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Purification and characterization of sea bream (*Sparus latus* Houttuyn) pepsinogens and pepsins

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Abstract

Four pepsinogens (PG-I, PG-II, PG-III, and PG-IV) were identified in sea bream (*Sparus latus* Houttuyn) stomach and were purified to homogeneity by 20–60% ammonium sulfate fractionation and several chromatographies. The molecular weights of the four purified PGs were 36, 32, 32, and 34 kDa, respectively. All the pepsinogens converted into pepsins within a few minutes under pH 2.0. The molecular weights of all the four pepsins were approximately 30 kDa as determined by SDS–PAGE. Optimum pHs of the four enzymes were 3.0–3.5, and optimum temperatures were 45–50 °C. Western blot analysis revealed that anti-PG-II antibody cross-reacted with all the four pepsins while scarcely any immunological cross-reaction existed between anti-PG-I antibody and other pepsins. From the Lineweaver–Burk double reciprocal plots, the K_ms of pepsins (P-I, P-II, P-III, and P-IV) for hemoglobin were calculated as 8.7×10^{-8} M, 1.0×10^{-7} M, 8.6×10^{-8} M, and 7.3×10^{-8} M, respectively.
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Keywords: Sea bream; Purification; Pepsinogens; Pepsins

1. Introduction

Pepsinogens (PGs), the precursors of 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. Pepsinogens are classified into five groups: pepsinogens A, B and F, progastricsin, and prochymosin (Kageyama, 2002). The five types of PGs differ from each other in their primary structures and enzymatic properties of their activated forms (Kageyama, 2002; Kageyama, Tanabe, & Koiwai, 1990). PGs have been purified from the gastric mucosa of various vertebrates, and their primary structures have been reported in different organisms, such as human (Sogawa, Fujii-Kuriyama, MIzukami, Ichihara, & Takahashi, 1983), Japanese monkey (Kageyama & Takahashi, 1976), pig (Nielsen & Foltmann, 1995), cow (Martin, Torieu-Cuot, Collin, & Ribadeau, 1982), goat (Suziki et al., 1999), rat (Muto & Tani, 1979;), rabbit (Kageyama et al., 1990), chicken (Donta & Van Vunakis, 1970), and bullfrog (*Rana catesbeiana*) (Yakabe et al., 1991). Pepsinogens and pepsins from reptiles, such as turtle (*Trionyx sinensis*) (Hirasawa, Athauda, & Takahashi, 1996), and snake (*Trimeresurus flavoviridis*) (Yonezawa et al., 2000), have also been studied.

On the other hand, so far, studies on pepsinogens and pepsins from fish are quite few. Only PGs from trout (*Salmo gairdneri*) (Twining, Alexander, Huibregtse, & Glick, 1983), tuna (*Thynuus orientalis*) (Tanji, Kageyama, & Takahashi, 1988), Atlantic cod (*Gadus morhua*) (Gildberg, Olsen, & Bjarnason, 1990), and shark (*Centroscymnus coelolepis*) (Nguyen et al., 1998) have been reported. From the viewpoint of structure–function relationships and molecular evolution of pepsinogens, it would be interesting to purify pepsinogens from other fish species and investigate their biochemical properties in detail.

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Sea bream (*Sparus latus* Houttuyn) is a popularly cultured marine fish in different provinces of China because of its good taste and texture. Pepsins are the most important digestive enzymes for animals and the activity of pepsins profoundly influences their digestion of proteins and many factors influence activity. Thus, a detailed study of pepsinogens and pepsins may be helpful for the manufacturers of fish feed and the development of aquaculture. In the present study, we purified pepsinogens and pepsins from the stomach of sea bream and investigated their characteristics.

2. Materials and methods

2.1. Materials

2.1.1. Fish

Eight sea bream *(S. latus* Houttuyn) cultured for 5–6 months, with body weights of about 200 g were purchased alive from the fish market of Jimei, Xiamen. The fish were sacrificed and stomach was obtained and washed for experimental use.

