 0.01 M phosphate buffer, pH 6.0 containing 0.1 M NaCl. The flow rate of 12 ml/hr. Each fraction was 6 ml. ○---○, absorbance at 280 nm; ●---●, activity with leu- β -naphthylamide.

FIG. 3. Chromatography of the Sardine Aminopeptidases on a Second DEAE-Cellulose Column.

Aminopeptidase fractions shown in parenthesis in Fig. 2 were dialyzed against 0.01 M phosphate buffer, pH 6.0. The dialyzed solution was applied to a DEAE-cellulose column (2×30 cm) and eluted as described in Fig. 1. Each fraction was 3 ml. ○---○, absorbance at 280 nm; ●---●, activity with leu- β -naphthylamide; ▲---▲, NaCl concentration.

FIG. 4. Isoelectric Focusing of the Sardine Aminopeptidases on Servalyt (AG. 3~7).

The enzyme fraction (17 mg) from the second DEAE-cellulose was dialyzed against 0.01 M phosphate buffer, pH 6.0, and subjected to an isoelectric focusing column (2.4×24 cm). The conditions for the electrophoresis are described under MATERIALS AND METHODS. ○---○, absorbance at 280 nm; ●---●, activity with leu- β -naphthylamide; ▲---▲, pH.

TABLE I. PURIFICATION OF SARDINE AMINOPEPTIDASE

Step	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)	Yield (%)	Purification (Fold)
Crude extract	9135	186	0.02	100	1
First DEAE-cellulose	203	147	0.72	79	36
Sephadex G-200	68	124	1.82	66	91
Second DEAE-cellulose	17	57	3.34	31	167
Isoelectric focusing column					
Aminopeptidase I	3	33	11.37	18	569
Aminopeptidase II	2	14	6.36	8	318

FIG. 5. Polyacrylamide Gel Electrophoresis of the Purified Sardine Aminopeptidase I in 7.5% Gel Containing Tris-HCl Buffer (pH 8.9).

(A) Enzyme activity was stained as described in MATERIALS AND METHODS.

(B) Protein was stained with Coomassie brilliant blue G-250. The arrow indicates the position of bromophenol blue.

FIG. 6. Estimation of Molecular Weight of the Purified Aminopeptidases I and II by Gel Filtration.

Molecular weight was estimated by a gel filtration method¹⁵⁾ on a column of Sephadex G-200 (1.6 × 90 cm) which was equilibrated with 0.01 M phosphate buffer, pH 7.0, containing 0.1 M NaCl. Reference proteins used for calibration of the column were ferritin (440,000), aldolase (158,000), bovine serum albumin (68,000) and chymotrypsinogen (25,000).

band but it had one major and one minor component in protein staining.

Molecular weight

The molecular weight determined by gel filtration on Sephadex G-200 was calculated to be 370,000 for aminopeptidase I and 320,000 for aminopeptidase II (Fig. 6).

FIG. 7. Effects of NaCl Concentration on Sardine Aminopeptidases I and II.

Enzymes I and II were preincubated for 15 min at 37°C in 0.1 M phosphate buffer, pH 7.0, containing different concentrations of NaCl. Each enzyme solution was added to substrate containing the same concentration of NaCl. Enzyme activities I (○—○) and II (●—●) were assayed in the respective NaCl concentrations with a substrate of leu-*p*-nitroanilide.¹²⁾

Isoelectric point

Isoelectric focusing also gave a single activity peak from each aminopeptidase. Isoelectric points of I and II were 4.1 and 4.8, respectively.

Effect of pH

The effect of pH on the activities and the stabilities of aminopeptidase I and II was examined. Both activities were maximal at 7.0~7.5 and were stable between pH 4.0 and 9.0 at 37°C for 15 min.

Heat stability

After incubating the enzymes in 0.1 M phosphate buffer, pH 6.0, for 15 min at various temperatures, we measured the residual activity against leu- β -naphthylamide at pH 7.0. The both enzymes were fairly stable up to 50°C under the above condition.

Effect of NaCl

The effect of NaCl on enzymes I and II were measured with leu-*p*-nitroanilide. I and II were preincubated with various concentration of NaCl for 15 min at 37°C, and then enzyme activities were assayed by the addition of leu-*p*-

nitroanilide. As shown in Fig. 7, I retained more than 70% of its original activity following the treatment with 15% NaCl, while II retained only 17% after the same treatment.

TABLE II. EFFECT OF INHIBITORS ON SARDINE AMINOPEPTIDASE ACTIVITY

The enzymes were preincubated in 0.1 M phosphate buffer, pH 7.0 containing various reagents for 15 min at 37°C. After preincubation, the activity was measured with leucine- β -naphthylamide.

Reagent	Conc. during reaction (mM)	Remaining activity	
		Enzyme I (%)	Enzyme II (%)
None	—	100	100
EDTA	1	20	22
DFP	1	109	99
<i>p</i> -CMB	1	79	80
Dithiothreitol	1	85	90
NEM ^a	1	87	92
TPCK ^b	1	97	92
Bestatin	0.01	38	107
	0.17	23	84

^a NEM, *N*-ethylmaleimide.

^b TPCK, *N*-tosyl-1-phenylalanine chloromethyl ketone.

Effects of inhibitor (Table II)

The effects of various inhibitors on enzymes I and II are shown in Table II. Both enzymes were inhibited strongly by a metal chelating agent, EDTA, but not by DFP, *p*-CMB, NEM, or dithiothreitol. While I was also inhibited strongly by 10 μ M bestatin, II was scarcely inhibited by this reagent.

