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Pepsinogens and Pepsins from Mandarin Fish (*Siniperca chuatsi*)

QIONG ZHOU,[†] GUANG-MING LIU,[†] YUAN-YUAN HUANG,[†] LING WENG,[†] KENJI HARA,[‡] WEN-JIN SU,[†] AND MIN-JIE CAO*,[†]

College of Biological Engineering, The Key Laboratory of Science and Technology for Aquaculture and Food Safety, Jimei University, Jimei, Xiamen, China, 361021, and Faculty of Fisheries, Nagasaki University, Nagasaki, Japan, 852-8521

Four pepsinogens (PG-I, PG-II, PG-III(a), and PG-III(b)) were highly purified from the stomach of the freshwater fish mandarin fish (*Siniperca chuatsi*) by ammonium sulfate fractionation, anion exchange, and gel filtration. The molecular masses of the four purified PGs were 36, 35, 38, and 35 kDa, respectively. All the pepsinogens converted into their active form pepsins within a few minutes under pH 2.0. The optimum pH and temperature of the four enzymes were 3.0−3.5 and 45−50 °C, using hemoglobin as a substrate. The N-terminal amino acid sequences of PG-I and PG-II were determined to the 12th and 17th amino acid residues, respectively. Western blot analysis using antisea bream polyclonal antibodies cross reacted with PG-I, PG-II, and PG-III(b) while no cross reaction with PG-III(a) was detected, suggesting the diversity of pepsinogens in fish.

KEYWORDS: Mandarin fish; purification; pepsinogen; pepsin; Western blot; antibody

INTRODUCTION

Pepsinogens (PGs), the precursor of the aspartic proteinase pepsin, are normally present in the gastric mucosa of animals. They are autocatalytically activated to pepsins under acidic conditions, releasing the NH_2-terminal activation segments. Pepsins perform important digestive functions in both invertebrates and vertebrates (*1*). To date, three major groups of pepsinogens have been classified, namely pepsinogen A, pepsinogen C (progastricsin), and prochymosin (or neonatal pepsinogen) (*2, 3*). These three types of PGs differ from each other not only in their primary structures but also in enzymatic properties of their activated forms (*2–4*).

As a nonmammalian species, digestive enzymes from fish have been paid much attention in recent decades. Pepsinogens and pepsins from rainbow trout (*5*), North Pacific bluefin tuna (*Thunnus thynuus orientalis*) (*6, 7*), Atlantic cod (*Gadus morhua*) (*8*), and shark (*Centroscymnus coelolepsis*) (*9*) have been well documented. More recently, pepsinogens and pepsins from Antarctic rock cod (*Trematomus bernacchii)* (*1*), Africa coelacanth (*Latimeria chalumnae*) (*3*), and pectoral rattail (*Corphaenoides pectoralis*) (*10*) were reported. In our recent study, four pepsinogens from marine fish sea bream (*Sparus latus*) were purified to high homogeneity, and polyclonal antibodies against these pepsinogens were prepared (*11*). However, all these studies were performed on marine fish. To our knowledge, no study concerning pepsinogens and pepsins from freshwater fish has been reported.

Mandarin fish (*Siniperca chuatsi*) is a popularly cultured freshwater fish in different provinces of China because of its good taste and muscle texture. As essential digestive enzymes, pepsins are most importantly involved in the digestion of proteins in fish feeds. Thus, a detailed study of these proteinases will be helpful to the manufacturing of fish feeds and the development of aquaculture. In the present study, we isolated pepsinogens from the stomach of mandarin fish and investigated their characteristics.

MATERIALS AND METHODS

Materials. Cultured mandarin fish (*Siniperca chuatsi*) with a body weight of about 500 g were purchased alive from the fish market of Jimei, Xiamen. Five fish were sacrificed, and the stomachs were collected and washed for use. DEAE-Sephacel and Sephacryl S-200 HR were purchased from Amersham Biosciences (Uppsala, Sweden); phenylmethanesulfonyl fluoride (PMSF) and bovine hemoglobin were from Sigma. L-3-carboxy-*trans*-2,3-epoxy-propionyl-L-leucin-4-guanidinobutylamide (E-64) was a product of Amresco (Solon, OH); pepstatin A was from Roche; protein marker for SDS-PAGE was from Fermentas (Lithuania); prestained protein marker for the Western blot was from New England Biolabs (Beverly, MA). Rat antisea bream (*Sparus latus*) pepsinogen I, II, IIII, and IV polyclonal antibodies were prepared in our own laboratory, as described (*11*). Other reagents are of analytical grade.

