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Identification of pepsinogens and pepsins from the stomach of European eel (*Anguilla anguilla*)

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ABSTRACT

Three pepsinogens (PG-I, PG-II and PG-III) were purified to homogeneity from the stomach of freshwater fish European eel (*Anguilla anguilla*) by ammonium sulphate fractionation, column chromatography on anion exchange and gel-filtration. Their molecular masses were determined as 36, 37 and 36 kDa, respectively, by SDS–PAGE, which were in agreement with the results obtained by Sephacryl S-200 and Superdex 75 gel-filtration. PG-I, PG-II and PG-III converted into corresponding pepsins with molecular masses of approximately 30 kDa at pH 2.0 and showed maximal activity at pH 3.5, 2.5 and 2.5. Optimal temperatures of these pepsins were 40, 40 and 35 °C, using bovine haemoglobin as substrate. Western blot analysis revealed that anti-sea bream PG-I and PG-II polyclonal antibodies cross-reacted with all three PGs of European eel. On the other hand, anti-sea bream PG-III and PG-IV antibodies cross-reacted with PG-II and PG-III of European eel, while no cross-reaction with PG-I was detected. The kinetic constants of K_m and k_{cat} of pepsins (P-I, P-II and P-III) for haemoglobin were calculated as 8.8×10^{-5} M, 23.7 s^{-1}; 9.2×10^{-5} M, 19.4 s^{-1} and 7.0×10^{-5} M, 34.4 s^{-1}, respectively.

1. Introduction

As aspartic proteinases, pepsins (Ps) are normally secreted in the gastric mucosa of animals from precursor pepsinogens (PGs) and perform important digestive functions in both invertebrates and vertebrates (Kageyama, 2002). Pepsinogens are synthesised in gastric mucosa and autocatalytically converted into their corresponding active form pepsins in the acidic environment of gastric juice, by releasing the NH_2-terminal activation segments. So far, five groups of pepsinogens have been classified, namely pepsinogens A, B and F, progastricsin, and prochymosin, which mainly exists in the gastric juice of foetal and newborn mammals (Kageyama, 2002; Tanji et al., 2007). These five groups of PGs are different from each other not only in their primary structures but also in their enzymatic properties (Kageyama, 2002; Kageyama, Tanabe, & Koiwai, 1990; Tanji et al., 2007).

Pepsins have in common the property to cleave protein substrates, such as denatured haemoglobin, serum albumin and suitable oligopeptides, under acidic pH conditions. As well-known enzymes, pepsinogens and pepsins have been extensively investigated in mammalian animals, including human (Sogawa, Fujii-Kuriyama, Mizukami, Ichihara, & Takahashi, 1983), Japanese monkey (Kageyama & Takahashi, 1976), pig (Nielsen & Foltmann, 1995), bovine (Martin, Torieu-Cuot, Collin, & Ribadeau, 1982), goat (Suzuki et al., 1999), rat (Muto & Tani, 1979) and rabbit (Kageyama et al., 1990).

Also pepsinogens and pepsins from fish have been purified and characterised from different species, such as rainbow trout (*Oncorhynchus mykiss*) (Twining, Alexander, Huibregtse, & Glick, 1983), North Pacific bluefin tuna (*Thunnus thynuus orientalis*) (Tanji, Kageyama, & Takahashi, 1988; Tanji, Yakabe, Kageyama, & Takahashi, 1996), Atlantic cod (*Gadus morhua*) (Gildberg, Olsen, & Bjarnason, 1990) and shark (*Centroscymnus coelolepsis*) (Nguyen et al., 1998). More recently, pepsinogens and pepsins from Antarctic rock cod (*Trematomus bernacchii*) (Brier et al., 2007), Africa coelacanth (*Latimeria chalumnae*) (Tanji et al., 2007), pectoral rat-tail (*Corphaenoides pectoralis*) (Klomklao, Kishimura, Yabe, & Benjakul, 2007) and smooth hound (*Mustelus mustelus*) (Bougatef, Balti, Zaied, Souissi, & Nasri, 2008) have been documented. In our previous study, four pepsinogens from the marine fish sea bream (*Sparus latus Houttuyn*) were purified to high homogeneity and polyclonal antibodies against these pepsinogens were prepared (Zhou, Fu, Zhang, Su, & Cao, 2007). So far, however, there is extremely limited information on characteristics of pepsinogens and pepsins from freshwater fish, except for our recent study on mandarin fish (*Siniperca chuatsi*) (Zhou et al., 2008).

