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STUDIES ON PROTEINASES FROM THE DIGESTIVE ORGANS OF SARDINE

II. PURIFICATION AND CHARACTERIZATION OF TWO ACID PROTEINASES FROM THE STOMACH

MINORU NODA and KAZUO MURAKAMI *

Institute of Applied Biochemistry, University of Tsukuba, Ibaraki 305 (Japan)

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Summary

Two fish acid proteinases designated acid proteinase I and II were found and isolated by $(NH_4)_2SO_4$ fractionation, CM-cellulose chromatography and gel filtration on Sephadex G-100. The final preparations were judged nearly homogeneous by multiple criteria. The molecular weights of the enzymes I and II were determined by the sedimentation equilibrium method to be 37 000 and 33 400, respectively. The sedimentation coefficients ($s^0_{20,w}$) were 3.06 and 3.09, respectively. Enzymes I and II contained similar amino acid composition except for the contents of histidine, arginine, threonine, serine and proline. Enzymes I and II differed from each other in optimal pH and stability at pH 7. Each enzyme could scarcely hydrolyze a synthetic pepsin substrate, *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine (APDT). Both of the enzymes were inhibited by acid proteinase specific reagents: pepstatin, diazoacetyl-DL-norleucine methyl ester (DAN), 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP) and *p*-bromophenacyl bromide. These results indicate fish enzymes are similar to mammalian pepsin and microbial acid proteinases in their active site structure having two different carboxyl groups, although they differ in regard to a number of molecular and enzymatic properties.

Abbreviations: APDT, *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine; DAN, diazoacetyl-DL-norleucine methyl ester; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane.

* To whom correspondence should be addressed.

Introduction

We previously reported [1] the purification and some properties of three alkaline proteinases from the pyloric caeca of sardine.

Fish pepsins from salmon [2], tuna [3] and bonito [4] or whale pepsin [5] have been purified and partially characterized. These pepsins seem to be different from porcine in many molecular properties [2—5]. However, no studies have been carried out on complete purification and characterization of fish acid proteinase. In this paper, we described the isolation and some of the properties of two acid proteinases in the stomach of sardine.

Materials and Methods

Materials. Bovine hemoglobin and *p*-bromophenacyl bromide were purchased from Wako Pure Chem. Ind. *N*-Acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine (APDT), diazoacetyl-DL-norleucine methyl ester (DAN) and porcine pepsin (twice crystallized) were obtained from Sigma Chemical Co. 1,2-Epoxy-3-(*p*-nitrophenoxy) propane (EPNP) was purchased from Eastman Kodak Co., and pepstatin was from the Protein Research Foundation. All other chemicals and materials were of reagent grade or prepared as described in the previous paper [1].

Assay for proteolytic activity. Proteinase activity was determined according to the following modification of Anson's method [6]. Urea-denatured hemoglobin was prepared in 0.05 N NaOH by the method of Konagaya and Amano [7] and freeze-dried. 0.6% of the freeze-dried hemoglobin was dissolved in 0.1 M acetate buffer, pH 4.0 for acid proteinase I and in 0.1 M acetate buffer, pH 2.0 for acid proteinase II. 1 ml enzyme solution was added to 5 ml of the substrate solution and incubated at 37°C, for 10 min. After incubation, proteinase activity was determined as described in the previous paper [1]. 1 proteinase unit was defined as the amount of enzyme to liberate 1 μg tyrosine from the denatured hemoglobin per min, under these conditions.

Toward a synthetic substrate, APDT. Hydrolytic activity toward APDT was determined according to the method of Tang [8]. Amino acid analysis, determinations of protein concentration, molecular weight and sedimentation coefficients, were conducted as described in the previous paper [1].

Results

Extraction and purification of acid proteinases. Acid proteinases were extracted from the stomachs of sardines and concentrated by $(NH_4)_2SO_4$ as described in the previous paper [1], except for the pH of the homogenization. The pH was adjusted to pH 5.0 by acetic acid immediately after the homogenization. The concentrated enzyme solution was applied to the first CM-cellulose column. Fig. 1 shows that two peaks having acid proteinase activity were eluted from this column. The two peaks are designated acid proteinase I and II. Each of the acid proteinases was further purified by the second CM-cellulose and/or by the gel filtration on Sephadex G-100 (Fig. 2).