Purification of Pepsinogens. All procedures were performed at 4 °C. Mandarin fish stomachs (23 g) that were nearly empty were washed and homogenized in 6-fold of 50 mM phosphate buffer (pH 7.0) containing 5 mM PMSF and 1 mM E-64, using a homogenizer (Kinematica, PT-2100, Switzerland). The homogenate was centrifuged at 18 000*g* for 90 min, the resulting supernatant was collected, and ammonium sulfate was added to 20% saturation and stood for 2 h. The mixture was then

* To whom correspondence should be addressed. Phone: +86-592-6180378. Fax: +86-592-6180470. E-mail: mjcao@jmu.edu.cn.
[†] Jimei University.
[‡] Nagasaki University.

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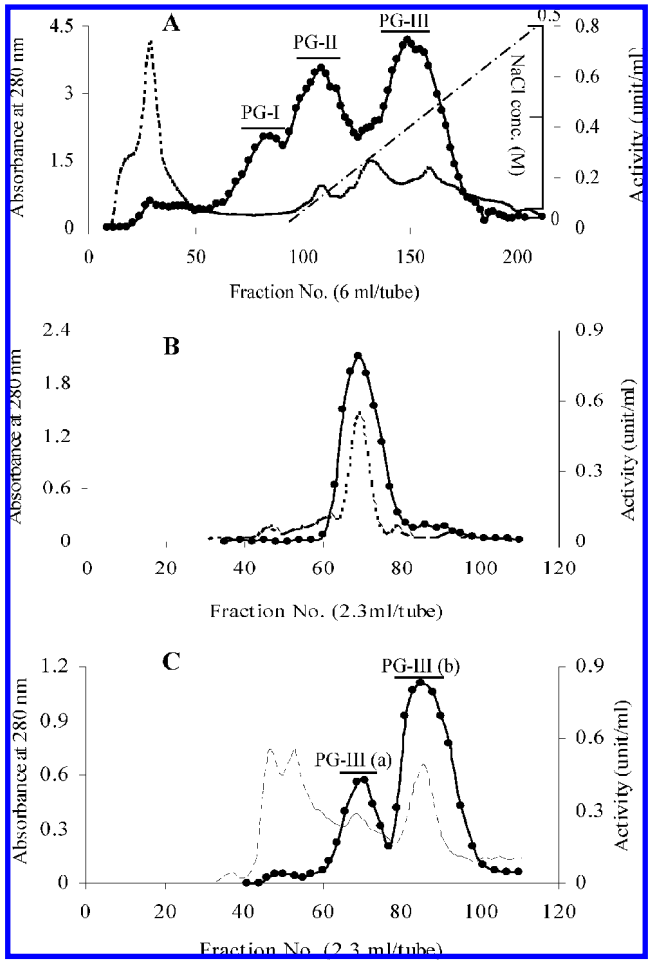

Figure 1. Column chromatography purification of mandarin fish pepsinogens: (**A**) DEAE-Sephacel chromatography; (**B**) Sephacryl S-200 gel-filtration purification of pepsinogen-I; (**C**) Sephacryl S-200 gel filtration of pepsinogen-III(a) and pepsinogen-III(b). Hemoglobin hydrolyzing activity (●); absorbance at 280 nm (---).

Table 1. Summary of Purification of Pepsinogens from Mandarin Fish Stomach

stage	total activity (units)	total protein (mg)	specific activity (units/mg)	purification (-fold)	yield (%)
crude extract	2340.0	450.0	5.2	1	100
ammonium sulfate	1901.6	237.7	8.0	1.5	81.3
DEAE-Sephacel					
PG-I	277.5	3.7	75.0	14.4	11.9
PG-II	468.3	12.1	38.7	7.4	20.0
PG-III	735.9	15.3	48.1	9.3	31.4
Sephacryl S-200					
PG-I	66.5	0.5	133.0	25.6	2.8
PG-II	312.0	5.2	60.0	11.5	13.3
PG-III(a)	153.1	1.9	80.6	15.5	6.5
PG-III(b)	328.0	3.9	84.1	16.2	14.0