2.1.2. Chemicals

DEAE–Sephacel, Sephacryl S-200 HR, and Protein A Sepharose CL-4B were purchased from Amersham Biosciences (Uppsala, Sweden), phenylmethanesulfonyl fluoride (PMSF), bovine hemoglobin, complete and incomplete Freund's adjuvants were from Sigma (St. Louis, MO, USA). L-3-carboxy-*trans*-2, 3- epoxy-propionyl-L-leucin-4-guanidinobutylamide (E-64) was a product of Amresco (Solon, OH, USA), pepstatin was from Roche (Mannhem, Germany), protein marker for SDS–PAGE was from Fermentas (Lithuania) and prestained protein marker for immunoblotting was from New England Biolabs (Richmond, CA, USA). The second antibody of rabbit anti-rat IgG-HRP was from DAKO (Denmark). DAB (3,3′-diaminobezindin tetrahydrochloride) was from Pierce (Rockford, IL, USA). Other reagents were of analytical grade.

2.2. Purification of pepsinogens

All procedures were performed at 4 °C. Sea bream stomach (20 g) was washed, and homogenized in sixfold 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 in a centrifuge (Avanti J-25, Beckman Coulter, USA), the supernatant was collected and the precipitate was further homogenized with threefold of the same buffer and centrifuged. The resulting supernatant was collected together and fractionated with ammonium sulfate from 20% to 60% saturation. After centrifugation at 10,000*g* for 30 min, the resultant precipitate was dissolved in a minimum amount of 25 mM of phosphate buffer (pH 7.0) and dialyzed against the same buffer for about 20 h with 4 times of buffer change. The dialyzed solution was subsequently applied to a DEAE–Sephacel column (2.5 × 10 cm), which was previously equilibrated with the dialysis buffer. After washing the column with the starting buffer until the absorbance at 280 nm reached baseline, binding proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M in a total volume of 600 ml. Fractions with pepsin activity in the unabsorbed or absorbed portions were pooled and concentrated by ultrafiltration, using a membrane of YM-10 (Millipore, MA, USA). The samples were then 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. Active fractions from the Sephacryl S-200 HR column were collected and used for electrophoresis analysis and enzymatic characterization.

2.3. Protein concentration determination

Protein concentration was determined by measuring the absorbance at 280 nm of the sample solution or by the method of Lowry, Rosebrough, Farr, and Randall (1951) with bovine serum albumin as standard.

2.4. Determination of enzyme activity

Potential pepsin activity was determined by the method of Anson (1938) with slight modification. Proteolytic activity assays were performed in duplicate and variation between duplicate samples was always <10%; the mean values were used. Briefly, 50 μl of appropriately diluted enzyme sample were mixed with 100 μl of solution consisting of 2.0% acid-denatured bovine hemoglobin and 350 μl of 0.5 M sodium acetate–HCl buffer (pH 3.0). After incubation of the mixture at 37 °C for 30 min, the reaction was immediately stopped by addition of 500 μl of 8.0% trichloroacetic acid (TCA). The mixture was centrifuged at 800*g* for 10 min and the absorbance of the supernatant at 280 nm was measured. The enzymatic activity was routinely determined at pH 3.0 and 37 °C unless otherwise described. One unit of pepsin activity was defined as an increase of absorbance of 1.0 during an incubation time of 30 min under the above assay conditions.

2.5. Electrophoresis and zymography

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method of Laemmli (1970), using a 12.0% gel, and the proteins were stained with Coomassie brilliant blue R-250 (CBB). Native-PAGE was performed according to the procedure of Laemmli (1970) without the addition of SDS. Zymography was performed, based on native-PAGE. Briefly, after electrophoresis, the gel was submerged in 1% acid-denatured bovine hemoglobin and incubated at 37 °C for 1 h. After washing, the gel was stained with Coomassie brilliant blue R-250 (CBB) for zymography analysis.

2.6. Characterization of the conversion from pepsinogen to pepsin

In order to convert pepsinogens to pepsins, the pH of purified PGs was adjusted to 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 tubes with a volume of 90 μl and incubated at 25 °C for different time intervals. At each reaction interval, 10 μl of 16% trichloroacetic acid (TCA) were immediately added to terminate the conversion reaction. Supernatant in each tube was collected after a short spin and subjected to SDS–PAGE. The gel was stained with CBB.