European eel is a popularly cultured freshwater fish in many provinces of China, because of its good taste and economic benefits. More than 90,000 tons of eel were processed in Fujian province in the year 2006. As one of the most essential digestive enzymes, pepsins play important roles in the digestion of proteins in fish

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feeds. Thus, a detailed study of such proteinases will be of benefit, not only for the manufacture of fish feeds and the development of aquaculture but also for the effective utilisation of byproducts during eel processing. In the present study, we reported the purification of pepsinogens from the stomach of European eel and investigated their characteristics.

2. Materials and methods

2.1. Materials

2.1.1. Fish

Cultured European eels (*Anguilla anguilla*) with body weight of about 400 g were obtained between winter 2007 and spring 2008 from the local fish market of Jimei, Xiamen. Eels were killed and stomach was removed and washed with cold distilled water thoroughly to prevent activation of pepsinogens by acidic gastric juice and immediately used for experiments.

2.1.2. Chemicals

DEAE-Sephacel, Sephacryl S-200 HR and Superdex 75 10/300 GL were from Amersham Biosciences (Uppsala, Sweden), bovine haemoglobin, bovine serum albumin and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO). L-3-carboxy-*trans*-2,3-epoxypropionyl-L-leucin-4-guanidinobutylamide (E-64) was the product of Amresco (Solon, OH). Pepstatin A was from Roche (Mannheim, Germany), protein marker for SDS–PAGE was from Fermentas UAB (Vilnius, Lithuania), prestained protein marker for Western blot was obtained from New England Biolabs (Beverly, MA). Rat anti-sea bream pepsinogen-I, -II, -III and -IV polyclonal antibodies were prepared in our own laboratory as described (Zhou et al., 2007). Horseradish peroxidase (HRP) labelled rabbit anti-rat immunoglobulin G (IgG-HRP) was from DAKO (Glostrup, Denmark). DAB (3,3′-diaminobenzidine tetrahydrochloride) was from Pierce (Rockford, IL). All other reagents were of analytical grade.

2.2. Purification of pepsinogens

All procedures were conducted at 0–4 °C, except Superdex 75 gel-filtration chromatography, which was performed at room temperature. European eel stomach (22 g) was minced and homogenised with 6-fold of 50 mM phosphate buffer (pH 7.0) containing 5 mM PMSF and 1 mM E-64. The homogenate was centrifuged at 18,000*g* for 90 min. The resulting supernatant, which was regarded as crude extract, was collected and fractionated with ammonium sulphate from 20% to 60% saturation. After centrifugation at 10,000*g* for 30 min, the resultant precipitate was suspended in a small amount of 25 mM phosphate buffer (pH 7.0) and dialysed against the same buffer extensively. The dialysate was subsequently applied to a DEAE-Sephacel column (2.5 × 15 cm), which was previously equilibrated with the dialysis buffer. Proteins in the flow-through fractions were collected by washing the column with 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 500 ml at a flow rate of 1 ml/min. Fractions showing pepsin activity were pooled and concentrated by ultrafiltration using a membrane of YM-10 (Millipore, Billerica, MA). Concentrated samples were respectively applied to a gel-filtration column of Sephacryl S-200 HR (1.5 × 98 cm), which was equilibrated with 25 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl and eluted at a flow rate of 0.6 ml/min. Active fractions from Sephacryl S-200 were collected and concentrated by ultrafiltration and further subjected to Superdex 75 10/300 GL gel-filtration column equilibrated with 25 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl at a flow rate of 0.4 ml/min. Active fractions were obtained and used for electrophoresis analysis and enzymatic characterisation.

2.3. Protein concentration determination

Protein concentration in each chromatographic purification step was determined by measuring the absorbance at 280 nm of the sample solution. The amount of each purified pepsinogen and pepsin was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as standard.

2.4. Enzyme activity assay

Pepsin activity against acid-denatured bovine haemoglobin was determined at pH 3.0 and 37 °C, according to the method of Anson (1938), with slight modification. Briefly, 50 μl of appropriately diluted enzyme sample was mixed with 350 μl of 0.25 M HCl–sodium acetate buffer (pH 3.0), 100 μl of 2.0% acid-denatured bovine haemoglobin was then added to the mixture to initiate the reaction. After incubation at 37 °C for 30 min, the reaction was terminated by addition of 500 μl of 8.0% trichloroacetic acid (TCA) and then centrifuged at 8000*g* for 10 min. The absorbance of the supernatant was measured at 280 nm using a Cary 50 UV spectrophotometer (Varian Inc., Palo Alto, CA). One unit of enzymatic activity was defined as the amount of pepsin that catalyses an increase of absorbance of 1.0 at 280 nm under the activity assay conditions.