centrifuged at 10000*g* for 20 min to remove precipitate. Ammonium sulfate was added to the supernatant to 60% saturation followed by standing for 2 h. After centrifugation at 10000*g* for 30 min, the resultant precipitate was dissolved in a minimum amount of 25 mM phosphate buffer (pH 7.0) and dialyzed against the same buffer extensively. The dialysate was subsequently applied to a DEAE-Sephacel column (2.5 cm × 10 cm), which was previously equilibrated with the dialysis buffer. Unbound proteins were collected by washing the column with starting buffer until the absorbance at 280 nm reached baseline. Thereafter, binding proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M in a total volume of 600 mL at a flow rate of 1 mL/min. Fractions with pepsin activity were pooled and concentrated by ultrafiltration using a membrane of YM-10 (Millipore). The samples were then respectively applied to a gel-filtration column of Sephacryl S-200 HR (1.5 cm × 98 cm) equilibrated with 25 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl at a flow rate of 0.5 mL/min. Active fractions from the Sephacryl S-200 were collected and used for electrophoresis analysis and enzymatic characterization. The numbering of all the enzymes was done according to their elution positions from the DEAE-Sephacel column sequentially.

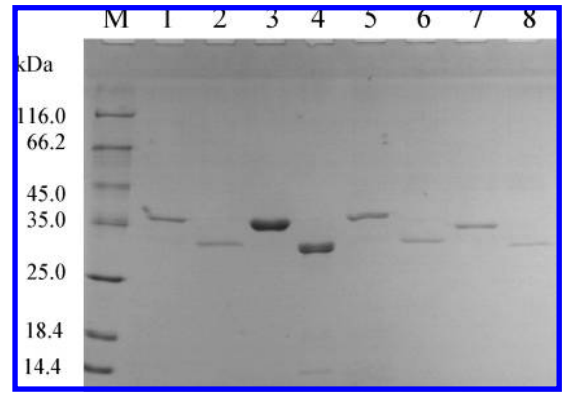

Figure 2. SDS-PAGE of purified mandarin fish pepsinogens (PGs) and pepsins. Lanes: M, molecular marker; 1, pepsinogen-I; 2, pepsin-I; 3, pepsinogen-II; 4, pepsin-II; 5, pepsinogen-III(a); 6, pepsin-III(a); 7, pepsinogen-III(b); 8, pepsin-III(b). The gel was stained with Coomassie brilliant blue.

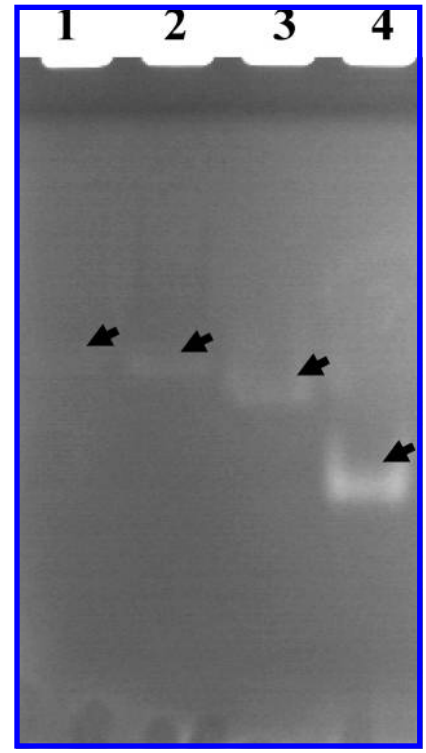

Figure 3. Zymogram of mandarin fish pepsins. Pepsins on native-PAGE were incubated at 37 °C with 1.0% hemoglobin as substrate for 1 h. Lanes: 1, pepsin-I; 2, pepsin-II; 3, pepsin-III(a); 4, pepsin-III(b). Approximately 20 μg of pepsin was applied on each lane. Gels were stained with Coomassie brilliant blue. Arrowheads indicate the position of the pepsins.

Protein Concentration Determination. Protein concentration was determined by measuring the absorbance at 280 nm of the sample solution or with the method of Lowry et al. (*12*) with bovine serum albumin as the standard.