2.7. Effects of pH and temperature on the activity

The effects of pH and temperature on the activity of sea bream pepsins were determined with 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 and 37 °C for 15 min. One hundred microliters of 2% acid-denatured bovine hemoglobin were added to 350 μl buffers of different pH and mixed with 50 μl of pepsin, followed by incubation at 37 °C for 15 min for activity assay. The buffers used were 0.5 M sodium acetate–HCl 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 were incubated with bovine hemoglobin in 0.5 M sodium acetate–HCl buffer (pH 3.5) in a total volume of 0.5 ml at different temperatures (10–60 °C) for 15 min and the activity was determined.

2.8. Effect of inhibitors on proteinase activity

Different kinds of proteinase inhibitors were added to the pepsin solution to obtain the final concentration designated (5 mM PMSF, 0.1 mg/ml E-64, 5 mM EDTA, and 0.1 mg/ml pepstatin). After incubation of the mixture at room temperature (26–28 °C) for 15 min, remaining activity was measured.

2.9. Determination of enzyme kinetics

Pepsins were prepared in appropriate concentrations and reacted with different concentrations of hemoglobin at 37 °C for 6 min to determine the enzymatic activities. The Michaelis constants (K_m) of different pepsins were then calculated according to the Lineweaver–Burk double reciprocal plots.

2.10. Preparation of polyclonal antibodies and Western blotting

Fifty micrograms of purified pepsinogens (PG-I and PG- II) in 300 μl solution, which were mixed with the same volume of complete Freund's adjuvant, were subcutaneously injected into rats (100 g body weight). Further injection with the same amount of purified pepsinogens mixed with incomplete Freund's adjuvant was carried out in a two-week period. After three times boostering, the rats were sacrificed and serum was collected. Imunoglobulin G was purified from the serum by Protein A–Sepharose affinity chromatography. Western blotting was carried out as described by Towbin, Staehelin, and Gordon (1979). Briefly, pepsinogens on the 12% acrylamide gels were electrophoretically transferred onto nitrocellulose membranes in transfer solution. Non-specific protein sites were blocked with 5% non-fat milk in Tris–HCl-buffered saline (TBS = 20 mM Tris–HCl, pH 7.5, containing 0.145 M NaCl). The blotted proteins were incubated with 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. Immuno-detection was carried out using DAB as a substrate.

3. Results and discussion

Four pepsinogens were purified from the stomach of sea bream by ammonium sulfate fractionation and column chromatographies of DEAE–Sephacel and Sephacryl S-200 HR. Four peaks (PG-I, PG-II, PG-III, and PG-IV) of hemoglobin-digesting activity were detected in the fractions from DEAE–Sephacel column chromatography (Fig. 1a). These peaks were pooled and further subjected to Sephacryl S-200 gel filtration (Fig. 1b and c, results not shown completely). The result of purification of different PGs is summarized in Table 1. After a series of column chromatographies, 2.1 mg of PG-I, 2.0 mg of PG-II, 2.1 mg of PG-III, and 0.9 mg of PG-IV were purified to high homogeneity with purification folds of and 9.0, 9.9, 9.9, and 9.6, respectively. The homogeneity of purified pepsinogens was checked by SDS–PAGE under reducing conditions. As shown in Fig. 2, all PGs gave single bands on SDS–PAGE with molecular weights of 36, 32, 32, and 34 kDa, respectively. Sea bream pepsinogens were treated, under pH 2.0, 25 °C for 6 min, to convert them into their active form pepsins. The purity of corresponding pepsins was also checked by SDS–PAGE under reducing conditions. As shown in Fig. 2, the molecular weights of the four corresponding pepsins were estimated to be 30, 29, 30, and 30 kDa. The minor difference between the PGs and their active form pepsins may suggest a difference in their signal peptides.

