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Purification and characterization of chymotrypsins from the hepatopancreas of crucian carp (*Carassius auratus*)

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ABSTRACT

Two chymotrypsins (chymotrypsin A and B) have been purified to homogeneity from the hepatopancreas of crucian carp (*Carassius auratus*) by ammonium sulphate fractionation and chromatographies on DEAE-Sepharose, Sephacryl S-200 HR, Phenyl-Sepharose and SP-Sepharose. The molecular masses of chymotrypsin A and B were approximately 28 and 27 kDa, respectively, by SDS–PAGE. Purified chymotrypsins also revealed single bands by native-PAGE. Optimum temperatures of chymotrypsin A and B were 40 and 50 °C, and optimal pHs were 7.5 and 8.0 using Suc-Leu-Leu-Val-Tyr-AMC as substrate. Both enzymes were effectively inhibited by serine proteinase inhibitors and slightly activated by metal ions such as Ca^{2+} and Mg^{2+}, while inactivated by Mn^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+} to different degrees. Apparent K_ms of chymotrypsin A and B were 1.4 and 0.5 μM, and K_{cat}s were 2.7 S^{-1} and 3.4 S^{-1}, respectively. Immunoblotting analysis using anti-chymotrypsin B weakly cross reacted with chymotrypsin A.

1. Introduction

Recently, strict environmental regulations have forced many aquatic product processors to find alternative ways to use secondary raw materials (Kristinsson & Rasco, 2000a). Large amounts of solid and liquid wastes are generated during fish processing. Fish viscera, accounting for 5% of the total fishery by-products, are rich potential sources of various enzymes that may have some unique properties of interest for both basic research and industrial applications (Simpson & Haard, 1987). However, fish viscera are usually discarded directly, causing environmental pollution (Kishimura, Tokuda, Klomklao, Benjakul, & Ando, 2005; Kristinsson & Rasco, 2000a, 2000b), or used as feeds and fertilizers with low economical value (Haard, 1998). Using proteinases from fish viscera has an advantage over commercial proteinases since their optimal temperatures and other enzymatic characteristics differ from homologous proteinases obtained from warm-blooded animals (Shahidi, Han, & Synowiecki, 1995). Therefore, there is a growing interest in obtaining higher value biochemicals and pharmaceuticals, notably proteinases from fishery wastes.

Fish are poikilothermic animals and their survival requires the adaptation of digestive enzymes to the temperature of the habitat. As a result, enzymes from cold-adapted fish species often have higher enzymatic activities at low temperatures than counterparts from warm-blooded animals (Ahsan & Watabe, 2001; Asgeirsson, Fox, & Bjarnason 1989; Kristjansson, 1991). High activity of fish enzymes at low temperatures is interesting for industrial applications, especially in certain food processing operations that require low temperatures (Haard, 1992). Furthermore, the relatively low thermal stability often observed in fish enzymes, may also be beneficial in such applications as the enzymes remained can be inactivated more readily, with less heat treatment (Simpson & Haard, 1987).

Main digestive proteinases detected in the hepatopancreas of fish are trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1). Trypsin and trypsin-like proteinases have received much interest and are well documented. However, information about chymotrypsin is less available (Kristjansson & Nielsen, 1992). Chymotrypsins, which cleave the peptides on the carboxyl side of phenylalanine, tyrosine and tryptophan residues, have been isolated and characterized from several species of fish. These fish include common carp (*Cyprinus carpio*) (Cohen, Gertler, & Birk, 1981), dogfish (*Squalus acanthias*) (Racicot & Hultin, 1987), cod (*Gadus morhua* L.) (Raae & Walther, 1989), Atlantic cod (*G. morhua*) (Asgeirsson & Bjarnason, 1991), rainbow trout (*Oncorhynchus mykiss*) (Kristjansson & Nielsen, 1992), anchovy (*Engraulis japonica*) (Heu, Kim, & Pyeun, 1995), grass carp (*Ctenopharyngodon idellus*) (Fong, Chan, M., Lau, & K. K., 1998) and Monterey sardine (*Sardinops sagax caeruleus*) (Castillo-Yanez, Pacheco-Aguilar, Garcia-Garreno, Toro, & Lopez, 2006). Although chymotrypsins from fish are basically similar to mammalian counterparts, differences both in structural and functional properties have been reported (Fong et al., 1998).

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Till now, most studies of fish chymotrypsin have focused on marine fish, while studies on chymotrypsins from freshwater fish are scarce. Crucian carp (*Carassius auratus*) is one of the most economically important freshwater fish species in China. Similar to other fish species, deterioration is also very rapid in muscle and especially in the digestive tract, suggesting that crucian carp hepatopancreas is a good source of proteinases. Our objective in the present study was to purify and study biochemical properties of chymotrypsins from crucian carp.

2. Materials and methods

2.1. Materials

2.1.1. Fish

Crucian carp (*C. auratus*) with body weight of about 300 g were purchased alive from the fish market of Jimei, Xiamen, China. Fish were obtained from February to September and subdued in iced water and sacrificed instantly. Hepatopancreas were collected and washed for experimental use immediately. The purification experiments were replicated three times.