Enzyme Activity Assay. Potential pepsin activity was determined by the method of Anson (*13*) with slight modification. Briefly, 50 μL of the appropriately diluted enzyme sample was added into 250 μL of 0.5 M HCl−sodium acetate buffer (pH 3.0) and mixed with 200 μL of

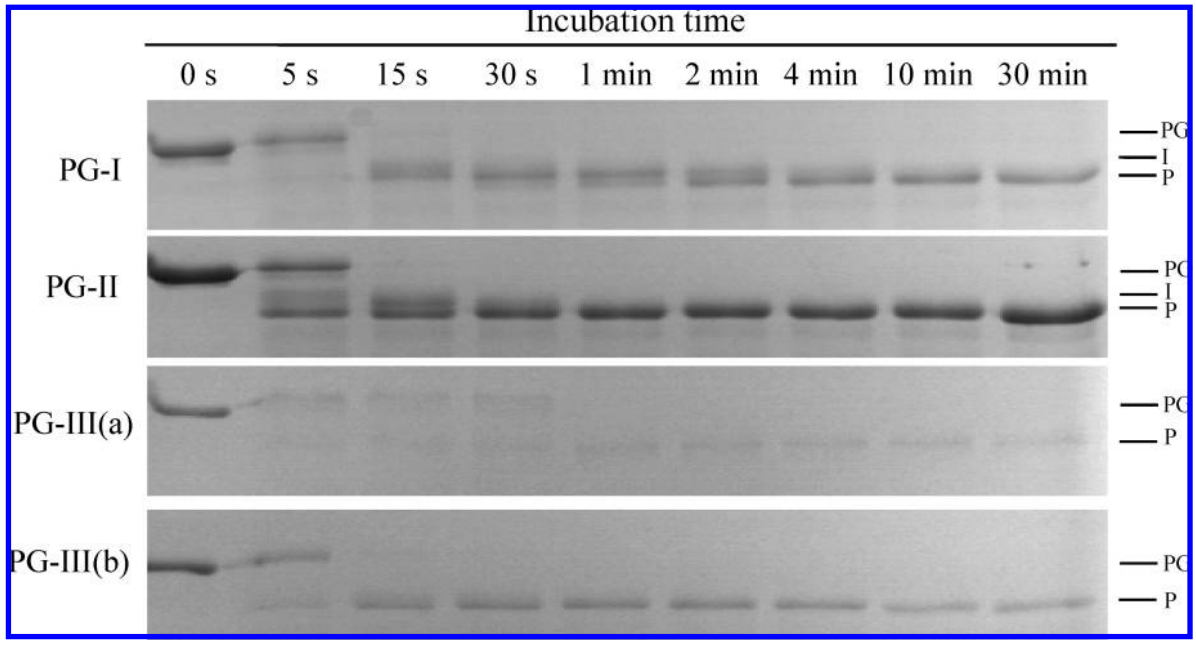

Figure 4. Conversion of mandarin fish pepsinogens to pepsins. Pepsinogens (PGs) were incubated at pH 2.0, 37 °C, respectively. Aliquots were removed, and the conversion reaction was stopped immediately at appropriate time intervals, as indicated above each lane, and subjected to SDS-PAGE. I, intermediate form; P, pepsin.

2.0% acid-denatured bovine hemoglobin, which had been extensively dialyzed against 0.05 M HCl. After incubation of the mixture at 37 °C for 30 min, the reaction was immediately stopped by the addition of 500 μL of 8.0% trichloroacetic acid (TCA) and centrifuged at 8000*g* for 10 min. The absorbance of the resulting supernatant at 280 nm was measured using a Cary 50 UV spectrophotometer (Varian). One unit of pepsin activity was defined as an increase of absorbance of 1.0 at 280 nm during an incubation time of 30 min under the above assay conditions. Proteolytic activity assays were performed in duplicate and the variation between duplicate samples was always less than 5%; the mean values were used.

SDS-PAGE, Western Blot, and Zymography. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (*14*) using 12.0% gel. Proteins were stained with Coomassie Brilliant Blue R-250 (CBB). A Western blot was performed as described by Towbin et al. (*15*). Briefly, pepsinogens on SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes in transfer solution. Nonspecific protein sites were blocked with 5% nonfat milk in Tris−HCl buffered saline (TBS = 20 mM Tris−HCl, pH 7.5, containing 0.145 M NaCl). Blotted proteins were incubated with corresponding polyclonal antibodies at room temperature for 2 h and washed with TBST (TBS, 0.05% Tween-20). After incubation for 1 h with horseradish peroxidase conjugated secondary antibody, the nitrocellulose membrane was washed extensively with TBST. Immunodetection was carried out using a detection kit from Bio-Rad.