Similar to PGs from various animals (Sogawa et al., 1983; Kageyama et al., 1990; Suziki et al., 1999; Martin et al., 1982), sea bream PGs also undergo limited hydrolysis to convert them into active form pepsins. The activation profile of PG was carried out at 25 °C, pH 2.0 and the active form of pepsins can be identified within 5 s to 10 min on SDS–PAGE (Fig. 3). The intermediate form of

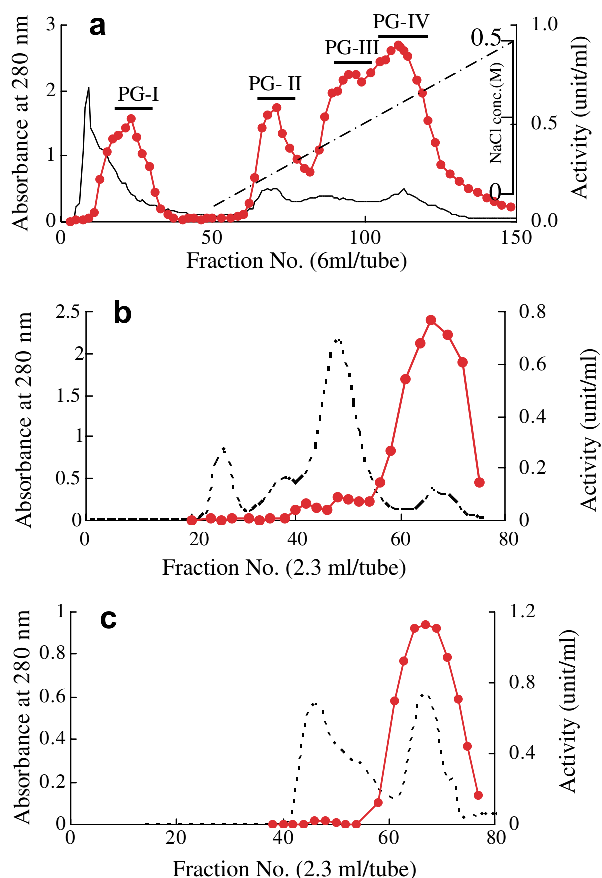

Fig. 1. Column chromatography purification of sea bream pepsinogens. (a) DEAE–Sephacel chromatography. (b) Sephacryl S-200 gel-filtration purification of pepsinogen-I. (c) Sephacryl S-200 gel-filtration of pepsinogen-III. Hemoglobin hydrolyzing activity (●); absorbance at 280 nm (—

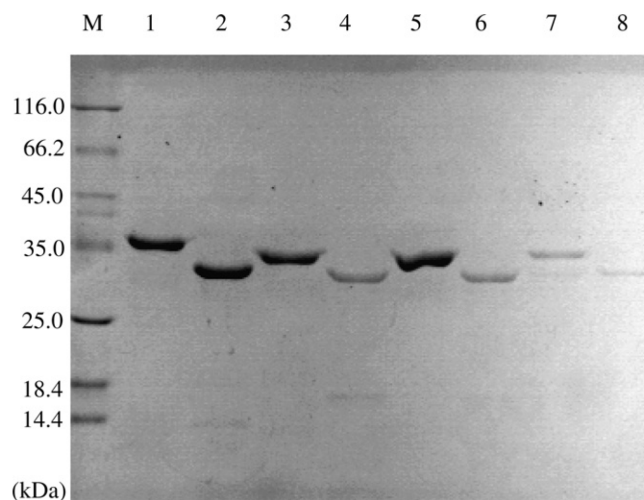

Fig. 2. SDS–PAGE of purified sea bream pepsinogens (PGs) and pepsins. M, molecular marker; lane 1, pepsinogen-I; lane 2, pepsin-I; lane 3, pepsinogen-II; lane 4, pepsin-II; lane 5, pepsinogen-III; lane 6, pepsin-III; lane 7, pepsinogen-IV; lane 8, pepsin-IV. The gel was stained with Coomassie brilliant blue.

PG-I could obviously be identified during acid activation, while those of PG-II, PG-III, and PG-IV could not be observed. Complete activation of PG-I was carried out for 10 min, while those of PG-II and PG-III and PG-IV were performed in a much short time interval.