2.5. SDS–PAGE, zymography and Western blot

Aliquots of purified pepsinogens were subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (1970) using 12.0% gel. Proteins were stained with Coomassie brilliant blue R-250 (CBB). Zymography was performed similar to SDS–PAGE without addition of SDS both to samples and gel. After electrophoresis, proteolytic activity was visualised by activity staining by incubation with 1% acid-denatured bovine haemoglobin (pH 2.5) at 37 °C for 1 h followed by CBB staining. Western blot was operated as described by Towbin, Staehelin, and Gordon (1979). Briefly, pepsinogens on SDS–PAGE 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). Blotted proteins were incubated with corresponding polyclonal antibodies at room temperature for 2 h, followed by washing with TBST (TBS, 0.05% Tween-20). After incubation for 1 h with horseradish peroxidase conjugated secondary antibody (IgG-HRP), the nitrocellulose membrane was rinsed extensively with TBST. Immunodetection was carried out using a substrate of DAB from Pierce.

2.6. Characterisation of the conversion from pepsinogen to pepsin

To convert pepsinogens to pepsins, purified PGs in solution were subjected to pH 2.0, respectively, to transform PGs into their activate form, pepsins. Conversion reaction was performed using purified PGs in different test tubes individually in a volume of 100 μl at pH 2.0 and 25 °C for corresponding reaction intervals as indicated. After reaction, 8 μl of 16% TCA and 2 μl of pepstatin A were immediately added to stop the reaction. Samples were then submitted to SDS–PAGE, followed by staining with CBB.

2.7. Effects of pH and temperature on the activity

The effect of pH on enzymatic activity was studied over the pH range of 1–6 at 37 °C with acid-denatured bovine haemoglobin as

substrate. In order to investigate the effect of pH on the activity of pepsin, pepsins were prepared by activation of pepsinogens at pH 2.0 and 37 °C for 15 min as described (Zhou et al., 2007). 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). The effect of temperature on the activity of pepsins was examined at different temperatures (10–70 °C) for 15 min.

2.8. Effect of inhibitors on proteinase activity

Different kinds of proteinase inhibitors were mixed with pepsin solutions (0.05 μM), to obtain the designated final concentrations (PMSF, 5 mM; E-64, 0.28 mM; EDTA, 5 mM). The inhibitory effect of pepstatin A on these pepsins was investigated in detail with different molar ratios. The inhibitor and pepsin mixture was preincubated at room temperature for 15 min, respectively, before incubating at 37 °C for 30 min for the activity assay, as described above.

2.9. Kinetic study of pepsins

Enzymatic activity was assayed with appropriate concentrations of pepsin and different concentrations of bovine haemoglobin at 37 °C for 6 min. The Michaelis constant, K_m, of different pepsins was then evaluated by plotting the data on a Lineweaver–Burk double-reciprocal graph (Lineweaver & Burk, 1934). The turnover number (k_{cat}) values were calculated based on the equation:

$$k_{cat} = V_{max}/[E],$$

where [E] is the pepsin concentration and V_{max} is the maximal velocity.

3. Results and discussion

In the present study, three pepsinogens were purified from the stomach of European eel by ammonium sulphate fractionation and column chromatography by DEAE-Sephacel, Sephacryl S-200 and Superdex 75. Upon DEAE-Sephacel ion-exchange chromatography, pepsinogens in European eel were separated into three peaks (PG-I, PG-II and PG-III), with PG-I in the unadsorbed fraction, and PG-II and PG-III in the adsorbed fraction (Fig. 1A). These three peaks were concentrated and subjected to Sephacryl S-200 gel-filtration (Fig. 1B). In order to obtain samples with homogeneity, pepsinogens from Sephacryl S-200 column were concentrated and further subjected to Superdex 75 high resolution gel-filtration column (Fig. 1C). Table 1 summarises the purification of these three PGs. As a result, 0.5 mg of PG-I, 0.2 mg of PG-II and 0.1 mg of PG-III were obtained, with purification folds of 28.3, 36.3 and 64.2, respectively. The homogeneity of purified pepsinogens was checked by SDS–PAGE. As shown in Fig. 2A, PG-I, PG-II, PG-III and their corresponding active form pepsins all gave single bands on SDS–PAGE under reducing conditions with molecular masses of 36 and 30; 37 and 30 and 36 and 30 kDa, respectively. The molecular masses of pepsinogens and pepsins of European eel were similar to those of marine fish African coelacanth (Tanji et al., 2007), sea bream (Zhou et al., 2007), pectoral rat-tail (Klomklao et al., 2007), and smooth hound (Bougatef et al., 2008) while slightly smaller than those from North Pacific bluefin tuna (*Thunnus thynuus orientalis*) (Tanji et al., 1996).