Zymography was performed similar to SDS-PAGE without the addition of SDS both to the samples and gel. After electrophoresis, the gel was submerged in 1% acid-denatured bovine hemoglobin (pH 3.0) and incubated at 37 °C for 1 h. After washing, the gel was stained with CBB. Enzymatic activity was detected by revealing clear bands against the dark background on the gel.

Characterization of the Conversion from Pepsinogen to Pepsin. In order to convert pepsinogens to pepsins, the solution of purified PGs was adjusted to pH 2.0 by adding 0.1 M HCl. Such acid treatment converted PG into the activated form, pepsin. The conversion reaction was carried out using purified PGs in different test tubes individually with a volume of 100 μL at pH 2.0 and 25 °C for corresponding reaction intervals. After reaction, 10 μL of 16% TCA was immediately added to stop the reaction, respectively. Samples were subjected to SDS-PAGE, and gels were stained with CBB.

Effects of pH and Temperature on the Activity. The effects of pH and temperature on the activity of mandarin fish pepsins were determined using acid-denatured bovine hemoglobin as a substrate. To investigate the effect of pH on the activity of pepsin, pepsinogens were first converted to pepsins at pH 2.0, 37 °C for 15 min followed by activity analysis. The buffers used were 0.5 M HCl−sodium acetate buffer (pH 1.0−4.0) and 0.5 M acetic acid−sodium acetate buffer (pH 4.0−6.0). To investigate the effect of temperature on the activity of pepsin, 50 μL of activated pepsins was incubated with 200 μL of 2.0% acid-denatured bovine hemoglobin in 0.5 M HCl−sodium acetate buffer (pH 3.0) in a total volume of 0.5 mL at different temperatures (10−65 °C) for 15 min, and the activity was determined, respectively.

Effect of Inhibitors on Proteinase Activity. Different kinds of proteinase inhibitors were allowed to react with pepsin solutions (0.05 μM) to reach their final concentrations designated (PMSF, 5 mM; E-64, 0.28 mM; EDTA, 5 mM and different concentrations of pepstatin A). After preincubation of the mixture at room temperature for 15 min, the remaining activity was measured at 37 °C for 30 min as described above.

Amino Acid Sequence Determination. The N-terminal amino acid sequences of PG-I and PG-II were determined using a protein sequencer (Applied Biosystems, Model 492).

RESULTS AND DISCUSSION

In the present study, pepsinogens were purified from the stomach of mandarin fish by ammonium sulfate fractionation and column chromatographies of DEAE-Sephacel and Sephacryl S-200. Three peaks (PG-I, PG-II, PG-III) of hemoglobin-digesting activity were detected in the fractions from the DEAE-Sephacel column chromatography (**Figure 1**A). These peaks were pooled respectively and subjected to Sephacryl S-200 gel filtration (**Figure 1**B). After Sephacryl S-200, PG-III was further separated into two parts, namely PG-III(a) and PG-III(b) (**Figure 1**C). As a result, four pepsinogens were purified to homogeneity in the present study. The result of the purification of different PGs is summarized in **Table 1**. After column chromatographies, 0.5 mg of PG-I, 5.2 mg of PG-II, 1.9 mg of PG-III(a), and 3.9 mg of PG-III(b) were obtained with purification folds of 25.6, 11.5, 15.5, and 16.2, respectively. The homogeneity of the purified pepsinogens was checked by SDS-PAGE under reducing conditions. As shown in **Figure 2**, all PGs gave relatively pure bands on SDS-PAGE with molecular masses of 36, 35,