2.1.2. Chemicals

DEAE-Sepharose, Sephacryl S-200 HR, SP-Sepharose and Phenyl-Sepharose were purchased from Amersham Biosciences (Uppsala, Sweden). Succinyl-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) and other synthetic fluorogenic peptide substrates (MCA-substrates) were obtained from Peptide Institute (Osaka, Japan). Chymostatin, phenylmethanesulfonyl fluoride (PMSF), *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), benzamidine, EDTA and bovine chymotrypsin were products of Sigma (St. Louis, MO, USA). Pepstatin and pefabloc SC were from Roche (Mannhem, Germany). Protein marker for SDS–PAGE was from Bio-Rad (Richmond, CA, USA) or Fermentas (Lithuania), prestained protein marker for immunoblotting was from New England Biolabs (Richmond, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (HRP-IgG) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) were from Pierce (Rock-ford, IL, USA). Other reagents were of analytical grade.

2.2. Determination of enzyme activity

Routinely, the proteolytic activity of chymotrypsin was measured using Suc-Leu-Leu-Val-Tyr-MCA as substrate, while the activity of trypsin was measured using Boc-Phe-Ser-Arg-MCA as substrate according to the method as described (Cao et al., 2000). Appropriately diluted enzyme (50 μl) was added to 900 μl of 0.1 M Tris–HCl buffer (pH 7.5). The reaction was immediately initiated by the addition of 50 μl of 10 μM substrate and incubated at 37 °C for 10 min. To stop the reaction, 1.5 ml of the stopping agent (methyl alcohol:n-butyl alcohol:distilled water = 35:30:35, v/v) was added. The fluorescence intensity of liberated 7-amino-4-methylcoumarin (AMC) was measured by a fluorescence spectrophotometer (Jasco, FP-6200, Japan) at an excitation wavelength of 380 nm and emission wavelength of 450 nm. One unit of chymotrypsin activity was defined as the amount of the enzyme to release 1 nmol AMC per minute. One unit of trypsin activity was defined as the amount of the enzyme to release 1 μmol AMC per minute. Proteolytic activity assay was performed in duplicate and variation between duplicate samples was always <5%. The mean values were used.

2.3. Purification of chymotrypsins

All procedures were performed under 4 °C. Crucian carp hepatopancreas (56 g) was homogenized in four-fold of 20 mM Tris–HCl buffer (pH 7.5) containing 5 mM $CaCl_2$ using a homogenizer (Kinematica, PT-2120, Switzerland). The homogenate was centrifuged at 10,000*g* for 30 min in a centrifuge (Avanti J-25, Beckman Coulter, USA). The supernatant was fractionated with ammonium sulphate from 30% to 60% saturation. After centrifugation at 10,000*g* for 30 min, the resulting pellet was dissolved in a minimum volume of 20 mM Tris–HCl buffer (pH 7.5) containing 5 mM $CaCl_2$ and dialyzed against the same buffer extensively. The dialysate was subsequently applied to a DEAE-Sepharose column (2.5 × 16 cm), which was previously equilibrated with the dialysis buffer. Unadsorbed fractions with chymotrypsin activity (cationic chymotrypsin) were collected for further experiment as described below. After 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 600 ml, fractions revealing chymotrypsin activity (anionic chymotrypsin) were pooled.

The two pooled fractions from DEAE-Sepharose column were individually concentrated by ultrafiltration using a membrane of YM-10 (Millipore, MA, USA) and applied to a Sephacryl S-200 HR gel-filtration column (1.5 × 98 cm) equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 0.2 M NaCl. Anionic chymotrypsin (designated as chymotrypsin A) fractions from Sephacryl S-200 were pooled, brought to 1 M $(NH_4)_2SO_4$ and subsequently applied to a phenyl-Sepharose column (1.5 × 6 cm) equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 1 M $(NH_4)_2SO_4$. The column was washed with the same buffer followed by a linear gradient of 1–0 M $(NH_4)_2SO_4$ in 20 mM Tris–HCl buffer (pH 7.5) in a total volume of 90 ml. Finally, the column was eluted with a linear gradient of ethylene glycol from 0% to 50% (v/v) in 20 mM Tris–HCl buffer (pH 7.5) in a total volume of 90 ml. Active fractions were pooled for further study.

The cationic chymotrypsin (designated as chymotrypsin B) fractions from Sephacryl S-200 were also pooled and dialyzed against 20 mM phosphate buffer (pH 5.5). The dialysate was applied to a cationic column SP-Sepharose column (1.5 × 6 cm) previously equilibrated with the dialysis buffer (pH 5.5) and eluted with a linear gradient of NaCl from 0 to 0.5 M in the same buffer in a total volume of 120 ml. Active fractions eluted were pooled for further study.

2.4. 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, Fan, and Randall (1951), using bovine serum albumin as standard.

2.5. SDS–PAGE and gelatin zymography

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method of Laemmli (1970), using a 12.0% gel and the proteins were silver stained. Native-PAGE was performed according to the procedure of Laemmli (1970), except that samples were not heated and no SDS or reducing agents were added. The gels were stained with Coomassie brilliant blue R-250 (CBB).