The pepsins of European eel revealed different mobility and enzymatic activity on zymographic analysis. The enzymatic activity of P-III was much higher than that of P-II and P-I (Fig. 2B).

Generally, conversion of pepsinogens into active form pepsins proceeds autocatalytically by the cleavage of a 3–5 kDa signal peptide at the N-terminal under acidic conditions by two different

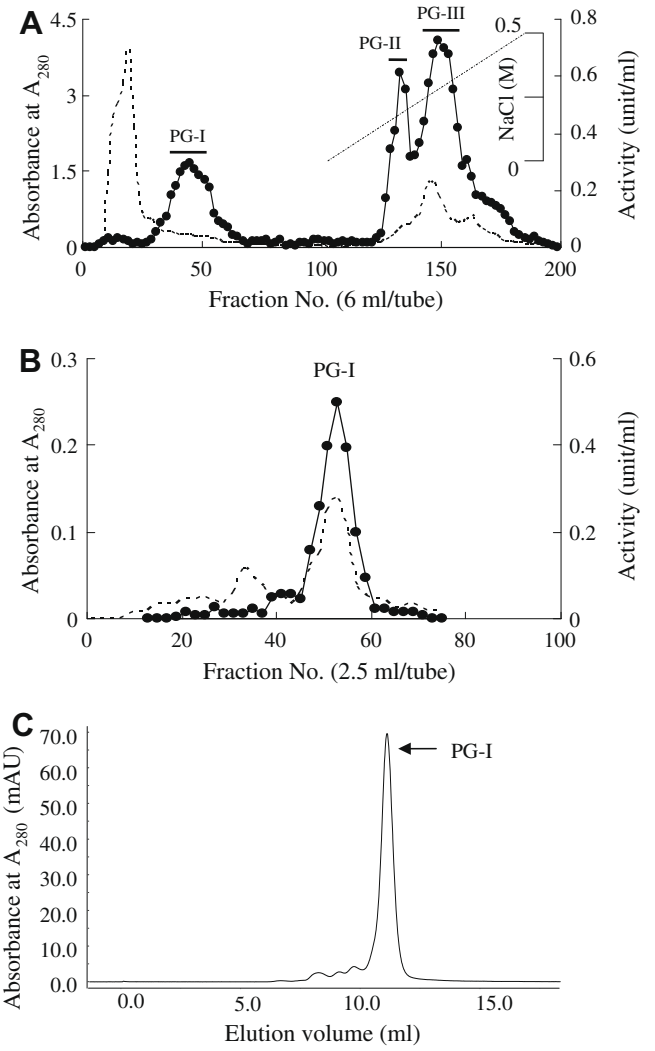

Fig. 1. Column chromatography purification of European eel pepsinogens. (A) DEAE-Sephacel chromatography; (B) Sephacryl S-200 gel-filtration purification of pepsinogen-I and (C) Superdex 75 gel-filtration of pepsinogen-I. Haemoglobin hydrolysing activity (●); absorbance at 280 nm (---).

pathways. The first is a one-step pathway to release the intact activation segment directly; the second is a stepwise pathway through a pseudopepsin (intermediate form) (Dykes & Kay, 1976; Kageyama, 2002). Time-course study of the activation of pepsinogens showed that active form pepsin of PG-I can be identified in as short a time as 5 s, while pepsins of PG-II and PG-III were detected after 1 min (Fig. 2C). Similar to pepsinogens from Japanese monkey (Kageyama & Takahashi, 1976), bovine (Martin et al., 1982), human (Sogawa et al., 1983), goat (Suzuki et al., 1999), turtle (Hirasawa, Athauda, & Takahashi, 1996) and bullfrog (Yakabe et al., 1991), European eel pepsinogens also undergo limited hydrolysis to convert into pepsins. The intermediate forms (I) of PG-I and PG-III could be detected during acid activation, while that of PG-II could not be recognised (Fig. 2C). Two major pepsinogens from African coelacanth (Tanji et al., 2007), a major pepsinogen from bluefin tuna (Tanji et al., 2007), PG-I from sea bream (Zhou et al., 2007) and PG-I, PG-II from mandarin fish (Zhou et al., 2008) also followed such a two-step conversion mechanism. Complete activation of PG-III occurred after 15 min while activation of PG-I, PG-II occurred in much shorter time intervals (Fig. 2C).

Optimal pH value of P-I was 3.5, while those of P-II and P-III were 2.5 and 2.5, respectively (Fig. 3A). These three pepsins lost

Table 1
Summary of purification of pepsinogens from European eel stomach.

