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Study on Pepsinogens and Pepsins from Snakehead (*Channa argus*)

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Three pepsinogens (PG1, PG2, and PG3) were highly purified from the stomach of freshwater fish snakehead (*Channa argus*) by ammonium sulfate fractionation, anion exchange, and gel filtration. Two-dimensional gel electrophoresis and native-PAGE analysis revealed that their molecular masses were 37, 38, and 36 kDa and their isoelectric points 4.8, 4.4, 4.0, respectively. All of the pepsinogens converted into their active form pepsins under pH 2.0 by one-step pathway or stepwise pathway. The three pepsins showed maximal activity at pH 3.0, 3.5, and 3.0 with optimum temperature at 45, 40, and 40 °C, respectively, using hemoglobin as substrate. All of the pepsins were completely inhibited by pepstatin A, a typical aspartic proteinase inhibitor. The N-terminal amino acid sequences of the three pepsinogens were determined to the 34th, 25th, and 28th amino acid residues, respectively. Western blot analysis of the three PGs exhibited different immunological reactions.

KEYWORDS: Snakehead fish; purification; pepsinogen; pepsin; Western blot

INTRODUCTION

Pepsinogens (PGs), the zymogens of pepsins, are normally derived from the stomach of animals. PGs autocatalytically convert into their corresponding active form pepsins under acidic conditions, releasing the activation segments from their NH_2-terminal region by two different pathways, a one-step pathway and a stepwise pathway (*1*, *2*). Pepsins (EC 3.4.23.1) are a family of aspartic proteinases that perform important digestive functions in both invertebrates and vertebrates (*2*). They have been studied extensively (*3*, *4*), and their three-dimensional structures have been determined at high resolutions (*5*). As commercial enzymes, pepsins have several applications in food industries, pharmaceutical industry, biotechnology, and other fields. Several types of gastric aspartic proteinases are known to be present in the stomach. They are mainly classified into five groups: pepsinogens A, B, and F; progastricsin (pepsinogen C); and prochymosin (*2*,*6*), which are different from each other in their primary structures and enzymatic properties of their activated forms.

To date, most knowledge of pepsinogens and pepsins is from studies on Japanese monkey (*7*), cow (*8*), human (*5*, *9*), pig (*10*), rabbit (*11*), and goat (*12*). On the other hand, aspartic proteinases from fish have also been research targets in these decades: pepsinogens and pepsins from Africa coelacanth (*6*), rainbow trout (*13*), Pacific bluefin tuna (*14*−*16*), Atlantic cod (*17*), shark (*18*), Antarctic rock cod (*19*), pectoral rattail (*20*), sea bream (*21*), and smooth hound (*22*) were reported. However, much less information is available on pepsinogens and pepsins from freshwater fish except for our recent study on mandarin fish (*Siniperca chuatsi*) (*23*) and European eel (*Anguilla anguilla*) (*24*).

Snakehead is a freshwater fish of the family Channidae that is native to the Yangtze River and can be found in many provinces of China. It is regarded as valuable fish because of its taste and even curative functions in traditional Chinese medicine. However, snakeheads are voracious predatory fish and when mature, they feed on other fish, amphibians, crustaceans, small reptiles, and even small mammals. Thus, snakeheads are regarded as invasive species causing potential ecological damages in many countries, especially in the United States.

As one of the most essential digestive enzymes, pepsins play important roles in their food digestion. Thus, a detailed study of pepsinogens and pepsins from the stomach of the freshwater top-level predator snakeheads is of much interest to us. In the present paper, we describe the purification and characterization of pepsinogens and pepsins from snakehead; the N-terminal amino acid sequences of the pepsinogens were also determined and analyzed.

MATERIALS AND METHODS

Materials. Cultured snakeheads (*Channa argus*) with body weight of about 1800 g were purchased alive from a fish market of Jimei, Xiamen. The fish were sacrificed, and stomachs were obtained and washed thoroughly for experimental use. DEAE-Sephacel, Sephacryl S-200 HR, and 7 cm IPG strips (pH 3−10) were from GE Healthcare; phenylmethanesulfonyl fluoride (PMSF) and bovine hemoglobin were from Sigma. L-3-Carboxy-*trans*-2,3-epoxypropionyl-L-leucine-4-guanidinobutylamide

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(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 Western blot was from New England Biolabs (Beverly, MA). Rat anti-sea bream (*Sparus latus*) pepsinogen I, II, and III polyclonal antibodies were prepared in our laboratory (*21*). Second antibody of rabbit anti-rat IgG−horseradish peroxidase (HRP) was from DAKO (Denmark). 3,3′-Diaminobenzidine tetrahydrochloride (DAB) was from Pierce (Rockford, IL). All other reagents were of analytical grade.