Gelatin zymography was performed according to the method of Kleiner and Steler-Stevenson (1994) with 1 mg/ml gelatin in the gel. Briefly, samples were mixed with one-fourth of SDS sample buffer (200 mM Tris–HCl, pH 6.8, containing 8% SDS, 0.4% bromophenol blue, and 40% glycerol) and then applied to 12% polyacrylamide gel and electrophoresed at 4 °C. After electrophoresis, the gels were washed with 50 mM Tris–HCl (pH 7.5) (buffer B) containing 2.5% (v/v) Triton X-100 for 30 min twice followed by gentle shaking to remove SDS and rinsed with deionized water. The gels

were then incubated at 37 °C for 2 h in buffer B followed by staining with CBB.

2.6. pH and temperature profiles

Enzymatic activity of the two chymotrypsins over the pH range of 4.0–11.0 was determined at the temperature of 37 °C for 10 min using 0.1 M of the following buffers: sodium citrate buffer (pH 4.0–5.0), sodium phosphate buffer (pH 5.5–7.0), Tris–HCl buffer (pH 7.5–9.0) and Na_2CO_3–$NaHCO_3$ buffer (pH 9.5–11.0). For temperature profile study, the activities were assayed at temperature range between 20 and 70 °C for 10 min at pH 7.5 using 0.1 M Tris–HCl buffer.

2.7. pH and thermal stability

Effect of pH on the stability of the two chymotrypsins was evaluated by measuring the residual activity at 37 °C for 10 min after incubation at various pH values for 30 min at room temperature (20–25 °C), using different buffers from pH 4.0 to 11.0 as described above. To investigate thermal stability, the two enzymes dissolved in 0.1 M Tris–HCl buffer (pH 7.5) was incubated at different temperatures from 20 to 70 °C for 30 min and immediately cooled in ice water. Thereafter, the residual activity was determined at 37 °C for 10 min at pH 7.5.

2.8. Effect of proteinase inhibitors and metal ions

To examine the effect of different proteinase inhibitors and metal ions on the two chymotrypsins, purified enzymes were preincubated with various inhibitors or metal ions at different final concentrations in 0.1 M Tris–HCl buffer (pH 7.5) at room temperature for 30 min and the remaining activity was measured. Control tests were performed without any addition of proteinase inhibitors and metal ions.

2.9. Kinetic studies

Crucian carp chymotrypsins and bovine chymotrypsin prepared at a concentration of 1 μg/ml were allowed to react with different concentrations of Suc-Leu-Leu-Val-Tyr-MCA at 37 °C for 10 min to determine the enzymatic activities. Kinetic parameters including V_{max} and K_m were evaluated based on the Lineweaver–Burk plots. The turnover number (K_{cat}) was calculated from the following equation: $K_{cat} = V_{max}/[E]$, where [E] is the active enzyme concentration and V_{max} is the maximal velocity.

2.10. Preparation of polyclonal antibody against chymotrypsin B

Purified chymotrypsin B was adjusted to the concentration of 200 μg/ml and inactivated by adding pefabloc SC to 5 mM, followed by emulsifying with an equal volume of complete Freund's adjuvant (Sigma–Aldrich, USA) as antigen. At initial immunization, an adult female rabbit was injected subcutaneously at several sites on the back with 100 μg antigen in a total volume of 1 ml. Two, 4 and 5 weeks later, the rabbit received three booster injections with the same amount of antigen as the initial immunization except that the antigen was emulsified with incomplete Freund's adjuvant. Five days after the final booster, the rabbit was bled from the retro-orbital plexus to obtain antiserum.

Immunoblotting was performed as described by Towbin, Staehelin, and Gordon (1979). Briefly, chymotrypsin A and B were electrophoresed in 12% gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk for 1 h, and incubated with the rabbit anti-chymotrypsin B serum (1:1000) 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.

3. Results and discussion

3.1. Purification of chymotrypsins

In the present study, two chymotrypsins were purified from the hepatopancreas of crucian carp by ammonium sulfate fractionation and column chromatographies of DEAE-Sepharose, Sephacryl S-200 HR, Phenyl-Sepharose or SP-Sepharose. As shown in Fig. 1a, two chymotrypsin active peaks were detected and the adsorbed portion (chymotrypsin A) eluted at NaCl concentration of 0.2 M was regarded as an anionic chymotrypsin. The unadsorbed portion (chymotrypsin B) was regarded as a cationic chymotrypsin.

Using ionic column DEAE-Sepharose, it is also efficient to separate chymotrypsins from trypsins (Fig. 1a). After gel-filtration on Sephacryl S-200, the two active portions were respectively applied to Phenyl-Sepharose and SP-Sepharose for further purification. The result of hydrophobic interaction chromatography Phenyl-Sepharose is shown in Fig. 1b. From fractions 0–60 washed by a linear gradient of 1–0 M $(NH_4)_2SO_4$, a large amount of contaminating proteins was removed. Finally, chymotrypsin A was eluted at 20% ethylene glycol with a yield of 6.5% (Table 1). On the other hand, cationic chymotrypsin B which was unadsorbed by DEAE-Sepharose was purified by a cationic-exchange column SP-Sepharose (Fig. 1c) with a yield of 13.3% (Table 1).