Stage	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purify (-fold)	Yield (%)
Crude extract	1275.1	562.1	2.3	1	100
Ammonium sulphate	951.6	366.6	2.6	1.1	74.6
DEAE-Sephacel					
PG-I	108.1	6.4	16.8	7.4	8.5
PG-II	221.1	9.8	22.5	9.9	17.3
PG-III	195.6	7.0	27.8	12.2	15.3
Sephacryl S-200					
PG-I	60.7	2.0	30.2	13.3	4.8
PG-II	67.3	1.6	41.4	18.2	5.3
PG-III	67.9	1.4	49.6	21.9	5.3
Superdex G-75					
PG-I	31.0	0.5	64.3	28.3	2.4
PG-II	15.7	0.2	82.4	36.3	1.2
PG-III	17.1	0.1	145.7	64.2	1.3

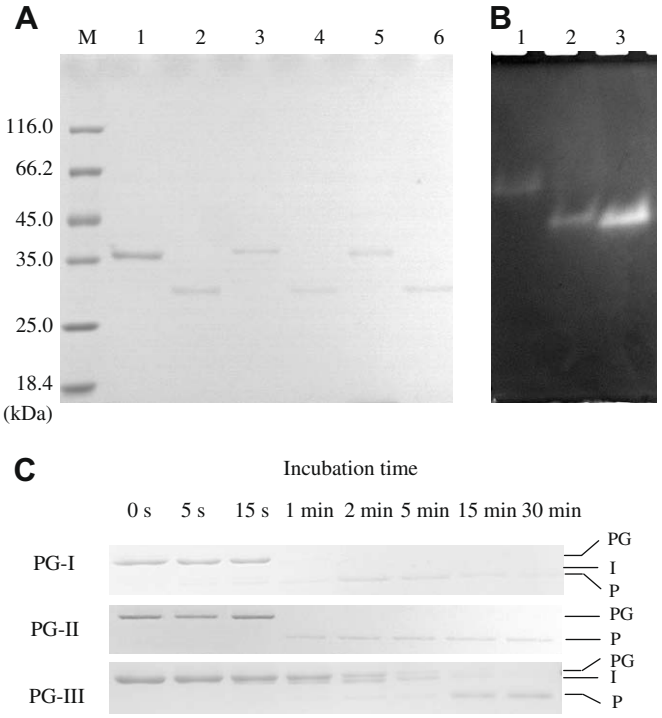

Fig. 2. (A) SDS–PAGE of purified pepsinogens (PGs) and pepsins. M, protein marker; 1, pepsinogen-I; 2, pepsin-I; 3, pepsinogen-II; 4, pepsin-II; 5, pepsinogen-III; and 6, pepsin-III. (B) Zymogram of pepsins. Pepsins on native-PAGE were incubated at 37 °C with 1.0% haemoglobin as substrate for 1 h. Lane 1, pepsin-I; 2, pepsin-II; and 3, pepsin-III. Approximately 20 μg of pepsin were applied on each lane. (C) Conversion of pepsinogens to pepsins. PGs were incubated at pH 2.0, 37 °C. Reaction was stopped immediately at appropriate time intervals as indicated on each lane and subjected to SDS–PAGE. I, intermediate form; and P, pepsin. All the gels were stained with Coomassie brilliant blue (CBB).

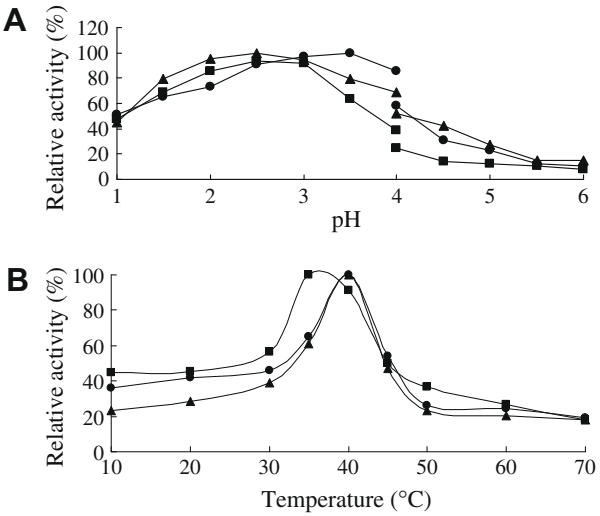

Fig. 3. Optimal pH and temperature of pepsins. (A) Effects of pH on the activities for haemoglobin hydrolysis by purified pepsins. (B) Effects of temperature on the activities for haemoglobin hydrolysis by purified pepsins. P-I (●), P-II (▲) and P-III (■).