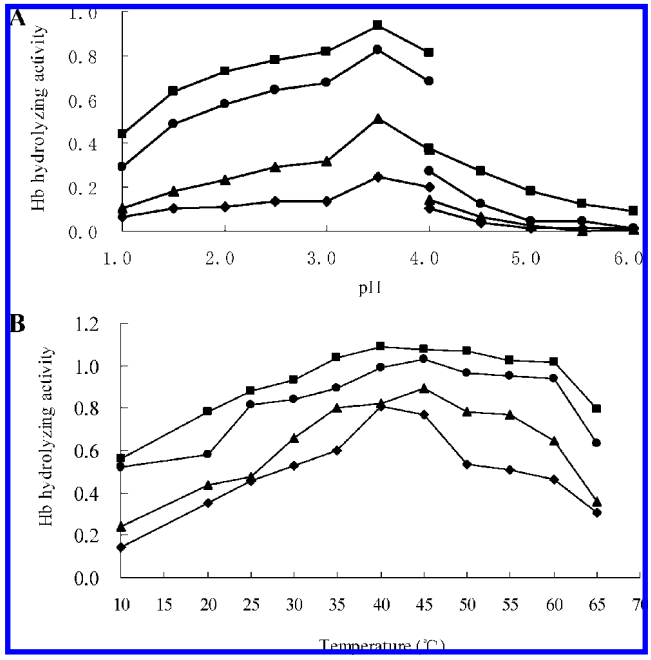

Figure 5. Optimum pH and temperature of pepsins. Enzymatic activity was evaluated using acid denatured bovine hemoglobin as substrate as described in the Materials and Methods. (**A**) Effect of pH on the activities of purified mandarin fish pepsins. Activity was assayed in the pH range 1.0−6.0 using 0.5 M HCl−sodium acetate buffer (pH 1.0−4.0) and 0.5 M acetic acid−sodium acetate buffer (pH 4.0−6.0) at 37 °C. (**B**) Effects of temperature on the activities of purified mandarin fish pepsins. Activity was assayed at temperatures from 10 to 65 °C at pH 3.0 using HCl−sodium acetate buffer. P-I (●); P-II (■); P-III (a) (▲); P-III (b) (◆). The errors of the assays were within ± 5%.

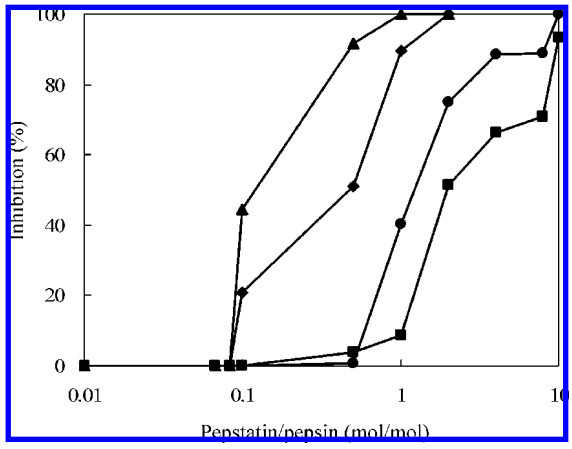

Figure 6. Effect of pepstatin A on mandarin fish pepsins. Pepsins were assayed in the presence of increasing concentrations of pepstatin A using a 2.0% hemoglobin as substrate. Pepsin activity was determined at pH 3.0 and 37 °C. P-I (●); P-II (■); P-III (a) (▲); P-III (b) (◆).

38, and 35 kDa, respectively. The purity of their corresponding active form pepsins after treatment at pH 2.0, 25 °C was also checked by SDS-PAGE under reducing conditions. The molecular masses of the four corresponding pepsins were estimated as 31, 30, 32, and 30 kDa (**Figure 2**). The minor molecular mass variation between mandarin fish PGs and their active form pepsins may suggest the difference in their signal peptides, which is similar to those from mammalians (*16–20*). For example, the activation segment of monkey pepsinogen A was 47 amino acid residues (*16*) while that of monkey progastricsin was 43 amino acid residues (*17*). This result is also comparable

Mandarin PG-I	LKQVPLEKGKTA
Mandarin PG-II	LIQVPLEKGKTARELLE
Coelacanth 1	IAKVPLIKEKPLRAILA
Coelacanth 2	IAKVPLIKEKPLRAILA
Coelacanth 3	LISVPLKRFKSIRESLX
Tuna 1	LIQVPLEKGQSAREYLQ
Tuna 2	FHKLPLIKGKTAREELQ
Tuna 3	INVPLTRHKSMRESLRE
Bullfrog PG1	IIKVPLKKFKSMREVMR
Porcine PG	LVKVPLVRKKSLRQNLI
Bovine PG	SVVKIPLVKKKSLRQNLI
Bovine prochymosin	AEITRIPLYKGKSLRKALK
Human PG-A	IMVKVPLIRKKSLRRTLS

Figure 7. Alignment of N-terminal amino acid sequences of pepsinogens from different animals. The sequences of mandarin fish pepsinogens were compared with those of African coelacanth (*3*), bluefin tuna (*6*), bullfrog (*22*), porcine (*23*), bovine (*24, 25*), and human (*3*).

to those from its counterpart marine fish including Africa coelacanth (*3*) and sea bream (*11*) while slightly smaller than those from North Pacific bluefin tuna (*6*).