Purification of Pepsinogens. All procedures for protein purification were performed at low temperature (0−4 °C). Snakehead stomachs (50 g) were washed and cut into small pieces and homogenized in 6-fold 50 mM phosphate buffer (pH 7.0) containing 5 mM PMSF, 5 mM EDTA, and 1 mM E-64 using a homogenizer (Kinematica, PT-2100, Switzerland). The homogenate was centrifuged at 10000*g* for 90 min, the resulting supernatant was collected, and the precipitate was further homogenized and centrifuged. All of the resulting supernatant was brought to 20% ammonium sulfate saturation and allowed to stand for 2 h. The mixture was then centrifuged at 10000*g* for 30 min to remove precipitate. Ammonium sulfate was added to the supernatant to 60% saturation and allowed to stand for another 2 h. After centrifugation at 10000*g* for 30 min, the resultant precipitate, which contained mostly snakehead pepsinogens, was dissolved in a minimum amount of 25 mM phosphate buffer (pH 7.0) and dialyzed against the same buffer extensively. The dialysate was then loaded on a DEAE-Sephacel anion-exchange column (2.5 × 13 cm). 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 0−0.5 M NaCl at a flow rate of 0.8 mL/min. Pepsin active fractions were pooled and concentrated by ultrafiltration using a membrane of YM-10 (Millipore). Samples were then respectively applied to a gel filtration column of Sephacryl S-200 HR (1.5 × 98 cm) equilibrated with 25 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl at a flow rate of 0.6 mL/min. Active fractions from Sephacryl S-200 were collected as purified pepsinogens and used for electrophoresis analysis and enzymatic characterization.

Protein Concentration Assay. Protein concentration was determined by measuring the absorbance at 280 nm of the sample solution or according to the method of Lowry et al. (*25*) with bovine serum albumin as standard.

Enzyme Activity Assay. Potential pepsin activity was determined according to the method of Anson (*26*) with slight modification as follows: 50 μL of appropriately diluted enzyme sample was added into 250 μL of 0.25 M HCl−sodium acetate buffer (pH 3.0) and mixed with 200 μL of 2.0% acid-denatured bovine hemoglobin, which had been extensively dialyzed against 0.05 M HCl. After 30 min of incubation at 37 °C, the reaction was stopped by the addition of 0.5 mL of 8.0% trichloroacetic acid (TCA) and centrifuged at 8000*g* for 10 min. The absorbance of the resulting supernatant containing proteolytic digested small peptides was measured at 280 nm using a Cary 50 UV−visible spectrophotometer (Varian). Control tests were performed by adding TCA prior to the addition of pepsin to the reaction mixture. All proteolytic activity assays were performed in duplicate, and variation between duplicate samples was always < 5%; the mean values were used. 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.

Isoelectric Focusing (IEF) and Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). IEF was performed on 7 cm ReadyStrip IPG strips with pH 3−10. Purified pepsinogens were resuspended in IEF buffer containing 2 M thiourea, 7 M urea, 2% (w/v) Chaps, 0.2% (v/v) pH 3−10 ampholines, 1 M dithiothreitol (DTT), and bromophenol blue. IEF was performed in Ettan IPGphor apparatus according to the instructions of the manufacturer (GE Healthcare) at 20 °C as follows: the strips were rehydrated in isoelectric focusing buffer at 50 V for 13 h, 30 min at 300 V, followed by 30 min at 1000 V; the voltage was then increased to 5000 until 6000 Vh was reached, and thereafter the voltage was decreased to 500 V and held at that voltage for 3 h. After IEF, the strips were equilibrated with 50 mM Tris-HCl (pH 8.8) containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 2% (w/v) DTT for 10 min followed by another 10 min using the same buffer with 2.5% iodoacetamide instead of DTT.

Electrophoresis in the second dimension was performed on MiniVE COMPLETE according to the method of Laemmli (*27*) using 12% gels. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (CBB). The molecular masses of the proteins were estimated by calibration of the gels with a protein marker consisting of β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), egg ovalbumin (45 kDa), pig muscle lactate dehydrogenase (35 kDa), restriction endonuclease (25 kDa), milk β-lactoglobulin (18.4 kDa), and egg white lysozyme (14.4 kDa).