Animal chymotrypsins usually have cationic and anionic forms. Our present results showed that there are two isoforms of chymotrypsin in the hepatopancreas of crucian carp, which is in agreement with studies on rainbow trout (Kristjansson & Nielsen, 1992), Atlantic cod (Asgeirsson & Bjarnason, 1991) and grass carp (Fong et al., 1998). Interestingly, during purification, both chymotrypsin A and B were fully activated when extracted, suggesting that rapid activation of zymogens were performed during dissection and extraction. This result is different from chymotrypsins from grass carp, which were activated by trypsin (Fong et al., 1998).

3.2. Electrophoresis and gelatin zymography

Both chymotrypsin A and chymotrypsin B gave a single band on SDS–PAGE and native-PAGE suggesting their high purity (Fig. 2a and b). The molecular masses of chymotrypsin A and B were 28 and 27 kDa, which were similar to those of mammalian chymotrypsins (22–30 kDa) and very close to fish chymotrypsins from cod (27 kDa) (Raae & Walther, 1989), Atlantic cod (26 kDa) (Asgeirsson & Bjarnason, 1991), rainbow trout (28.2 kDa and 28.8 kDa) (Kristjansson & Nielsen, 1992), anchovy (26.1 kDa) (Heu et al., 1995), grass carp (28 kDa and 27 kDa) (Fong et al., 1998) and Monterey sardine (26 kDa) (Castillo-Yanez et al., 2006).

In accordance to chymotrypsins from other fish (Asgeirsson & Bjarnason, 1991; Kristjansson & Nielsen, 1992; Raae & Walther, 1989), the two chymotrypsins also gave only a single band even in the presence of β-mercaptoethanol (data not shown), which was different from bovine chymotrypsin that was split into three polypeptide chains (chains A, B and C) under reducing conditions (Bender & Killheffer, 1973). Initial N-terminal sequencing by Edman degradation revealed that native Atlantic cod chymotrypsins consist of two polypeptide chains (Leth-Larsen, Asgeirsson, Thorolfsson, Norregaard-Madsen, & Hojrup, 1996; Raae, Flengsrud, & Sletten, 1995), chain A (13 amino acid residues) and chain B (230 amino acid residues), suggesting that fish chymotrypsins may potentially have two polypeptide chains. In the case of grass