nearly all activity above pH 5.5. Similar optimal pH of P-I profiles were also reported in pepsins from pectoral rat-tail (Klomklao et al., 2007) and sea bream (Zhou et al., 2007), while most pepsins, such as those from Antarctic rock cod (Brier et al., 2007), African coelacanth (Tanji et al., 2007), bluefin tuna (Tanji et al., 2007) and bullfrog (Yakabe et al., 1991) are similar to P-II and P-III. Different from porcine pepsin, which exhibited optimal temperature at 60 °C (Nielsen & Foltmann, 1995), optimal temperatures of European eel P-I and P-II were 40 °C while that of P-III was 35 °C. Similar to aspartic proteinase nepenthesin II (Athauda et al., 2004), the activity of these three pepsins had largely disappeared at 70 °C, suggesting their susceptibility to higher temperature (Fig. 3B).

The effect of various inhibitors on the activity of pepsins was also investigated. Pepstatin A, a typical aspartic proteinase inhibitor almost completely inhibited the activity of these enzymes. Pepsin-II was most sensitive to pepstatin A with a pepstatin:pepsin molar ratio of 2:1, whereas pepsins-I and -III were not so sensitive to pepstatin A, a 8-fold and 4-fold molar excess of pepstatin A was necessary for the complete inhibition of these two enzymes (data not shown). On the other hand, specific inhibitors of cysteine proteinases (E-64), serine proteinases (PMSF) and metalloproteinases (EDTA) had no inhibitory effect on the activity of the three pepsins (data not shown). These results strongly suggested that the three enzymes purified in the present study were aspartic proteinases.

Kinetic constants, K_m and k_{cat} for hydrolysis of acid-denatured bovine haemoglobin of these three pepsins were determined, based on a Lineweaver–Burk plot (Table 2). The K_m of each pepsin (P-I, P-II and P-III) was estimated to be 8.8×10^{-5} M, 9.2×10^{-5} M and 7.0×10^{-5} M, and the k_{cat} was 23.7 s^{-1}, 19.4 s^{-1} and 34.4 s^{-1}, respectively. The K_m of P-III was lower than that of P-II and P-I, indicating that P-III has a higher affinity to substrate haemoglobin.

Table 2
Kinetic properties of European eel pepsins for the hydrolysis of haemoglobin.

Enzyme	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
Pepsin I			
European eel	8.8×10^{-5}	23.7	2.7×10^5
Sea bream[a]	8.7×10^{-5}	ND	ND
Atlantic cod[b]	1.56×10^{-4}	18	1.2×10^5
Pepsin II			
European eel	9.2×10^{-5}	19.4	2.1×10^5
Sea bream[a]	1.0×10^{-4}	ND	ND
Pepsin IIa			
Atlantic cod[b]	4.4×10^{-5}	33	2.8×10^5
Pepsin A			
Pectoral rat-tail[c]	9.8×10^{-5}	50	5.1×10^5
Pepsin III			
European eel	7.0×10^{-5}	34.4	4.9×10^5
Sea bream[a]	8.6×10^{-5}	ND	ND
Pepsin B			
Pectoral rat-tail[c]	1.52×10^{-4}	32	2.1×10^5

The kinetic parameters of European eel were compared with these of sea bream ([a]Zhou et al., 2007), Atlantic cod ([b]Gildberg et al., 1990) and pectoral rat-tail ([c]Klomklao et al., 2007). Reactions were carried out at 37 °C, pH 3.0–3.5 using bovine haemoglobin as substrate.
ND, not determined.

The kinetic constant K_m values of these three European eel pepsins were similar to these from sea bream (Zhou et al., 2007), Atlantic cod (Gildberg et al., 1990) and pectoral rat-tail (Klomklao et al., 2007). In addition, the turnover number (k_{cat}) value of P-III was higher than that of P-II and P-I, suggesting this isoenzyme may play a more important role in digestion. The k_{cat} values of these three pepsins were close to that from Atlantic cod (Gildberg et al., 1990) and pectoral rat-tail (Klomklao et al., 2007). The catalytic efficiencies (k_{cat}/K_m) of these three pepsins were $2.7 \times 10^5\,M^{-1}\,s^{-1}$, $2.1 \times 10^5\,M^{-1}\,s^{-1}$ and $4.9 \times 10^5\,M^{-1}\,s^{-1}$ (Table 2), further indicating that P-III works more efficiently than the other two pepsins.