Western Blot and Zymography. Western blot was performed as described by Towbin et al. (*28*). 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 rat anti-sea bream PG-I, PG-II, and PG-III polyclonal antibodies (*21*) at room temperature for 2 h and washed with TBST (TBS, 0.05% Tween-20). After incubation for 1 h with HRP conjugated rabbit anti-rat IgG secondary antibody, membranes were washed extensively with TBST. Immunodetection was carried out using DAB as substrate.

Zymography was performed similarly to SDS-PAGE without the addition of SDS to both samples and gel. Electrophoresis was performed at 4 °C. After electrophoresis, the gel was incubated in 1% acid-denatured bovine hemoglobin (pH 3.0) at 37 °C for 1 h. After washing, the gel was stained with CBB. Enzymatic activity was detected by revealing clear bands against dark background on the gel.

Conversion of Pepsinogen to Pepsin. The solution of purified pepsinogen was acidified to pH 2.0 by adding 0.1 M HCl, which converted pepsinogen into the activated form, pepsin. The conversion reaction was carried out using purified pepsinogens in different test tubes individually at pH 2.0 and 25 °C. At corresponding reaction intervals, 10 μL of 16% TCA was added to the 100 μL reaction mixture to stop the reaction. Samples were subjected to SDS-PAGE, and gels were stained with CBB.

Determination of pH and Temperature Optimum. To investigate the effect of pH on the activity of pepsin, pepsinogens were first converted to pepsins at pH 2.0 and 37 °C for 30 min followed by activity analysis. The buffers used were 0.25 M HCl−sodium acetate buffer (pH 1.0−4.0) and 0.25 M acetic acid−sodium acetate buffer (pH 4.0−6.0). Optimum temperature of pepsins was determined by incubating pepsins with 2.0% hemoglobin in 0.25 M HCl−sodium acetate buffer (pH 3.0) at different temperatures (10−65 °C) for 30 min, and the activity was determined, respectively, as described above.

Inhibitory Effect of Pepstatin A. Pepsin activity was analyzed in the presence of different molar ratios of pepstatin A to pepsin in the range from 1:12 to 4:1. After preincubation of the mixture at room temperature for 20 min, the remaining pepsin activity was measured using 2% hemoglobin as substrate at 37 °C for 30 min as described above.

Amino Acid Sequence Determination. Purified pepsinogens were subjected to SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was briefly stained by CBB, and the protein band was excised. The N-terminal amino acid sequences of the pepsinogens were determined using a protein sequencer (Shimadzu, PPSQ-33A).

Kinetic Parameters. For kinetic analysis, hemoglobin was used as substrate, and the activity was determined as described above. Kinetic constants K_m and V_{max} were determined by Lineweaver−Burk plots using the reciprocal of the initial velocity versus the reciprocal of the concentration of hemoglobin. The catalytic constant k_{cat} values were calculated from the equation $V_{max} = k_{cat}[E]$, where [E] is the pepsin concentration. All of the kinetic values were obtained from the average of three independent determinations.

RESULTS AND DISCUSSION

In the present study, three pepsinogens (PGs) from the stomach of the freshwater fish snakehead were purified by ammonium sulfate fractionation and column chromatographies of DEAE-Sephacel and Sephacryl S-200. In the first anion-exchange column DEAE-Sephacel, three peaks (PG1, PG2, and PG3) of hemoglobin-digesting activity were detected (**Figure 1A**). These peaks were pooled respectively and applied to gel filtration column Sephacryl S-200 for further purification (**Figure 1B**). As a