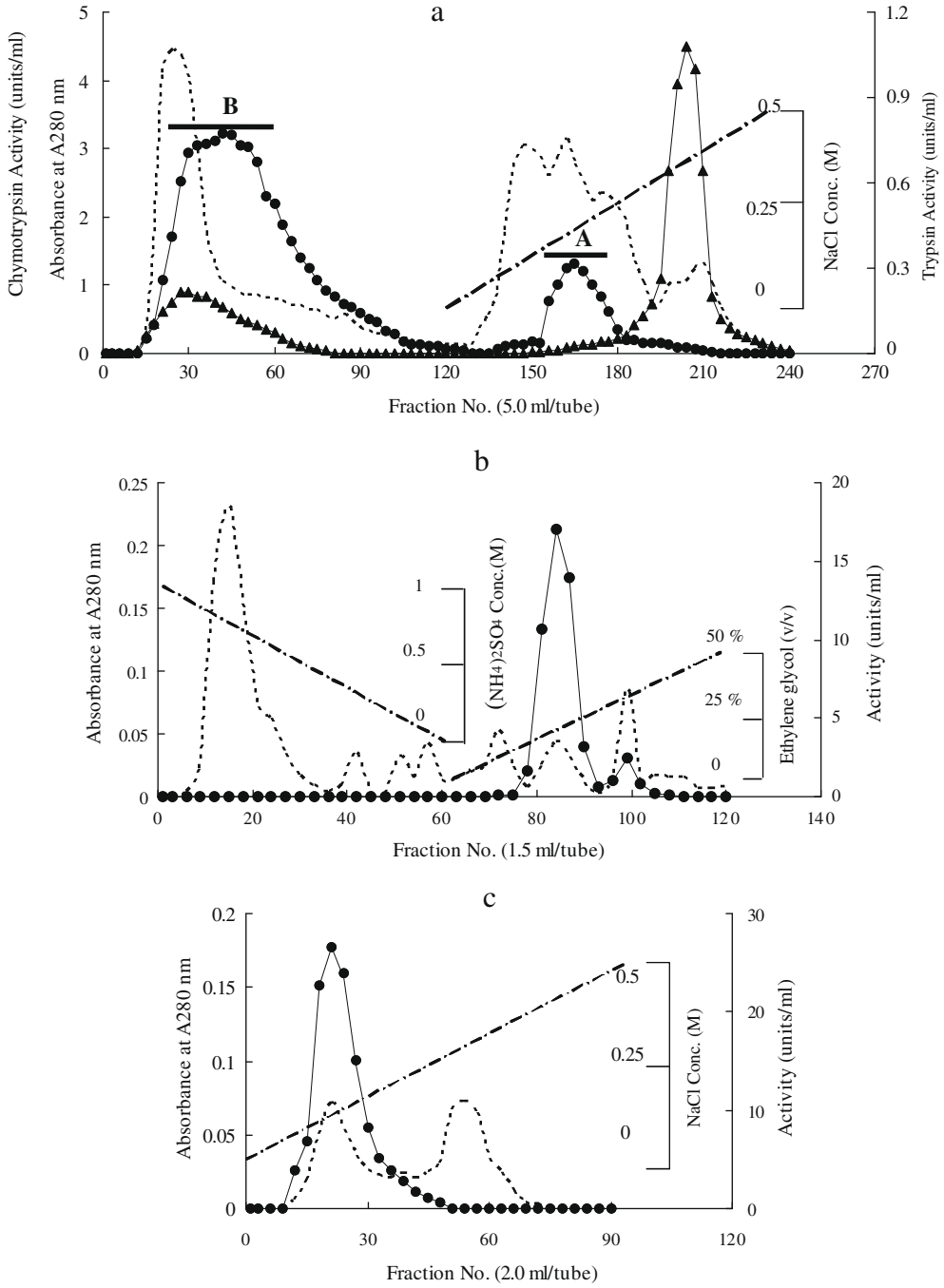

Fig. 1. Chromatographic purification of crucian carp chymotrypsins. (a) DEAE-Sepharose chromatography. (b) Phenyl-Sepharose chromatography. (c) SP-Sepharose chromatography. Absorbance at 280 nm (—); Suc-Leu-Leu-Val-Tyr-AMC hydrolyzing activity (●); Boc-Phe-Ser-Arg-MCA hydrolyzing activity (▲).

Table 1
Purification of chymotrypsin A and chymotrypsin B from the hepatopancreas of crucian carp

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purity (fold)	Yield (%)
Crude enzyme	5378.2	3328	0.62	1	100
Ammon. sulfate	696.9	2828	4.1	6.6	85.0
Chymotrypsin A					
DEAE-Sepharose	24.8	415	16.7	26.9	12.5
Sephacryl S-200	3.2	314	98.1	158.3	9.4
Phenyl-Sepharose	0.22	216	981.8	1583	6.5
Chymotrypsin B					
DEAE-Sepharose	53.5	1276	23.8	38.4	38.3
Sephacryl S-200	7.8	788.7	101.1	163.1	23.7
SP-Sepharose	0.76	442	581.6	938.1	13.3

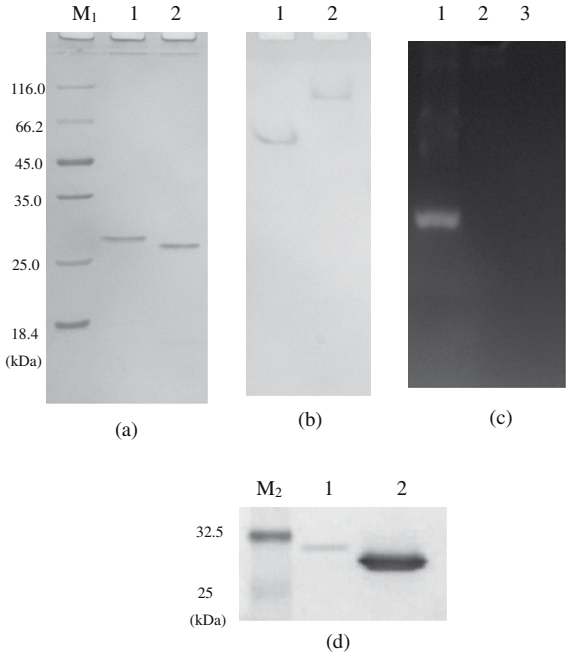

Fig. 2. SDS-PAGE, native-PAGE, gelatin zymography and immunoblotting of crucian carp chymotrypsin A and B. (a), SDS–PAGE in 12% gel followed by silver staining. (b), native-PAGE followed by CBB staining. (c), gelatin zymography followed by CBB staining. (d), immunoblotting. M_1, protein marker; M_2, prestained protein marker; lane 1, chymotrypsin A; lane 2, chymotrypsin B; lane 3, bovine chymotrypsin.

carp chymotrypsins, chain A was too short to be identified by SDS–PAGE, leaving the longer B chain as the only polypeptide chain detected (Fong et al., 1998). Thus, quite possibly, differently to bovine chymotrypsin, crucian carp chymorypsins, like chymotrypsins from Atlantic cod and common carp, are likely to remain in either the π or δ form and do not transform into α form during the activation process.

Interestingly, chymotrypsin A is also active in degrading gelatin as shown by zymography, while chymotrypsin B and bovine chymotrypsin did not reveal activity against gelatin (Fig. 2c). To our knowledge, similar behavior has not been reported in chymotrypsin from any other fish. However, a chymotrypsin-like proteinase from *Tenebrio molitor* larvae revealed a similar characteristic (Elpidina et al., 2005). For substrate specificity, chymotrypsin A showed much lower catalytic efficiency to substrate Suc-Ala-Ala-Pro-Phe-MCA than chymotrypsin B (date not shown). These results implied that crucian carp chymotrypsin A similar to grass carp chymotrypsin I was a less typical chymotrypsin (Fong et al., 1998). In order to make a further comparison of the two enzymes, particularly their structural relationship between each other, molecular cloning of the full length sequences of them is necessary.