Native-PAGE assay indicated that pepsins of sea bream have a high activity on acid-denatured bovine hemoglobin (Fig. 4). Optimum pHs of P-I, P-III, and P-IV for bovine hemoglobin were 3.5 while that of P-II was 3.0. Optimum temperatures of P-II, P-III, and P-IV for bovine hemoglobin were 50 °C while that of P-I was 45 °C (Fig. 5). It is clear that all four pepsins were completely inhibited by pepstatin, a typical asparatic proteinase inhibitor (Fig. 6). Moreover, pepstatin binds to P-I, P-III, and P-IV in a 1:1 stoichiometry, which is similar to that of porcine pepsin (Athauda et al., 2004), but it binds to P-II in a 2:1 stoichiometry. On the other hand, inhibitors for serine (PMSF), cysteine (E-64) and metallo proteinases (EDTA) did not reveal any effect (results not shown).

The Lineweaver–Burk plots of the pepsins, using acid-denatured bovine hemoglobin as substrate, are shown in Fig. 7. The apparent K_ms of different pepsins (P-I, P-II, P-III, and P-IV) for hemoglobin were estimated to be 8.7×10^{-8} M, 1.0×10^{-7} M, 8.6×10^{-8} M, and 7.3×10^{-8}M, respectively.

Table 1
Summary of purification of pepsinogens from sea bream stomach

Stage	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	998	475	2.1	1	100
Ammon. sulfate	639	213	3.0	1.4	64.0
DEAE–Sephacel					
PG-I	55.3	9.7	5.7	2.7	5.5
PG-II	46.4	4.3	10.8	5.1	4.6
PG-III	48.7	5.8	8.4	4.0	4.9
PG-IV	36.8	4.0	9.2	4.4	3.7
Sephacryl S-200					
PG-I	39.5	2.1	18.8	9.0	4.0
PG-II	41.6	2.0	20.8	9.9	4.2
PG-III	43.5	2.1	20.7	9.9	4.4
PG-IV	18.2	0.9	20.2	9.6	1.8

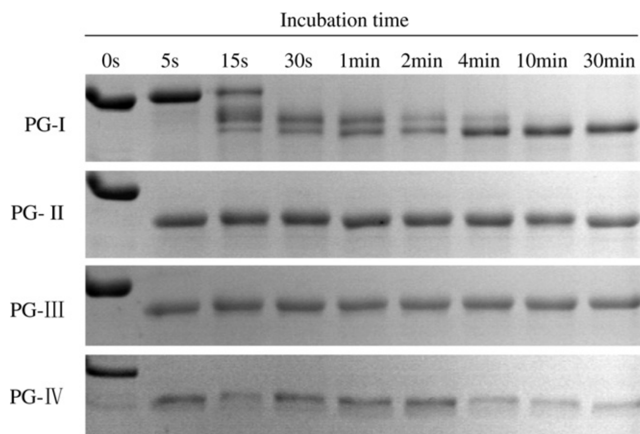

Fig. 3. Conversion of sea bream pepsinogens to pepsins. Pepsinogen solution was incubated at pH 2.0, 25 °C, as described in Section 2. Conversion reactions were stopped at appropriate intervals and subjected to SDS–PAGE. Gels were stained with CBB.

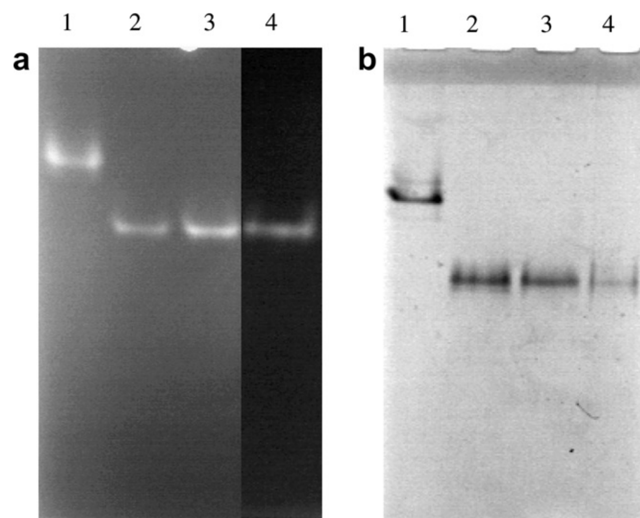

Fig. 4. Zymography (a) and native-PAGE (b) of sea bream pepsins. (a) Pepsins on native-PAGE were incubated with 1.0% hemoglobin at 37 °C for 1 h. (b) Native-PAGE. Lane 1, pepsin-I; lane 2, pepsin-II; lane 3, pepsin-III; lane 4, pepsin-IV. Gels were stained with CBB.