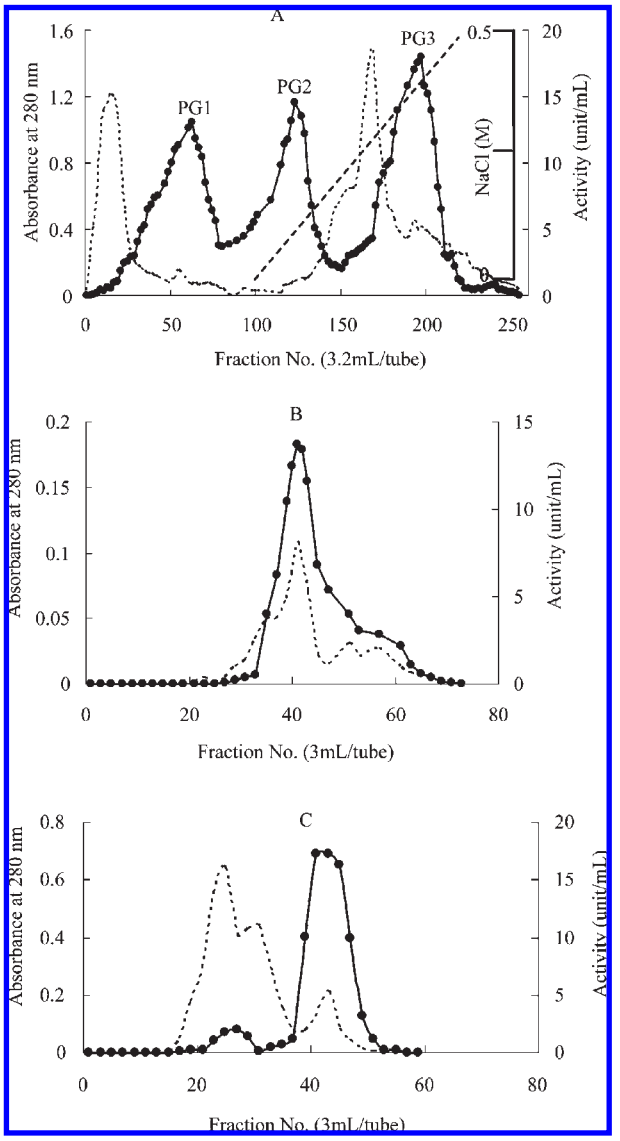

Figure 1. Column chromatography purification of snakehead fish pepsinogens: (**A**) DEAE-Sephacel chromatography; (**B**) Sephacryl S-200 gel filtration purification of PG2; (**C**) Sephacryl S-200 gel filtration of PG3; (●) hemoglobin hydrolyzing activity; (- - -) absorbance at 280 nm.

Table 1. Summary of Purification of Pepsinogens from Snakehead Stomach

stage	total protein (mg)	specific activity (units/mg)	purification (-fold)	yield (%)
crude extract	1022.8	4.2	1	100
ammonium sulfate	435.6	6.7	1.6	66.8
DEAE-Sephacel				
PG1	4.0	142.5	33	13.2
PG2	5.2	114.9	27.4	13.8
PG3	32.1	35.4	8.4	26.2
Sephacryl S-200				
PG1	0.8	225.0	53.6	4.1
PG2	1.7	125.0	29.8	4.8
PG3	2.2	139.0	33.1	7.0

result, 0.8 mg of PG1, 1.7 mg of PG2, and 2.2 mg of PG3 were obtained with purification folds of 53.6, 29.8, and 33.1, respectively (**Table 1**).

The homogeneity of purified pepsinogens was checked by 2D-PAGEs. All pepsinogens gave single spots on 2D-PAGEs, and the estimated molecular masses were approximately 37, 38, and