3.3. pH optimum and stability

The pH dependence of the two chymotrypsins is shown in Fig. 3a. Chymotrypsin A and B exhibited maximal activity toward Suc-Leu-Leu-Val-Tyr-MCA at pH 7.5 and 8.0, respectively. Considerable loss of activities for the two enzymes was observed below pH 5.0. For pH stability, both chymotrypsin A and B showed high stability over a broad pH range of 6.0–11.0 (Fig. 3b). However, the two chymotrypsins were unstable at pH below 5.0. Only about 10% activity was found for the enzymes at pH 4.0. The stability of chymotrypsins at particular pH may be relevant to the net charge of the enzyme at that pH (Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, & Toro, 2005). Likewise, a loss in stability below pH 5.0 had been detected in chymotrypsins from common carp (Cohen et al., 1981), Atlantic cod (Asgeirsson & Bjarnason, 1991) and rainbow trout (Kristjansson & Nielsen, 1992).

3.4. Temperature optimum and stability

The temperature profiles of the two chymotrypsins are presented in Fig. 3c. The maximal activity of chymotrypsin A and B were at 40 and 50 °C, respectively. The optimum temperatures of chymotrypsins of crucian carp are similar to anchovy chymotrypsin (45 °C) (Heu et al., 1995). The difference in optimal temperatures might be related to the fish inhabiting environments. Relative activity of chymotrypsin A at 20 °C exhibited more than 60% of its maximum activity, which was much higher than chymotrypsin B (27%), implying that chymotrypsin A is more active at low temperatures. For the thermal stability study, slight changes in activity were observed for both enzymes at 37 °C (Fig. 3d). However, relative activities of the two enzymes decreased sharply at above 45 °C. These results are in accordance with chymotrypsins from anchovy (Heu et al., 1995), rainbow trout (Kristjansson & Nielsen, 1992), cod (Raae & Walther, 1989), Atlantic cod (Asgeirsson & Bjarnason, 1991), and dogfish (Racicot & Hultin, 1987). Although thermal stabilities of both chymotrypsins were not distinctively different, chymotrypsin A was slightly thermal stable and which may be related to disulphide linkages as well as hydrophobic interactions in the interior of proteinases (Kim, Meyers, & Godber, 1992; Simpson & Haard, 1984).

3.5. Effect of proteinase inhibitors and metal ions

The effect of various proteinase inhibitors and metal ions was shown in Table 2. Activities of the two enzymes were strongly inhibited by chymostatin and serine proteinase inhibitors such as PMSF, pefabloc SC and partially inhibited by benzamidine. Significant inhibition of a chymotrypsin specific inhibitor TPCK was also observed in suppressing the activity of these enzymes, especially to chymotrypsin B. On the other hand, asparatic proteinase inhibitor pepstatin and metalloproteinase inhibitor EDTA showed minimal

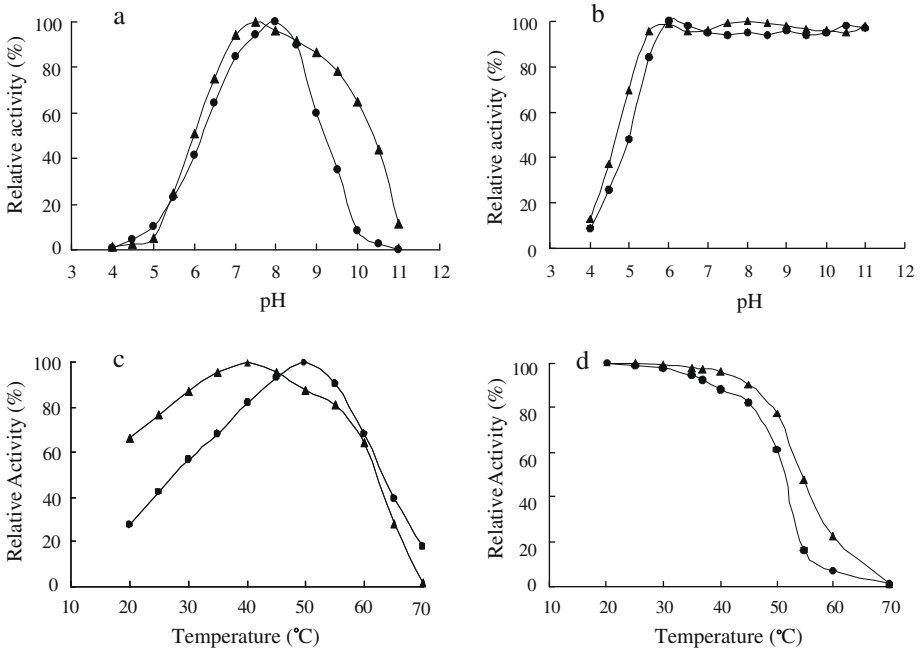

Fig. 3. Effect of pH and temperature on chymotrypsin A and B. pH profiles (a) and pH stability (b). Temperature profiles (c) and thermal stability (d). Chymotrypsin A (▲) and chymotrypsin B (●).

Table 2
Effects of various proteinase inhibitors and metal ions on the activity of chymotrypsin A and B.