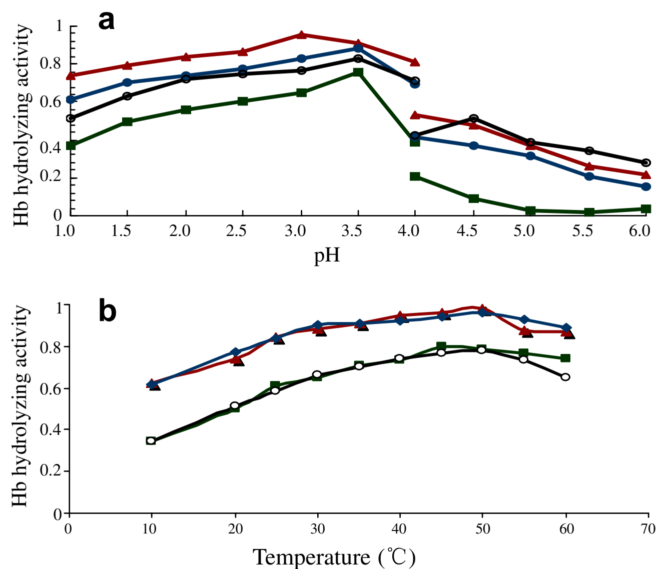

Fig. 5. Optimum pH and temperature of pepsins. (a) Effects of pH. (b) Effects of temperature. P-I (■); P-II (▲); P-III (□); and P-IV (○).

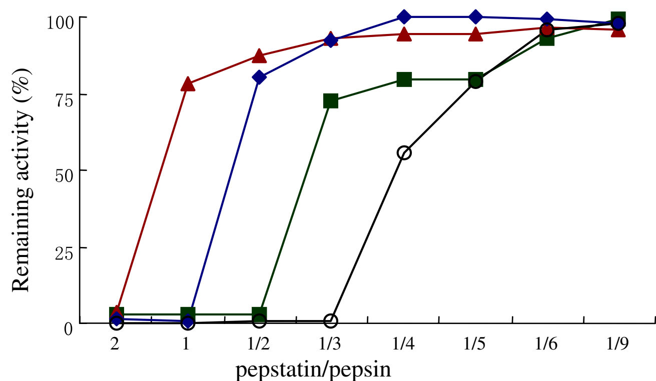

Fig. 6. Pepstatin inhibition of pepsins. P-I (■); P-II (▲); P-III (■); and P-IV (○).

In this study, we tried to purify pepsinogens from sea bream as this kind of fish is popularly cultured in many provinces in China and is economically important. Previous studies on fish pepsins have revealed that, generally, 2–3 kinds of isozyme exist (Kageyama et al., 1990; Tanji et al., 1988; Gildberg, 1988). In the present study, 4 kinds of isozyme were identified and all of them (PG-I, PG-II, PG-III, and PG-IV) were purified to homogeneity and polyclonal antibodies against PG-I and PG-II were prepared. Western blotting of pepsins using anti-PG-I and anti-PG-II polyclonal antibodies revealed that anti-PG-I antibody gave only positive reaction with P-I and a weak reaction with P-II while no cross-reactions with P-III or P-IV were identified. However, anti-PG-II antibody cross-reacted with all the four pepsins (Fig. 8). These results suggested that PG-I share a relatively high primary structure homology with PG-II and low homology with PG-III and PG-IV. However, PG-II may share relatively high homology with the other three PGs.