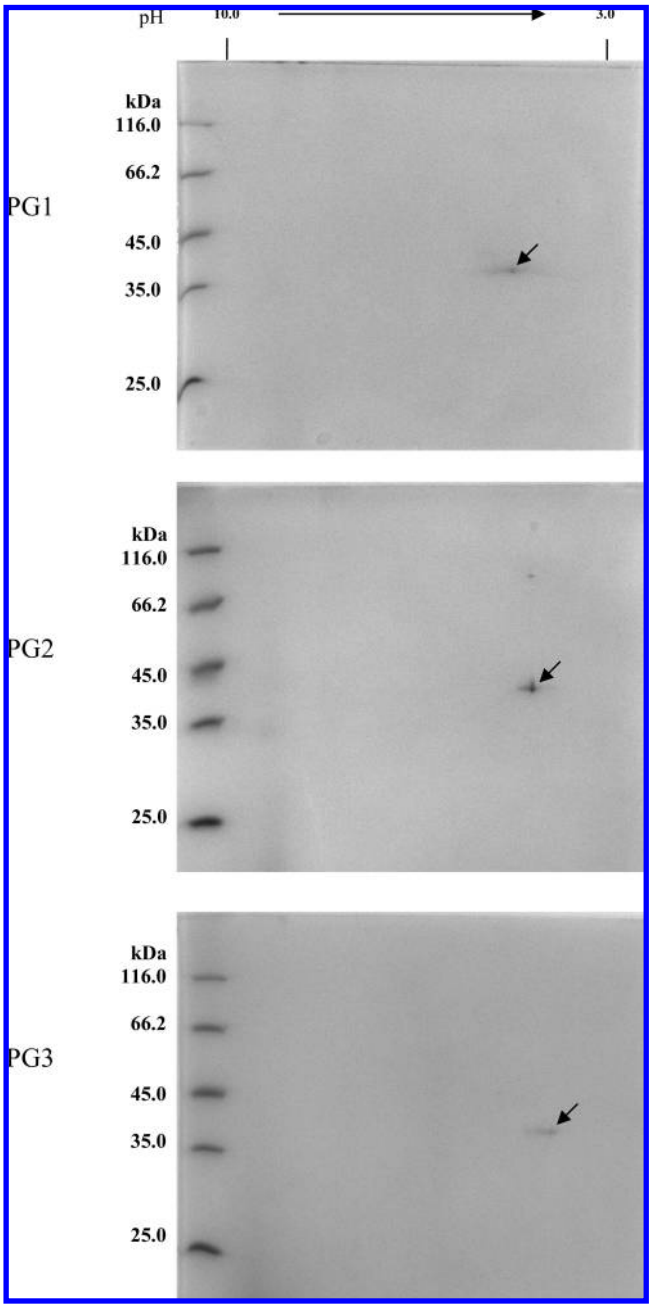

Figure 2. Two-dimensional gel electrophoresis of purified snakehead pepsinogens. The gels were stained with Coomassie Brilliant Blue.

36 kDa; their p*I* values were approximately 4.8, 4.4, and 4.0, respectively (**Figure 2**). It should be noted that although the p*I* value of PG1 was 4.8, the binding of this protein to the DEAE-Sephacel ion-exchange column was weak, and only an elution position delay of PG1 compared with the main unbound protein peak could be observed (**Figure 1**). Quite possibly, because of the steric structure of the protein, the p*I* value may not be in complete accordance with its performance under chromatographic pH conditions (pH 7.0). p*I* values of other fish, such as three pepsinogens from Pacific bluefin tuna (5.69, 4.95, and 4.66) (*16*) and pepsinogens A1 and A2 from orange-spotted grouper (4.41 and 5.17) (*29*), have been reported, whereas all of these p*I* values were deduced from amino acid sequence analysis based on molecular cloning. Compared with these, the p*I* values of snakehead pepsinogens were relatively lower.

Zymography analysis indicated that pepsins of snakehead revealed degrading activity against acid-denatured bovine

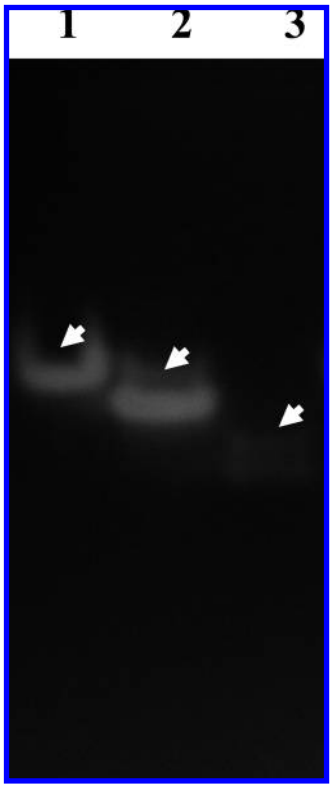

Figure 3. Zymogram of snakehead pepsins: lane 1, pepsin 1; 2, pepsin 2; 3, pepsin 3. Pepsinogens were converted to pepsins at pH 2.0 and 25 °C. Electrophoresis of pepsins was performed at 4 °C on native-PAGE. After electrophoresis, pepsins on the gel were incubated at 37 °C with 1.0% acid-denatured bovine hemoglobin (pH 3.0) as substrate for 1 h. Approximately 20 μg of pepsin was applied on each lane. Gels were stained with Coomassie Brilliant Blue. Arrowheads indicate the position of pepsins.

hemoglobin (pH 3.0). The three pepsins are quite different in mobility and enzymatic activity under native conditions (**Figure 3**), which further suggested that they are different from each other.

The conversion of pepsinogens to pepsins is expected to have a decrease in molecular mass of approximately 5 kDa because of the loss of the N-terminal propart (*19*). This process involves a series of conformational changes and bond cleavage steps that result in the unveiling of the active site and ultimately the removal and dissociation of the prosegment from the active center of the enzyme (*30*). The activation reaction initiates autocatalytically at acidic pH by two different pathways: a direct pathway to release the intact activation segment and a sequential pathway through a pseudopepsin, an intermediate form between pepsinogen and pepsin (*2*). In the present study, the activation profile of PGs was carried out at pH 2.0 and 37 °C, and the active form of pepsins can be identified with corresponding molecular masses of 32, 33, and 31 kDa (**Figure 4**). Similar to PGs from various animals such as monkey (*7*), bullfrog (*31*), turtle (*32*), and goat (*12*), snakehead pepsinogens also undergo limited hydrolysis to convert into active pepsins. The intermediate form (I) of PG1 and PG2 can be observed during acid activation, whereas conversion of PG3 to P3 was performed by a direct pathway (**Figure 4**). A direct activation pathway has been demonstrated in PG3 from tuna (*14*), PG-III(a) and PG-III(b) from mandarin fish (*23*), and PG-II from European eel (*24*). On the other hand, a sequential conversion mechanism was also observed in PGs from other fish, such as two major pepsinogens from African coelacanth (*6*), a major pepsinogen from bluefin tuna (*14*), PG-I from sea bream (*21*), PG-I and PG-II from mandarin fish (*23*), and PG-I and PG-III from European eel (*24*).