Fig. 2 shows an elution profile of acid proteinases I and II on the first G-100

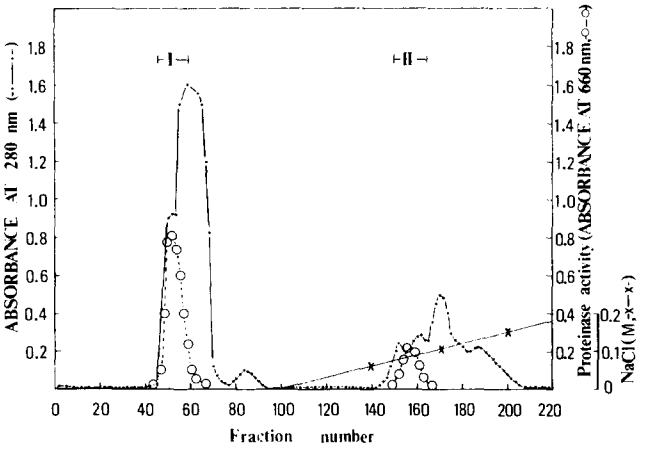

Fig. 1. Chromatography of acid proteinases on CM-cellulose. 25 ml of the extract prepared as described in Results in the text were equilibrated with 0.01 M acetate (pH 5) by dialysis with the same buffer and applied to a CM-cellulose column (3 X 100 cm, Brown Co.), previously equilibrated with the same buffer. This column was washed with the same buffer and further eluted using a 750 ml linear gradient of 0—0.6 M in the same buffer.

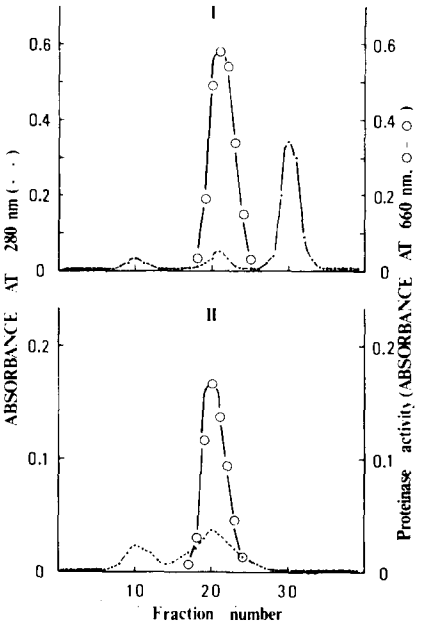

Fig. 2. Gel filtration of acid proteinases on Sepahdex G-100. I: Acid proteinase I fractions from the first CM-cellulose chromatography (Fig. 1) were concentrated to 10 ml by ultrafiltration with a membrane of Diaflo-UM 10, applied to a Sephadex G-100 (Pharmacia 2 X 100 cm) previously equilibrated with 0.01 M acetate buffer (pH 5.0) and eluted with the same buffer. II: Acid proteinase II fractions from the second CM-cellulose chromatography (rechromatography with the same condition as in the first one) were concentrated, applied and eluted as described in I.

TABLE I

PURIFICATION OF SARDINE ACID PROTEINASES I AND II

Procedure	I		II	
	Spec. act. (unit/mg protein)	Yield (%)	Spec. act. (unit/mg protein)	Yield (%)
Homogenate	0.62	100	0.50	100
Crude extract	2.05	61.9	1.64	61.9
$(NH_4)_2SO_4$ fraction	8.43	23.8	6.74	23.8
First CM-cellulose	53.6	14.9	40.24	4.7
Second CM-cellulose	—	—	48.14	4.1
First Sephadex G-100	122.9	5.9	56	2.9
Second Sephadex G-100	133.3	3.4	62.5	1.9

column. In each instance, only one peak of proteinase activity coinciding with protein concentration was noted. The outline and the result of the purification of acid proteinases are summarized in Table I. Two acid proteinases were isolated in reasonable quantities by the present purification procedure. Starting with 100 g of digestive organ, 7.79 mg of enzyme I and 7.2 mg of enzyme II were isolated, at a combined yield of 5.3%. Enzyme I was a major acid proteinase. A 215-fold purification was achieved.