Though the molecular weights of all four PGs and their active form pepsins are quite similar, some differences in enzymatic characteristics of PG-I, PG-II, PG-III, and PG-IV were identified, such as their binding properties to an ion-exchanger column, DEAE–Sephacel, zymograms of their active forms and optimum pHs and temperatures. Thus, it is quite possible that these four PGs are physiologically not the same. The presence of multiple pepsinogens in sea bream may be advantageous in the efficient digestion of different foods. 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 (Kageyama, 2002).

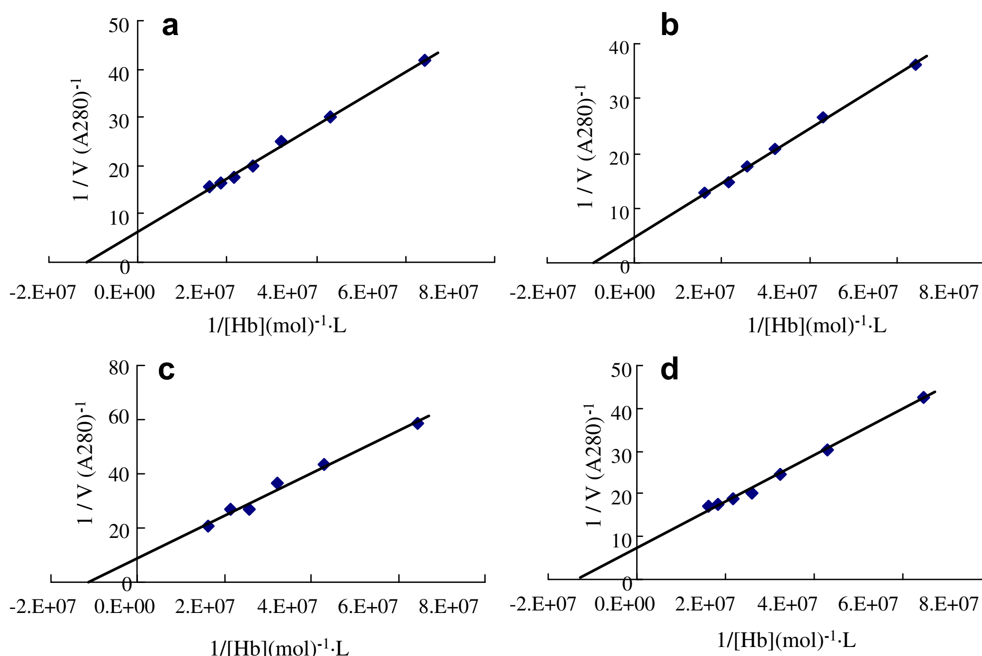

Fig. 7. Lineweaver–Burk double reciprocal plot of pepsins. (a) P-I; (b) P-II; (c) P-III; and (d) P-IV.

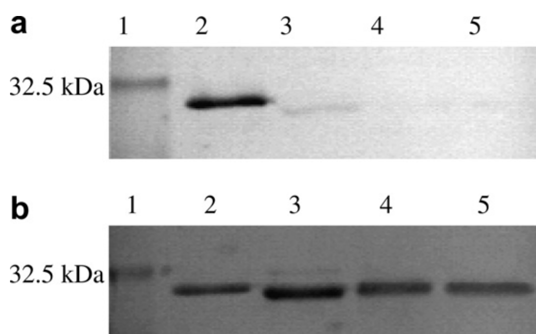

Fig. 8. Immunoblotting of pepsins developed with anti-sea bream pepsinogen polyclonal antibodies. (a) Reacted with anti-PG-I antibody. (b) Reacted with anti-PG-II antibody. Lane 1, prestained protein marker; lane 2, P-I; lane 3, P-II; lane 4, P-III; lane 5, P-IV.

In the present study, PG-I to P-I was carried out in a stepwise pathway while other PGs were converted to active forms in one-step pathway. Previously, it has been reported that tuna pepsins had relatively high activity (Norris & Mathies, 1953). Similar results were observed in the present study. Western blotting has simply proved the relationship of these four pepsinogens in the evolvement. However, further characterization of these pepsinogens, including the determination of their full-length primary structures and which kind of pepsinogen they are, is necessary.

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