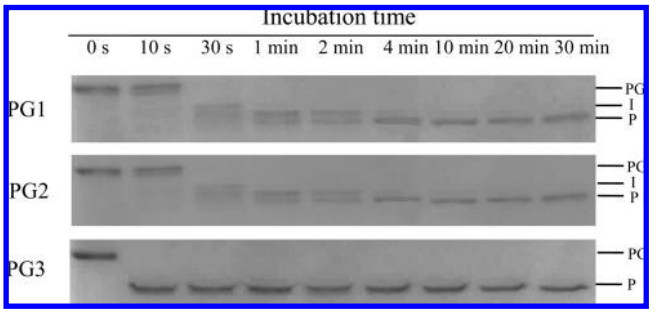

Figure 4. Conversion of snakehead pepsinogens to pepsins. Pepsinogens were incubated at pH 2.0 and 37 °C, respectively. Aliquots were removed, and conversion reaction was stopped immediately at appropriate time intervals as indicated above each lane and subjected to SDS-PAGE. I, intermediate form; P,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

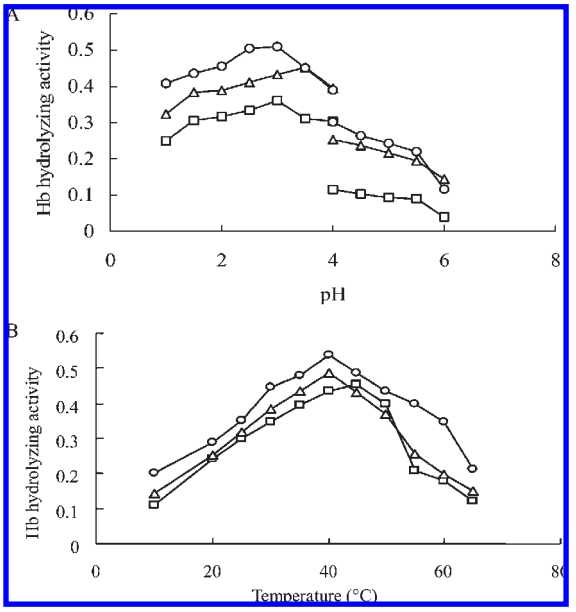

Figure 5. Optimum pH and temperature of pepsins. Enzymatic activity was evaluated using acid-denatured bovine hemoglobin as substrate as described under Materials and Methods: (**A**) effect of pH on the activities of purified snakehead pepsins [activity was assayed in the pH range of 1.0−6.0 using 0.25 M HCl−sodium acetate buffer (pH 1.0−4.0) and 0.25 M sodium acetate buffer (pH 4.0−6.0) at 37 °C]; (**B**) effect of temperature on the activities of purified snakehead pepsins [activity was assayed at temperatures from 10 to 65 °C at pH 3.0 using HCl−sodium acetate buffer buffer]; P1 (□); P2 (△); P3 (○).

As shown in **Figure 5A**, the optimum pH value of P1 and P3 was 3.0, whereas that of P2 was 3.5, which reflects the fact that pepsins are endopeptidases and maximally active at acidic pH. Similar optimum pH values of pepsin profiles were also identified in pepsins from sea bream (*21*), Antarctic rock cod (*19*), African coelacanth (*6*), bluefin tuna (*14*), mandarin fish (*23*), and European eel (*24*). The denaturation of substrate hemoglobin at low pH was proposed to be responsible for their efficient hydrolysis (*2*).