Zymography analysis indicated that pepsins of mandarin fish revealed degrading activity against acid-denatured bovine hemoglobin. P-I and P-II are similar in migration under native conditions (**Figure 3**). Interestingly, though PG-III(a) and PG-III(b) were eluted at the same fraction size from the DEAE-Sephacel anion exchange column, their mobility on zymography is quite different, and the enzymatic activity of PG-III(b) is much higher than that of PG-III(a).

Generally, mammalian pepsinogens convert into active forms of pepsin by cleavage of 3−5 kDa signal peptides at the N-terminal. The conversion of pepsinogens to pepsins proceeds autocatalytically at acidic pH by two different pathways: a one-step pathway to release the intact activation segment directly and a stepwise pathway through pseudopepsins (*2*). In the present study, the activation profile of PG was carried out at 37 °C, pH 2.0, and the active form of pepsins can be identified as short as 5 s (**Figure 4**). Similar to PGs from various animals (*16, 19–22*), mandarin fish PGs also undergo limited hydrolysis to convert into active form, pepsins. Though the intermediate form (I) of PG-I and PG-II can be observed during acid activation, those of PG-III(a) and PG-III(b) could not be recognized (**Figure 4**). Such a two-step conversion mechanism has also been reported in two major pepsinogens from African coelacanth (*3*), a major pepsinogen from bluefin tuna (*6*), and pepsinogen I from sea bream (*11*). Complete activation of PG-I was carried out after 10 min, while that of PG-II, PG-III(a), and PG-III(b) was performed in much short time intervals (**Figure 4**).

Optimum pHs of all the four pepsins were 3.5 and showed a slow decline of activity at pH values above their optimum (**Figure 5**). The enzymes nearly completely lost activity at pH 5.5. Similar optimum pH profiles were also identified in pepsins from pectoral rattail (*10*) and sea bream (*11*), while they are higher than most pepsins such as those from Antarctic rock cod (*1*), African coelacanth (*3*), bluefin tuna (*6*), and bullfrog (*22*). However, it should be noticed that because the activity assay at different pH was performed at 37 °C for 30 min, the data shown here not only reflected the enzymatic activity at that pH but also its stability to some degree. Different from porcine pepsin, which exhibited optimum temperature at 60 °C (*1*),

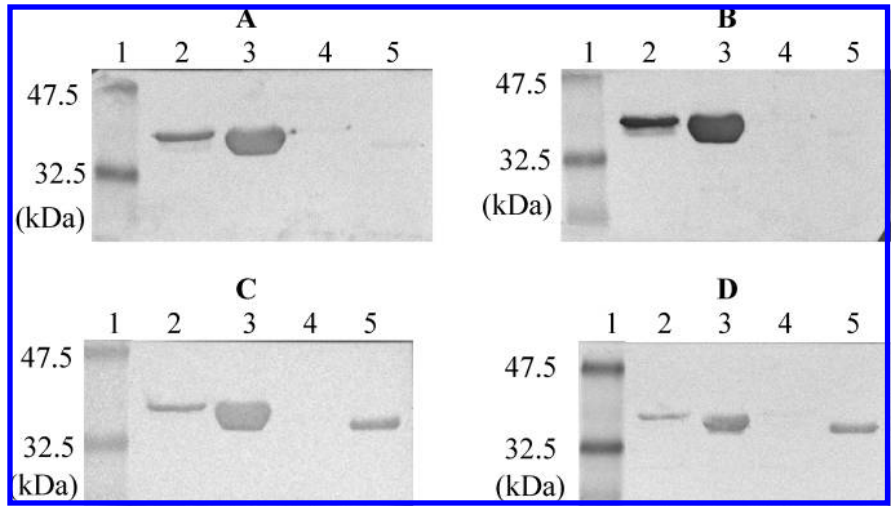

Figure 8. Western blot of pepsinogens using antisea bream pepsinogen polyclonal antibodies. (**A**) reacted with anti-PG-I antibody; (**B**) reacted with anti-PG-II antibody; (**C**) reacted with anti-PG-III antibody; (**D**), reacted with anti-PG-IV antibody. Lanes: 1, prestained protein marker; 2, PG-I; 3, PG-II; 4, PG-III(a); 5, PG-III(b).