Evidence for purity of acid proteinases I and II. When the purified proteinases were examined by an analytical ultracentrifuge, each enzyme gave rise to a single symmetrical sedimentation peak (Fig. 3) from which sedimentation

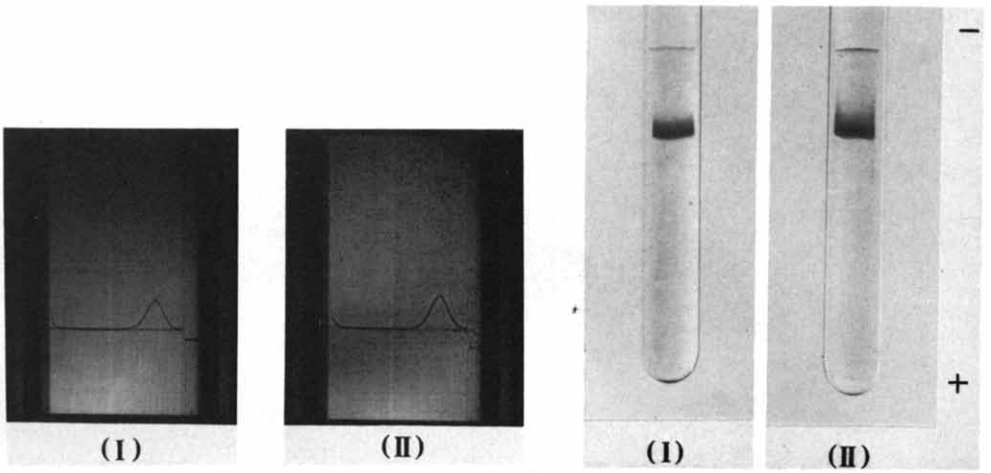

Fig. 3. Sedimentation patterns of acid proteinase I and II. The purified enzyme I and II preparations were dissolved in 0.01 M acetate buffer (pH 5.0)/0.1 M NaCl to give the concentration of 0.85% for I and 0.94% for II. The photographs were taken at 100 min after attainment of full speed (55 430 rev./min, Hitachi rotor No. RA-72T). Temperature 5°C, bar angle: 70°.

Fig. 4. Polyacrylamide gel electrophoresis of acid proteinases I and II. The electrophoresis was performed at pH 6.6 at 4 mA/tube, 5°C for 40 min in 0.5 × 7 cm columns of 7.5% polyacrylamide gel according to the method of Taber and Sherman [14].

TABLE II

AMINO ACID COMPOSITION OF SARDINE ACID PROTEINASES I, II AND PORCINE PEPSINS

Based on a molecular weight of acid proteinase I (37 000) and II (33 000), values were calculated as residues/protein molecule. The values in parentheses are the nearest integer.

	Sardine acid proteinase		Porcine pepsin	
	I	II	A	C
Lysine	6.9 (7)	6.3 (6)	1	4
Histidine	3.8 (4)	5.7 (6)	1	1
Arginine	3.4 (3)	6.4 (6)	2	4
Aspartic acid	39.5 (40)	32.5 (33)	40	28
Threonine	36.6 (37)	21.8 (22)	25	25
Serine	28.9 (29)	19.6 (20)	43	35
Glutamic acid	29.2 (29)	27.2 (27)	26	41
Proline	27.5 (28)	17.5 (18)	16	18
Glycine	34 (34)	36.3 (36)	34	32
Alanine	27 (27)	22.5 (23)	16	21
Half cystine	8.6 (9)	6.6 (7)	6	6
Methionine	8.5 (9)	5.8 (6)	4	4
Valine	22 (22)	25.9 (26)	20	20
Leucine	16.4 (16)	16.2 (16)	28	34
Isoleucine	14.1 (14)	14.2 (14)	23	14
Tyrosine	8.8 (9)	10.5 (11)	16	18
Phenylalanine	8 (8)	10 (10)	14	21
Tryptophan	4.6 (5)	4.2 (4)	6	6

coefficients ($s^0_{20,w}$) were found to be 3.06 (I) and 3.09 (II). Likewise, electrophoresis of each preparation on SDS-polyacrylamide gel electrophoresis yielded a single protein band (see below). Polyacrylamide disc-gel electrophoresis of each preparation at pH 6.6 also yielded only one main band (Fig. 4), and this band coincided with proteinase activity.