TABLE III. EFFECT OF CATIONS ON SARDINE AMINOPEPTIDASE ACTIVITY

The enzyme activity on leucine- β -naphthylamide was measured in 0.1 M phosphate buffer (pH 7.0) containing 1 mM metal ions at 37°C.

Divalent cation (1 mM)	Remaining activity (%)	
	Aminopeptidase I	Aminopeptidase II
None	100	100
Mg ⁺⁺	105	99
Mn ⁺⁺	100	100
Zn ⁺⁺	105	91
Ba ⁺⁺	103	92
Co ⁺⁺	166	150
Cd ⁺⁺	55	45
Fe ⁺⁺	40	42
Cu ⁺⁺	31	11
Hg ⁺⁺	50	69

TABLE IV. HYDROLYSIS OF SYNTHETIC SUBSTRATES AND PEPTIDES BY SARDINE AMINOPEPTIDASES

Substrate	Relative activity (%)		Peptide	Relative activity (%) ^b	
	Enzyme I	Enzyme II		Enzyme I	Enzyme II
L-Leucyl- β -naphthylamide	100 ^a	100 ^a	(Ala) ₂	100 ^a	100 ^a
L-Alanyl- β -naphthylamide	105	104	(Ala) ₃	92	69
L-Glycyl- β -naphthylamide	52	66	(Ala) ₄	70	58
L-Glutamyl- β -naphthylamide	0	0	Ala-Val	57	61
L-Isoleucyl- β -naphthylamide	5	4	Ala-Tyr	46	43
L-Seryl- β -naphthylamide	11	9	Ala-Ser	25	22
L-Phenyl- β -naphthylamide	19	21	Ala-Phe	5	3
L-Arginyl- β -naphthylamide	0	0	Leu-Ala	0	0
L-Valyl- β -naphthylamide	6	5	Gly-Leu	50	45
			Gly-Phe	25	21
L-Leucyl- <i>p</i> -nitroanilide	100 ^a	100 ^a	Gly-Ala-Ala	30	24
L-Alanyl- <i>p</i> -nitroanilide	340	430	Leu-Gly	0	0
L-Glutamyl- <i>p</i> -nitroanilide	10	9	Tyr-Ala	19	25
			Leu-Tyr	0	0

^a Substrate concentration was 0.2 mM, and the rate of hydrolysis of L-leu- β -naphthylamide, L-leucyl-*p*-nitroanilide and (Ala)₂ was taken to be 100%.

^b The enzymatic hydrolysis of peptide substrates was measured by the colorimetric Ninhydrin method.²⁰⁾

Effect of metal

Enzymes I and II (Table III) were activated 1.5 fold by 1 mM Co^{++} . But they are inhibited strongly by 1 mM each of Cu^{++} , Fe^{++} , Cd^{++} , and Hg^{++} . Especially, enzyme II was inhibited almost completely by 1 mM Cu^{++} .

Substrate specificities (Table IV)

The substrate specificities were studied with aminoacyl- β -naphthylamide, aminoacyl-*p*-nitroanilide, and di- or tri-peptides. Leucyl- β -naphthylamide, leucyl-*p*-nitroanilide, and Leu-Leu were used as standards for comparison of the hydrolysis rate. The naphthylamide or nitroanilide containing leucine and alanine were hydrolyzed well by enzymes I and II. Especially, alanine-*p*-nitroanilide was hydrolyzed by the both enzymes 3.4- to 4.3-fold faster than leucine-*p*-nitroanilide. Di-, tri- or tetra-alanine were also hydrolyzed rapidly by enzymes I and II compared with other peptides. Di-alanine was the best substrate among the peptides we tested.

DISCUSSION

The present paper is concerned with the first study on the detailed properties of aminopeptidases which were highly purified from the internal organs of fish. The properties of two enzymes from sardines are acidic isoelectric points, instability at low pH regions, high molecular weight ($M_r = 320,000 \sim 370,000$), and inhibition by EDTA. In these respects they resemble mammalian leucine aminopeptidase^{16,17)} but are different from the aminopeptidase in marine bacteria⁵⁾ or microorganisms.¹⁸⁾

However, sardine aminopeptidases are different from mammalian leucine aminopeptidase,^{21,22)} because sardine enzymes can not hydrolyze Leu-Gly, Leu-Tyr, and Leu-Ala (Table IV) which are the best substrates for leucine aminopeptidase.

Furthermore, sardine aminopeptidases are similar to human liver alanine aminopeptidase^{19,20)} in terms of optimum pH, activation by cobaltous ion, and substrate specificity.

Enzyme I is distinct from enzyme II in terms of isoelectric point, molecular weight, salt sensitivity, and bestatin inhibition. Bestatin, an inhibitor of aminopeptidase B,²³⁾ inhibits enzyme I but not enzyme II, suggesting enzyme I is an aminopeptidase B-like enzyme. However, enzyme I is not the same as aminopeptidase B because the amino-terminal lysyl or arginyl residue of a peptide substrate is liberated by aminopeptidase B but not by enzyme I (Table IV).

Recently, mammalian aminopeptidase has been described as a zinc metalloenzyme containing one atom of zinc per molecule of enzyme.²⁴⁾ It will be interesting to learn whether sardine aminopeptidase contain zinc. Sardine aminopeptidase was stable for three months after the beginning of fish sauce production.⁴⁾ The present investigation shows that enzyme I retained high activity in the presence of 15% NaCl (Fig. 7). These results indicate that enzyme I participates in hydrolyzing proteins and peptides in fish sauce production.

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