Chemicals	Concentration (mM)	Relative activity (%)	
		Chymotrypsin A	Chymotrypsin B
None		100	100
TPCK	0.05	35.6	17.5
Chymostatin	0.01	0	0
Benzamidine	5	60.3	62.5
Pefabloc SC	1	1.2	1.1
PMSF	1	0.4	0.3
Pepstatin	0.03	95.9	92.3
EDTA	1	99	97
	10	98	100
$CaCl_2$	1	107	101
	5	115	103
$MgCl_2$	1	102	105
	5	111	110
$MnCl_2$	1	98.5	51.1
	5	85.3	48.9
$CdCl_2$	1	86.8	59.1
	5	76.8	49.7
$FeSO_4$	1	26.4	27.8
	5	14.6	21.4
$CuCl_2$	1	19.8	21.1
	5	0	2.5

Purified crucian carp chymotrypsins were preincubated with different inhibitors and metal ions individually at room temperature for 30 min and the residue activity was determined. Control tests were performed under identical conditions in the absence of chemicals.

inhibitory effects. Similar inhibition pattern was reported in Atlantic cod (Asgeirsson & Bjarnason, 1991), anchovy (Heu et al., 1995) and rainbow trout (Kristjansson and Nielsen, 1992). These results suggested that the two purified enzymes are chymotrypsin type serine proteinases.

For metal ions, both chymotrypsins were slightly activated by Ca^{2+} and Mg^{2+} at concentrations of 1 and 5 mM. On the other hand, both enzymes were strongly inhibited by Fe^{2+}, Cu^{2+} and partially inhibited by Mn^{2+}, Cd^{2+} at the concentration of 1 and 5 mM, suggesting the adverse effect of heavy metal ions on chymotrypsins.

3.6. Kinetic studies

Kinetic constants, K_m and K_{cat}, of the two chymotrypsins for hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA were determined based on Lineweaver–Burk plots (Table 3). K_ms of chymotrypsin A and B were 1.4 μM and 0.5 μM, respectively. K_{cat} s of chymotrypsin A and B were 2.7 and 3.4 S^{-1}, respectively. For turnover number (K_{cat}), chymotrypsin B had a higher value than chymotrypsin A, and the catalytic efficiency (K_{cat}/K_m) value of chymotrypsin B was also higher (3.6 folds) than that of chymotrypsin A. Compared with bovine α-chymotrypsin, both crucian carp chymotrypsins revealed higher catalytic efficiency (K_{cat}/K_m). This result strongly suggests that fish chymotrypsins are more efficient in hydrolyzing native protein substrates physiologically than mammal ones.

3.7. Immunoblotting

Immunoblotting analysis showed that the polyclonal antibody was specific in detecting chymotrypsin B while only a faint band was detected against chymotrypsin A (Fig. 2d), suggesting that the homogeneity between these two enzymes was not high. The specific polyclonal antibody against chymotrypsin B prepared in the present study will help us to make an immunoaffinity column for more efficient purification of chymotrypsin B in the future. Also, it may help us to detect the tissue distribution of chymotrypsin B in crucian carp hepatopancreas immunohistochemically.

Table 3
Kinetic properties of crucian carp chymotrypsins for the hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA.

Enzyme	K_m (μM)	K_{cat} (S^{-1})	K_{cat}/K_m (S^{-1} μM^{-1})
Chymotrypsin A	1.4	2.7	1.9
Chymotrypsin B	0.5	3.4	6.8
Bovine chymotrypsin	0.8	0.9	1.1

The K_m and K_{cat} values were determined at pH 7.5 and 37 °C. The final concentration of crucian carp chymotrypsins and bovine chymotrypsin for the assay were 1 μg/ml.

4. Conclusions

Two chymotrypsins from the hepatopancreas of crucian carp were purified and identified based on molecular masses, inhibitor sensitivity and substrate specificity. A specific polyclonal antibody against chymotrypsin B was prepared. The two enzymes showed maximal activity at pH 7.5–8.0 and 40–50 °C respectively. Especially chymotrypsin A revealed about 60% of the maximum activity at 20 °C and both enzymes lose activity significantly at above 50 °C. These characteristics suggested the potential application value of the two chymotrypsins where low processing temperature and higher enzymatic activity is needed.

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References

Ahsan, M. N., & Watabe, S. (2001). Kinetic and structural properties of two isoforms of trypsin isolated from the viscera of Japanese anchovy (*Engraulis japonicus*). *Journal of Protein Chemistry, 20*, 49–58.

Asgeirsson, B., & Bjarnason, J. B. (1991). Structural and kinetic propertises of chymotrypsin from Atlantic cod (*Gadus morhua*). Comparison with bovine chymotrypsin. *Comparative Biochemistry and Physiology, 99B*, 327–335.

Asgeirsson, B., Fox, J. W., & Bjarnason, J. B. (1989). Purification and characterization of trypsin from the poikilotherm *Gadus morhua*. *European Journal of Biochemistry, 180*, 85–94.

Bender, M. L., & Killheffer, J. V. (1973). Chymotrypsins. *CRC Critical Reviews in Biochemistry, 1*(2), 149–199.

Cao, M. J., Osatomi, K., Suzuki, M., Hara, K., Tachibana, K., & Ishihara, T. (2000). Purification and characterization of two anionic trypsins from the hepatopancreas of carp. *Fisheries Science, 66*, 1172–1179.

Castillo-Yanez, F. J., Pacheco-Aguilar, R., Garcia-Carreno, F. L., & Toro, M. A. N. (2005). Isolation and characterization of trypsin from pyloric caeca of Monterey sardine (*Sardinops sagax caerulea*). *Comparative Biochemistry and Physiology, 140B*, 91–98.