The effect of temperature on enzyme activity was also examined (**Figure 5B**). Different from porcine pepsin, which exhibited optimum temperature at 60 °C (*10*), the optimum temperature of snakehead P1 was 45 °C, whereas that of P2 and P3 was 40 °C. All of the enzymatic activity of the three pepsins decreased above 50 °C, suggesting their susceptibility to higher temperature (**Figure 5B**). To avoid autolysis of pepsins at low pH, the stability

of all three pepsins was thus examined at 50 °C and pH 7.0. **Table 2** shows that the half-lives of snakehead P1, P2, and P3 were 110, 40, and 17 min, respectively. Compared to P1, the half-lives of P2 and P3 were 2.7 and 6.5 times more labile under the test condition. Similar results were also reported in pepsins from Antarctic rock cod, although their pH was set at 5.3 (*19*).

The activity of all three pepsins could be completely inhibited by pepstatin A, a pentapeptide from *Streptomyces*, which is a specific inhibitor against aspartic proteinases, although its affinity differs between enzymes. P1 and P2 were nearly completely inhibited by pepstatin with a pepstatin/pepsin molar ratio of 1:1, whereas P3 was not so sensitive: a 4-fold molar excess of pepstatin A was necessary for the complete inhibition (**Figure 6**). On the other hand, inhibitors for serine proteinase (PMSF), cysteine proteinase (E-64), and metalloproteinases (EDTA) did not reveal any effect (data not shown). These results strongly indicated that the three enzymes of snakehead purified in the present study are indeed pepsins.

Table 2. Stability of Snakehead Pepsins at 50 °C and pH 7.0[a]

enzyme	half-life (min)
pepsin 1	110 ± 1.4
pepsin 2	40 ± 0.6
pepsin 3	17 ± 0.5

[a] Aliquots of enzyme were removed at different time intervals, and the remaining activity was measured at pH 3.0 and 37 °C.

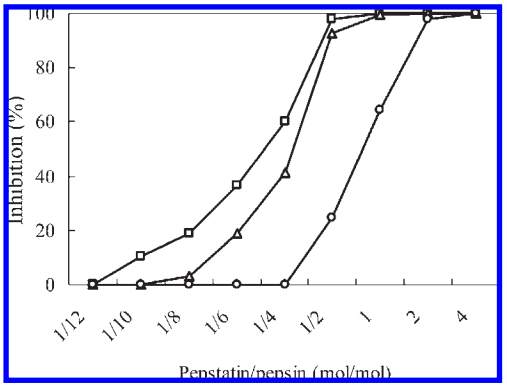

Figure 6. Effect of pepstatin A on snakehead pepsins. Pepsin was preincubated with different molar ratios of pepstatin A at room temperature for 20 min; the remaining pepsin activity was measured using 2.0% hemoglobin as substrate at pH 3.0 and 37 °C: P1 (□); P2 (△); P3 (○).

The N-terminal amino acid sequences of the three pepsinogens were determined, respectively (**Figure 7**). The results indicate a high degree of sequence homology among the three pepsiongens. Similar to pepsinogens 1 and 2 from African coelacanth (*6*), snakehead PG1 and PG2 were identical, and PG1 revealed 67.9% identity to PG3. It is of interest to note that PG1 and PG2 shared high identities of 82.4 and 76% to PG-1 from tuna (*14*). Relatively higher identities to PGs from African coelacanth (*6*) and bullfrog (*31*) could also be identified (**Figure 7**). The low sequence homology (21−36%) of snakehead PGs to these of porcine (*10*), bovine (*8*, *33*), and human (*9*) indicated that fish pepsinogens reveal a significant evolutionary gap between fish and mammalian pepsinogens.

Western blot using anti-sea bream PG-I and PG-II polyclonal antibodies positively reacted with snakehead PG1 and PG2, whereas only weak cross-reaction could be observed toward PG3 (**Figure 8**). However, anti-sea bream PG-III polyclonal antibody gave no positive reaction to all three PGs from snakehead (**Figure 8**). These results further confirmed that PG1 and PG2 are closely related pepsinogens and share high identity with each other, whereas PG3 is different.

The results of the K_m and k_{cat} of different pepsins, which were determined by Lineweaver−Burk plots using acid-denatured bovine hemoglobin as substrate, are shown in **Table 3**. The kinetic parameter K_m values of the three snakehead PGs were comparable to those from European eel (*24*), Atlantic cod (*17*), and pectoral rattail (*20*), whereas the specificity constants (k_{cat}/K_m), which are measures of catalytic efficiency, were several fold lower, suggesting that the steric structures of snakehead pepsins may be different around their active site, which ultimatetly affected their catalytic performances.