optimum temperatures of mandarin fish P-I and P-III(a) were identified as 40 °C while that of P-II and P-III(b) was 45 °C and a sharp drop of enzymatic activity to all the four pepsins at above 60 °C was observed, suggesting their susceptibility to higher temperature (**Figure 5**).

The effect of different inhibitors on the activity of pepsins from mandarin fish is investigated. All the four pepsins were almost completely inhibited by pepstatin A, a typical asparatic proteinase inhibitor, which works by binding the active cleft of pepsins. Pepsins III(a) and III(b) were strongly inhibited by pepstatin with a pepstatin/pepsin molar ratio of approximately 1:1, whereas pepsins I and II were not so sensitive to pepstatin A; a 10-fold molar excess of pepstatin A was necessary for the complete inhibition of these two enzymes (**Figure 6**). A similar result was also found in pepsin 3 from bluefin tuna (*6*) and two pepsins from bullfrog (*22*) where about 17-fold molar excess of pepstatin was required for complete inhibition. On the other hand, inhibitors for serine (PMSF), cysteine (E-64), and metalloproteinases (EDTA) did not reveal any effect (data not shown). These results strongly suggested that the four enzymes purified in the present study are indeed pepsins.

The N-terminal amino acid sequences of PG-I and PG-II were determined to the 12th and 17th residues, respectively, as the following: PG-I, LKQVPLEKGKTA; and PG-II, LIQVPLEKGKTARELLE. Sequence homology alignment revealed that the two PGs shared extremely high identity (91.7%) with each other and high identities (66.7% for PG-I; 70.6% for PG-II) to PG-1 from tuna (*6*). Relatively higher identities to PGs from other animals such as bullfrog (*22*) and porcine (*18*) could also be identified (**Figure 7**).

Western blot using antisea bream PG-I and PG-II polyclonal antibodies revealed that they positively reacted with mandarin fish PG-I and PG-II while only very weak cross reaction could be observed toward PG-III(a) and PG-III(b) (**Figure 8**). On the other hand, antisea bream PG-III and PG-IV antibodies both cross reacted with PG-I, PG-II, and PG-III(b). None of the four antibodies used gave positive reaction to PG-III(a) (**Figure 8**). These results strongly suggested that PG-I and PG-II are closely related pepsinogens and share high primary structure identity with each other, as shown in **Figure 7**. Though the N-terminal amino acid sequences of PG-III(a) and PG-III(b) were not successfully determined in the present study, they may be distant in primary structures, as two positive antibodies to PG-III(b) did not react with PG-III(a). Thus, it is quite possible that PG-III(a) and PG-III(b) may play different roles physiologically.

Previous studies on fish pepsins have revealed that more than two kinds of isozyme exist (*1, 3, 6, 10, 11*). In the present study, four pepsinogens from mandarin fish were detected, and these enzymes are, to our knowledge, the first pepsins purified from freshwater fish. Though the molecular masses of all the four PGs and their active form pepsins are quite similar, some differences in the enzymatic characteristics of PG-I, PG-II, PG-III(a), and PG-III(b) were identified, such as their binding properties to the ion-exchanger column DEAE-Sephacel, zymograms of their active forms, especially their immunological reaction to polyclonal antibodies against sea bream pepsinogens. Similar to its counterpart marine fish such as North Pacific bluefin tuna (*6*) and sea bream (*11*), the presence of multiple pepsinogens in mandarin fish may also be advantageous to accomplish efficient digestion of different foods. Taken together, our present data indicate that the characteristics of the four mandarin fish pepsins are similar but not identical to each other and these differences should be contributed to the genetic evolution of pepsins. Thus, further characterization of these pepsinogens, including the determination of their full-length primary structures and which kind of pepsinogen they are belonging to, is needed.

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