Castillo-Yanez, F. J., Pacheco-Aguilar, R., Garcia-Garreno, F. L., Toro, M. A. N., & Lopez, M. F. (2006). Purification and biochemical characterization of chymotrypsin from the viscera of Monterey sardine (*Sardinops sagax caeruleus*). *Food Chemistry, 99*, 252–259.

Cohen, T., Gertler, A., & Birk, Y. (1981). Pancreatic proteolytic enzymes from carp (*Cyprinus carpio*)-I. Purification and physical properties of trypsin, chymotrypsin, elastase and carboxipeptidase B. *Comparative Biochemistry and Physiology, 69B*, 639–646.

Elpidina, E. N., Tsybina, T. A., Dunaevsky, Y. E., Belozersky, M. A., Zhzhikov, D. P., & Oppert, B. (2005). A chymotrypsin-like proteinase from the midgut of *Tenebrio molitor* larvae. *Biochimie, 87*, 771–779.

Fong, W. P., Chan, Y. M., & Lau, K. K. (1998). Isolation of two chymotrypsins from grass carp. *Biochemistry and Molecular Biology International, 45*, 409–418.

Haard, N. F. (1992). A review of proteolytic enzymes from marine organisms and their application in the food industry. *Journal of Aquatic Food Product Technology, 1*(1), 17–35.

Haard, N. F. (1998). Specialty enzymes from marine organisms. *Food Technology, 52*, 64–67.

Heu, M. S., Kim, H. R., & Pyeun, J. H. (1995). Comparison of trypsin and chymotrypsin from the viscera of anchovy (*Engraulis japonica*). *Comparative Biochemistry and Physiology, 112B*, 557–568.

Kim, H. R., Meyers, S. P., & Godber, J. S. (1992). Purification and characterization of anionic trypsins from the hepatopancreas of crayfish, *Procambarus clarkii*. *Comparitive Biochemistry and Physiology, 103 B*, 391–398.

Kishimura, H., Tokuda, Y., Klomklao, S., Benjakul, S., & Ando, S. (2005). Comparative study of enzymatic characteristics of trypsin from the Pyloric ceca of yellow tail (*Seriola quinqueradiata*) and brown hakeling (*Physiculus japonicus*). *Journal of Food Biochemistry, 30*, 521–534.

Kleiner, D. E., & Steler-Stevenson, W. G. (1994). Quantitative zymography: detection of picogram quantities of gelatinases. *Analytical Biochemistry, 218*, 325–329.

Kristinsson, H. G., & Rasco, B. A. (2000a). Fish protein hydrolysates: Production, biochemical and functional properties. *Critical Reviews in Food and Nutrition, 40*(1), 43–81.

Kristinsson, H. G., & Rasco, B. A. (2000b). Biochemical and functional properties of Atlantic Salmon (*Salmo salar*) muscle proteins hydrolyzed with various alkaline proteases. *Journal of Agricultural and Food Chemistry, 48*, 657–666.

Kristjansson, M. M. (1991). Purification and characterization of trypsin from the pyloric caeca of rainbow trout (*Oncorhynchus mykiss*). *Journal of Agriculture and Food Chemistry, 39*, 1738–1742.

Kristjansson, M. M., & Nielsen, H. H. (1992). Purification and characterization of two chymotrypsin-like proteases from the pyloric ceca of rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology, 101B*, 247–253.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature, 277*, 680–685.

Leth-Larsen, R., Asgeirsson, B., Thorolfsson, M., Norregaard-Madsen, M., & Hojrup, P. (1996). Structure of chymotrypsin variant B from Atlantic cod, *Gadus morhu*. *Biochimca et Biophysica Acta, 1297*, 49–56.

Lowry, O. H., Rosebrough, N. J., Fan, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 256–275.

Raae, A. J., Flengsrud, R., & Sletten, K. (1995). Chymotrypsin isoenzymes in Atlantic cod; differences in kinetics and substrate specificity. *Comparative Biochemistry and Physiology, 112B*, 393–398.

Raae, A. J., & Walther, B. T. (1989). Purification and characterization of chymotrypsin, trypsin and elastase like proteinases from cod (*Gadus morhua* L.). *Comparative Biochemistry and Physiology, 93B*, 317–324.

Racicot, W. F., & Hultin, H. O. (1987). A comparison of dogfish and bovine chymotrypsins. *Archives of Biochemistry and Biophysics, 256*, 131–143.

Shahidi, F., Han, X. Q., & Synowiecki, J. (1995). Production and characteristics of protein hydrolysates from capelin (*Mallofus villosus*). *Food Chemistry, 53*, 285–293.

Simpson, B. K., & Haard, H. F. (1984). Trypsin from Greenland cod, *Gadus ogac*. Isolation and comparative properties. *Comparative Biochemistry and Physiology, 79B*, 613–622.

Simpson, B. K., & Haard, N. F. (1987). Cold-adapted enzymes from fish. In D. Knorr (Ed.), *Food biotechnology* (pp. 495–528). New York: Marcel Dekker.

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 Acadamy of Sciences USA, 76*, 4350–4354.