Similar to its counterpart marine fish such as Pacific bluefin tuna (*14*) and sea bream (*21*), the presence of multiple pepsinogens in snakehead may also be advantageous to accomplish efficient digestion of different foods. As an invasive freshwater fish, it is of interest to note that all three pepsins of snakehead exhibited 2−4-fold higher specific activity as compared with those from mandarin fish (*23*) and European eel (*24*), suggesting

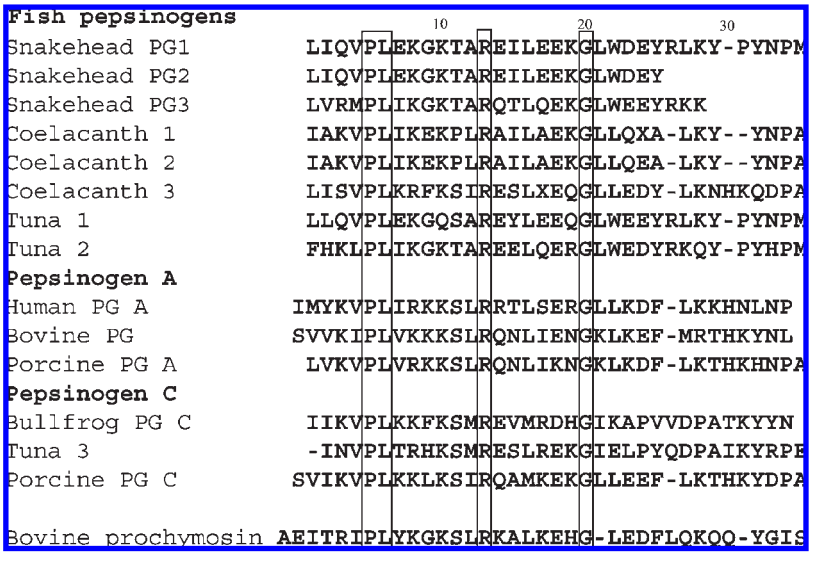

Figure 7. Alignment of N-terminal amino acid sequences of pepsinogens from different animals. The sequences of three snakehead pepsinogens were compared with those of African coelacanth (*6*), bluefin tuna (*16*), bullfrog (*31*), pig (*10*), cow (*8*, *33*), and human (*9*). Identical amino acid residues are boxed.

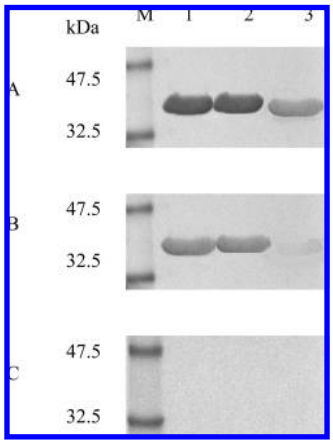

Figure 8. Western blot of pepsinogens using anti-sea bream pepsinogen polyclonal antibodies: (**A**) reacted with anti-PG-I antibody; (**B**) reacted with anti-PG-II antibody; (**C**) reacted with anti-PG-III antibody. Lanes: M, prestained protein marker; 1, PG1; 2, PG2; 3, PG3.

Table 3. Kinetic Properties of Snakehead Pepsins for the Hydrolysis of Hemoglobin[a]

enzyme	K_m (M)	k_{cat}(s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
snakehead			
pepsin 1	9.8×10^{-5}	8.24	8.4×10^4
pepsin 2	6.0×10^{-5}	4.02	6.7×10^4
pepsin 3	1.4×10^{-4}	13.2	9.5×10^4
pepsin I			
European eel	8.8×10^{-5}	23.7	2.7×10^5
Atlantic cod	1.56×10^{-4}	18	1.2×10^5
pepsin II			
European eel	9.2×10^{-5}	19.4	2.1×10^5
pepsin IIa			
Atlantic cod	4.4×10^{-5}	33	2.8×10^5
pepsin III			
European eel	7.0×10^{-5}	34.4	4.9×10^5
pepsin A			
pectoral rattail	9.8×10^{-5}	50	5.1×10^5
pepsin B			
pectoral rattail	1.52×10^{-4}	32	2.1×10^5

[a] The kinetic constants of other fish species including European eel (*24*), Atlantic cod (*17*), and pectoral rattail (*20*) are also listed in the table for comparison.

pepsin-specific activity and eating habits may have some relationship. Such a phenomenon was also found in PG2 from tuna, for which migratory behavior and much more active protein digestion in the stomach were ascribed (*6*, *14*). Our present results indicate that the characteristics of the three snakehead pepsinogens are similar but not identical to each other, and these differences should contribute to the genetic evolution of pepsins. Thus, further characterization of the primary structures, function, and expressional regulation of these pepsinogens is needed.

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