All three pepsinogens positively reacted with anti-sea bream PG-I and PG-II polyclonal antibodies, as detected by Western blot (Fig. 4). Anti-sea bream PG-III and PG-IV antibodies positively reacted with PG-II and PG-III, while no cross-reaction toward PG-I could be detected. These results indicated that PG-I shares low primary structure homology with PG-II and PG-III, while PG-II and PG-III are closely related in primary structures with each other.

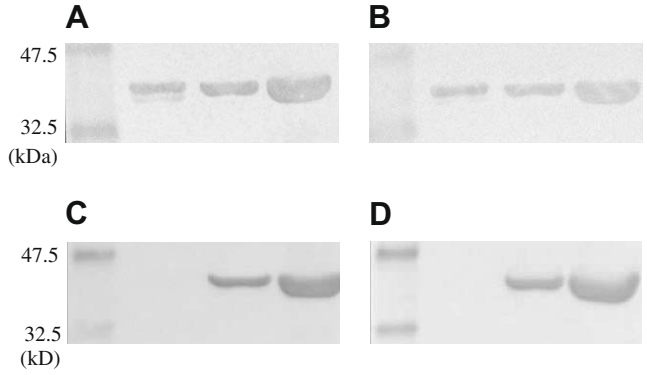

Fig. 4. Western blot of pepsinogens using anti-sea bream pepsinogen polyclonal antibodies. (A) Reacted with anti PG-I antibody; (B) reacted with anti-PG-II antibody; (C) reacted with anti-PG-III antibody; and (D) reacted with anti-PG-IV antibody. Lane 1, prestained protein marker; lane 2, PG-I; lane 3, PG-II; and lane 4, PG-III.

Though pepsinogens in non-mammalian vertebrate fish appeared much earlier in evolution than those in mammalians, positive immunological reactions between PGs from marine fish sea bream and freshwater fish European eel strongly suggested that PGs are relatively conserved proteins.

In the present study, we tried to purify pepsinogens from freshwater fish European eel, as it is one of the most popularly cultured fish in many provinces of China. Generally, more than two kinds of pepsin isozymes have been reported in different fish species (Gildberg et al., 1990; Kageyama et al., 1990; Klomklao et al., 2007; Tanji et al., 1988; Zhou et al., 2007, 2008) and in this study, three pepsinogens from freshwater fish European eel were identified. The molecular masses of all the three PGs and their corresponding pepsins are quite similar. However, differences in enzymatic characteristics were identified; for example, their binding properties to ion-exchange column DEAE-Sephacel, and zymograms of their active forms, especially their immunological reaction to polyclonal antibodies against sea bream PGs. It is still unknown why different isoforms of pepsinogens and pepsins exist in the stomach of different species of animals. Quite possibly, multiple pepsinogens in European eel might be advantageous in digestion of different foods. On the other hand, digestion with multiple pepsins may also increase the utilisation efficiency of amino acids from foods, which is beneficial for the development of muscle. If the existence of different pepsinogens is the product of different genes or the result of post-translational modification such as phosphorylation, glycosylation, which generate a variety of pepsinogen species with more or less different catalytic properties (Kageyama, 2002), needs to be further examined by revealing their primary structures.

Acknowledgments

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References

Anson, M. L. (1938). The estimation of pepsin, trypsin, papain, and cathepsin with haemoglobin. *The Journal of General Physiology, 22*, 79–89.
Athauda, S. B. P., Matsumoto, K., Rajapakshe, S., Kuribayashi, M., Kojima, M., Kubomura-Yoshida, N., et al. (2004). Enzymic and structural characterization of nepenthesin, a unique member of a novel subfamily of aspartic proteinases. *Biochemical Journal, 381*, 295–306.
Bougatef, A., Balti, R., Zaied, S. B., Souissi, N., & Nasri, M. (2008). Pepsinogen and pepsin from the stomach of smooth hound (*Mustelus mustelus*): Purification, characterization and amino acid terminal sequences. *Food Chemistry, 107*, 777–784.
Brier, S., Maria, G., Carginale, V., Capasso, A., Wu, Y., Taylor, R. M., et al. (2007). Purification and characterization of pepsins A1 and A2 from the Antarctic rock cod (*Trematomus bernacchii*). *FEBS Journal, 274*, 6152–6166.
Dykes, C. W., & Kay, J. (1976). Conversion of pepsinogen into pepsin is not a one-step process. *Biochemical Journal, 153*, 141–144.
Gildberg, A., Olsen, R. L., & Bjarnason, J. B. (1990). Catalytic properties and chemical composition of pepsins from Atlantic cod (*Gadus morhua*). *Comparative Biochemistry and Physiology, 96B*, 323–330.
Hirasawa, A., Athauda, S. B. P., & Takahashi, K. (1996). Purification and characterization of turtle pepsinogen and pepsin. *Journal of Biochemistry (Tokyo), 120*, 407–414.
Kageyama, T. (2002). Pepsinogens, progastricsins, and prochymosins: Structure, function, evolution, and development. *Cellular and Molecular Life Science, 59*, 288–306.
Kageyama, K., & Takahashi, T. (1976). Pepsinogens and pepsins from gastric mucosa of Japanese monkey. Purification and characterization. *Journal of Biochemistry (Tokyo), 79*, 455–468.
Kageyama, T., Tanabe, K., & Koiwai, O. (1990). Structure and development of rabbit pepsinogens, stage-specific zymogens, nucleotide sequences of cDNAs, molecular evolution, and gene expression during development. *Journal of Biological Chemistry, 265*, 17031–17038.
Klomklao, S., Kishimura, H., Yabe, M., & Benjakul, S. (2007). Purification and characterization of two pepsins from the stomach of pectoral rattail

