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Purification and characterisation of trypsins from the pyloric caeca of mandarin fish (*Siniperca chuatsi*)

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Abstract

Two trypsins of anionic form (trypsin A) and cationic form (trypsin B) from the pyloric caeca of mandarin fish (*Siniperca chuatsi*) were highly purified by a series of chromatographies, including DEAE-Sephacel, Sephacryl S-200 HR, Q-Sepharose or SP-Sepharose. Purified trypsins revealed a single band on native-PAGE. The molecular weights of trypsin A and B were 21 kDa and 21.5 kDa, respectively, as estimated by SDS–PAGE, both under reducing and non-reducing conditions. Zymography analysis showed that both trypsins were active in degrading casein. Trypsin A and B exhibited maximal activity at 35 °C and 40 °C, respectively, and shared the same optimal pH of 8.5, using Boc-Phe-Ser-Arg-MCA as substrate. The two trypsins were stable up to 45 °C and in the pH range from 4.5 to 11.0. Trypsin inhibitors are effective on these two enzymes and their susceptibilities were similar. Both trypsins were activated by metal ions such as Ca^{2+} and Mg^{2+} and inactivated by Fe^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Al^{3+}, Ba^{2+} and Co^{2+} to different degrees. Apparent K_m values of trypsin A and B were 2.18 μM and 1.88 μM, and K_{cat} values were 81.6 S^{-1} and 111.3 S^{-1} for Boc-Phe-Ser-Arg-MCA, respectively. Immunoblotting analysis using anti-common carp trypsin A positively cross-reacted with the two enzymes, suggesting their similarity. The N-terminal amino acid sequence of trypsin B was determined as IVGGYECEAH, which is highly homologous with trypsins from other species of fish.

Keywords: Trypsins; *Siniperca chuatsi*; Purification; Immunoblotting; Homology

1. Introduction

The rapid development of the fisheries industry will depend on the effective utilisation of the available raw materials. Fish viscera, accounting for approximately 5% of total mass, are byproducts of the fisheries industry and represent both waste disposal and potential pollution problems. These materials, however, 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).

As poikilothermic animals, the survival of fish requires the adaptation of their digestive enzymes to the temperatures of their habitats. Enzyme from cold-adapted fish species thus often have higher enzymatic activities at low temperatures, than counterparts from warm-blooded animals (Ahsan & Watabe, 2001; Kristjansson, 1991). High activity of fish enzymes at low temperature may be interesting for many biotechnological and food processing applications (Haard, 1992).

One of the main digestive proteinases detected in the pyloric caeca and intestine of fish is trypsin (EC 3.4.21.4). Trypsin is a member of a large family of serine proteinases which specifically hydrolyse proteins and peptides at the carboxyl group of arginine and lysine residues and play a

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critical role in protein digestion. Because of their potential industrial usage, trypsins and trypsin-like serine proteinases have been isolated from different species of fish, including Atlantic cod (*Gadus morhua*) (Asgeirsson, Fox, & Bjarnason, 1989), rainbow trout (*Oncorhynchs mykiss*) (Kristjansson, 1991), common carp (*Cyprinus carpio*) (Cao et al., 2000), Japanese anchovy (*Engraulis japonicus*) (Ahsan & Watabe, 2001), tongol tuna (*Thunnus tonggol*) (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2006), true sardine (*Sardinops melanostictus*) and arabesque greenling (*Pleuroprammus azonus*) (Kishimura, Hayashi, Miyashita, & Noami, 2006), Atlantic bonito (*Sarda sarda*) (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007) and walleye Pollock (*Theragra chalcogramma*) (Kishimura, Klomklao, Benjakul, & Chun, 2008).

Mandarin fish (*Siniperca chuatsi*) is one of the most important farmed freshwater fish in China. Similar to other fish species, deterioration is also very rapid in muscle and especially in the digestive tract, which suggests that mandarin fish viscera might be a good source of enzymes. So far, most research concerning fish trypsins has been done on marine fish, while very little information regarding trypsins or trypsin-like enzymes from byproducts of freshwater fish has been documented.

Thus, in the present study, we describe the purification and characterisation of trypsins from the pyloric caeca of mandarin fish and provide basic information about their main biochemical and kinetic characteristics.

2. Materials and methods

2.1. Materials

2.1.1. Fish

Cultured mandarin fish (*Siniperca chuatsi*) with body weight about 500 g were purchased alive from the fish market of Jimei, Xiamen, China. The fish were subdued in iced water and sacrificed instantly. Immediately afterwards, pyloric caeca were collected and washed for experimental use.