Molecular weight. The molecular weights determined by the sedimentation equilibrium method were calculated to be 37 000 and 33 400 for acid proteinases I and II, respectively. Electrophoresis in SDS-polyacrylamide gel columns in the presence of mercaptoethanol produced a single discrete band at a position corresponding to the molecular weights of 35 000 (I) and 33 000 (II). In the estimate of the molecular weight good agreement was obtained between the sedimentation equilibrium and the SDS-polyacrylamide gel electrophoresis methods.

Amino acid composition. The amino acid compositions of acid proteinases I and II (Table II) show similarity, but they differ mainly in terms of histidine, arginine, threonine, serine and proline. Their composition shows a overwhelming predominance of acidic over basic amino acid residues. Of the residues in enzyme I, 4.3% are basic and 21.0% are acidic. The corresponding percentages in enzyme II are 6.4 and 20.6%.

Proteinase activities with protein substrates and N-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine (APDT). Conditions for maximum rates of proteolysis of hemoglobin were 55°C and pH 4.0 for enzyme I, and 40°C and pH 2.0 for enzyme II. The highest rates of proteolysis among proteins examined were ob-

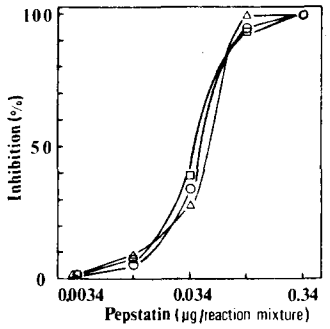

Fig. 5. Effects of pepstatin on the proteolytic activity of acid proteinases I, II and porcine pepsin. 5 μg enzyme in 0.5 ml of the acetate buffer were mixed with 0.5 ml of 10^{-6} M—10^{-8} M pepstatin solution. After incubation for 20 min at 37°C, the residual activity was measured. △———△, pepsin; □———□, I; ○———○, II.

tained with hemoglobin and myoglobin. Sarcoplasmic sardine-muscle protein was hydrolyzed by enzymes I and II at about one-quarter the rate found for hemoglobin.

Activity against APDT. This synthetic pepsin substrate [8] was hydrolyzed very slowly by both proteinases, the activity (absorbance at 570 nm/min per mg enzyme) being $3.9 \cdot 10^{-2}$ for both enzymes. This activity is about one-tenth of the value obtained under the same conditions with porcine pepsin.

Effect of NaCl. The proteinase activity of acid proteinases I and II toward milk casein and hemoglobin decreased to 30—40% of their maximum activity in the presence of 18—20% NaCl. However, when sardine muscle-proteins i.e., sarcoplasmic, myofibril and stroma proteins were used as substrates, enzymes I and II indicated as high as 80% of their maximum activity in the presence of 20% NaCl.