(*Coryphaenoides pectoralis*). *Comparative Biochemistry and Physiology, 147B*, 682–689.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature, 277*, 680–685.

Lineweaver, H., & Burk, D. (1934). The determination of enzyme dissociation constants. *Journal of the American Chemical Society, 56*, 665–666.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Martin, P., Torieu-Cuot, P., Collin, J. C., & Ribadeau, D. B. (1982). Purification and characterization of bovine gastricsin. *European Journal of Biochemistry, 122*, 31–39.

Muto, N., & Tani, S. (1979). Purification and characterization of rat pepsinogens whose contents increase with developmental progress. *Journal of Biochemistry (Tokyo), 85*, 1143–1149.

Nguyen, A., Nungaray, J., Martel, A., Le Goffic, F., Molle, D., & Leonil, J. (1998). Purification and characterization of the main pepsinogen from the shark, *Centroscymunus coelolepis*. *Journal of Biochemistry (Tokyo), 124*, 287–293.

Nielsen, P. K., & Foltmann, B. (1995). Purification and characterization of porcine pepsinogen B and pepsin B. *Archives of Biochemistry Biophysics, 322*, 417–422.

Sogawa, K., Fujii-Kuriyama, Y., Mizukami, Y., Ichihara, Y., & Takahashi, K. (1983). Primary structure of human pepsinogen gene. *Journal of Biological Chemistry, 258*, 5306–5311.

Suzuki, M., Narita, Y., Oda, S., Moriyama, A., Takenaka, O., & Kageyama, T. (1999). Purification and characterization of goat pepsinogens and pepsins. *Comparative Biochemistry and Physiology, 122B*, 453–460.

Tanji, M., Kageyama, T., & Takahashi, K. (1988). Tuna pepsinogens and pepsins. Purification, characterization, and amino-terminal sequence. *European Journal of Biochemistry, 177*, 251–259.

Tanji, M., Yakabe, E., Kageyama, T., & Takahashi, K. (1996). The primary structure of the major pepsinogen from the gastric mucosa of tuna stomach. *Journal of Biochemistry (Tokyo), 120*, 647–656.

Tanji, M., Yakabe, E., Kageyama, T., Yokobori, S., Ichinose, M., Miki, K., et al. (2007). Purification and characterization of pepsinogens from the gastric mucosa of African coelacanth, *Latimeria chalumnaem*, and properties of the major pepsins. *Comparative Biochemistry and Physiology, 146B*, 412–420.

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America, 76*, 4350–4354.

Twining, S. S., Alexander, P. A., Huibregtse, K., & Glick, D. M. (1983). A pepsinogen from rainbow trout. *Comparative Biochemistry and Physiology, 75B*, 109–112.

Yakabe, E., Tanji, M., Ichinose, M., Goto, S., Miki, K., Kurokawa, K.,, et al. & Takahashi, K. (1991). Purification, characterization, and amino acid sequences of pepsinogens and pepsins from the esophageal mucosa of bullfrog (*Rana catesbeiana*). *Journal of Biological Chemistry, 266*, 22436–22443.

Zhou, Q., Fu, X. P., Zhang, L. J., Su, W. J., & Cao, M. J. (2007). Purification and characterization of sea bream (*Sparus latus Houttuyn*) pepsinogens and pepsins. *Food Chemistry, 103*, 795–801.

Zhou, Q., Liu, G. M., Huang, Y. Y., Weng, L., Hara, K., Su, W. J., et al. (2008). Pepsinogens and pepsins from mandarin fish (*Siniperca chuatsi*). *Journal of Agricultural and Food Chemistry, 56*, 5401–5406.