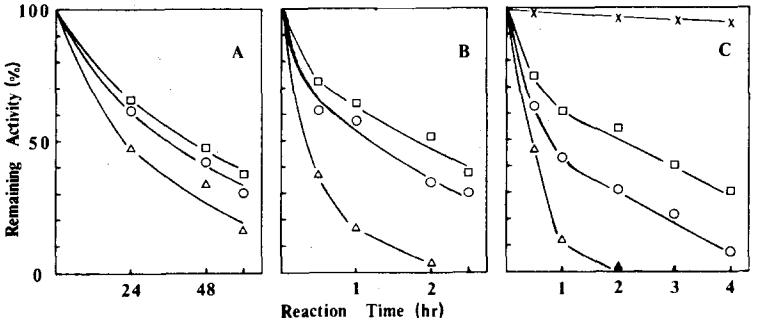

Fig. 6. Time courses of inhibitors on acid proteinases I, II and porcine pepsin. △———△, pepsin; □———□, acid proteinase I; ○———○, acid proteinase II. A. Enzyme (10 μg) in 1.0 ml of the above acetate buffer, pH 5.5 was mixed with 1.0 ml 0.1 M citrate buffer, pH 4.6 and 500 μg EPNP in 50 μl ethanol. The reaction mixture was maintained at 25°C with gentle stirring. Aliquots of 200 μl were withdrawn and used for the assay of enzymatic acitivity. B. The reaction mixture consisted of: 10 μg enzyme in 0.1 ml of the acetate buffer (pH 5.5); 1.0 ml 0.1 M Na_2HPO_4/citrate buffer (pH 2.8); 500 μg *p*-bromophenacyl bromide in 50 μl ethanol. The reaction was performed at 15°C with gentle stirring and residual enzymatic activity was determined. C. Each (10 μg) of the purified enzymes in 1.0 ml of 0.01 M sodium acetate buffer, pH 5.5 was mixed with 1.0 ml 0.1 M sodium phosphate buffer, pH 6.0, and 25 μl 0.01 M cupric sulfate. After incubation of the mixture at 25°C for 10 min, 25 μg DAN in 50 μl methanol was added to the mixture. The reaction was allowed to proceed at 25°C for 4 h. At the indicated intervals, 100 μl aliquots were withdrawn and used for the assay of enzymatic activity. X———X, without Cu^{2+}.

TABLE III

PROPERTIES OF SARDINE ACID PROTEINASES I AND II

1. Sedimentation equilibrium method. 2. SDS-polyacrylamide gel electrophoresis. Inhibitors are pepstatin, DAN, EPNP and *p*-bromophenacyl bromide.

	I	II
Specific activity for hemoglobin (unit/mg protein)	133.3	62.5
Optimum pH	4	2
Optimum temperature	55°C	40°C
pH stability	2~6	2~6
Temperature stability	pH4 80%	80%
	pH2 10%	10%
Inhibitors	+	+
Sedimentation coefficient (s^0_{20} w)	3.06	3.09
Molecular weight (1)	37 000	33 400
Molecular weight (2)	35 000	33 000

Stability. No loss of proteinase activity of the purified enzymes was noted when stored at −15°C for 1 month after freeze-drying of the purified enzyme solution, at pH 5.0. Almost no loss of activity of the enzymes was observed at pH 2—6 when they were incubated at 37°C, for 2 h. Both of the enzymes became unstable beyond pH 7 and lost their activities completely at pH 8 during their incubation at 37°C for 2 h. However, at pH 7 acid proteinase II shows 80% of the maximum activity and acid proteinase I only 5%.

In an incubation at 60°C for 5 min, both enzymes were very unstable at pH 2.0, but stable at pH 4.0.

Inhibitors. Pepstatin inactivated acid proteinases I and II to almost the same extent as porcine pepsin (Fig. 5): 50% inhibition of I and II was $5—6 \cdot 10^{-8}$ M and that of porcine pepsin was $7 \cdot 10^{-8}$ M under the condition used. The time-courses of inactivation of the two acid proteinases and pepsin, by EPNP, *p*-bromophenacyl bromide and DAN are shown in Fig. 6. Pepsin was more sensitive than the two acid proteinases to these inhibitors. DAN was not inhibitory in absence of Cu^{2+}. The molecular and enzymatic properties of the two acid proteinases are summarized in Table III.

Discussion

Our present detailed knowledge of acid proteinases comes from studies of the mammalian and bacterial enzymes. Previously described fish pepsin-like enzymes differ from porcine pepsin in substrate specificity and optimum pH [2—5]. Until now there have been no reports of acid proteinases from fish being purified to homogeneity. We found two acid proteinases in the stomach of sardine and purified them to a nearly homogeneous state. The two acid proteinases in the stomach of sardine were similar to porcine pepsins [8] in molecular weight, sedimentation coefficient, acidic pH optimum, instability in alkaline pH, inhibition of pepstatin, DAN, EPNP and *p*-bromophenacyl bromide. The inhibition of acid proteinases I and II by EPNP and DAN indi-

cates that the fish enzymes contain two different carboxyl group(s) as their active site like all other gastric and microbial acid proteinases [9].

However, there are several clear differences in properties between fish acid proteinases and all other major acid proteinases from mammals and microorganisms. First, acid proteinases I and II hydrolyze APDT much more slowly than does pepsin A [8]. In this respect enzymes I and II show activities comparable to a minor form of pepsin, pepsin C [10—12], human gastricsin [13] and Bonito pepsin [4]. Secondly, the amino acid composition of acid proteinases I and II differed from porcine pepsins A and C especially in the content of basic, aromatic and hydroxy amino acid.

All these results indicate fish acid proteinases are basically similar to those from other species but distinct in their molecular structure at minute points.

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