2.1.2. Chemicals

DEAE-Sephacel, Sephacryl S-200 HR, Q-Sepharose and SP-Sepharose were purchased from Amersham Biosciences (Uppsala, Sweden). *t*-Butyloxy-carbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (Boc-Phe-Ser-Arg-MCA) and other synthetic fluorogenic peptide substrates (MCA-substrates) were obtained from Peptide Institute (Osaka, Japan). Lima bean trypsin inhibitor (LBTI), phenylmethanesulfonyl fluoride (PMSF), 1,10-phenanthroline monohydrate, EDTA and benzamidine were products of Sigma (St. Louis, MO). Pepstatin and Pefabloc SC were purchased from Roche (Mannheim, Germany). Protein markers for SDS–PAGE were from Bio-Rad (Richmond, CA) or Fermentas VAB (Vilnius, Lithuania). Prestained protein marker for immunoblotting was from New England Biolabs (Richmond, CA). Rat anti-common carp trypsin A polyclonal antibody was prepared as described (Cao et al., 2000). Horseradish peroxidase (HRP) labelled rabbit anti-rat IgG-HRP was a product of DAKO (Glostrup, Denmark). 3,3′-Diaminobenzidine tetrahydrochloride (DAB) was from Pierce (Rockford, IL). Soybean trypsin inhibitor (STI) was prepared in our laboratory. Other reagents were of analytical grade.

2.2. Determination of enzyme activity

Routinely, proteolytic activity was measured, using Boc-Phe-Ser-Arg-MCA as substrate. Appropriately diluted enzyme (50 μl) was added to 900 μl of 0.1 M Tris–HCl buffer (pH 8.0). 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) were added. The fluorescence intensity of liberated 7-amino-4-methylcoumarin (AMC) was measured by a fluorescence spectrophotometer (JASCO, FP-6200, Japan) at the excitation wavelength of 380 nm and the emission wavelength of 450 nm. One unit of enzyme activity was defined as the amount of the enzyme to release 1 nmol 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 trypsins

All procedures were performed below 4 °C. Mandarin fish pyloric caeca (34 g) were homogenised in six equivalents of 20 mM Tris–HCl buffer (pH 7.5) containing 5 mM $CaCl_2$. The homogenate was centrifuged at 15,000*g* for 20 min and the supernatant was fractionated with ammonium sulfate from 30% to 70% saturation. After centrifugation at 15,000*g* for 20 min, the resulting pellet was dissolved in a minimum volume of 20 mM Tris–HCl buffer (pH 7.5) containing 50 mM NaCl, 5 mM $CaCl_2$ and dialysed against the same buffer extensively. The dialysate was subsequently applied to a DEAE-Sephacel column (2.5 × 17 cm), which was previously equilibrated with the dialysis buffer. During column washing, unadsorbed fractions with trypsin activity (cationic trypsin) were collected for further experiment. Binding proteins were eluted with a linear gradient of NaCl from 0.05 to 0.5 M in 20 mM Tris–HCl buffer (pH 7.5); a total volume of 500 ml. Fractions revealing trypsin activity (anionic trypsin) were pooled for further study.

The two pooled trypsin active fractions from DEAE-Sephacel column were individually concentrated by ultrafiltration, using a membrane of YM-10 (Millipore Corporation, Billerica, MA), and applied to a gel-filtration column of Sephacryl S-200 (1.5 × 98 cm), equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 0.2 M NaCl. Anionic trypsin fractions from Sephacryl S-200 were

pooled, dialysed against 20 mM Tris–HCl buffer (pH 7.5) and applied to a Q-Sepharose column (1.5 × 4 cm). Elution was performed in a step-wise process, using dialysis buffer containing 0.05, 0.075, 0.1 and 0.125 M NaCl, respectively. Active fractions designated as trypsin A were eluted by 0.075 M NaCl and used for further study.

Cationic trypsin fractions from Sephacryl S-200 were also pooled and dialysed against 20 mM phosphate buffer (pH 6.0) before applying to an SP-Sepharose column (1.5 × 4 cm). Elution was performed with a linear gradient of NaCl from 0 to 0.2 M. An active fraction designated as trypsin B was collected.

2.4. Protein concentration determination

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

2.5. Electrophoresis and zymography

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method of Laemmli (1970), using 12.0% gels. Proteins were stained with Coomassie brilliant blue R-250 (CBB). Native-PAGE was performed according to the procedure of Laemmli (1970); samples were not heated and there was no addition of SDS or any reducing agent.

Zymography was carried out according to the method of Garcia-Carreno, Dimes, and Haard (1993), with slight modification. Briefly, the enzymes were submitted to native-PAGE. Gels after electrophoresis were immersed in 20 mM Tris–HCl buffer (pH 8.0) containing 2.5% Triton X-100 at 4 °C, with shaking for 30 min to remove SDS in the gel. Remaining Triton X-100 was removed by washing the gels three times with 0.1 M Tris–HCl buffer (pH 8.0). Gels were then pre-incubated with 3% (w/v) casein in 20 mM Tris–HCl buffer (pH 8.0) at 4 °C for 30 min followed by increasing the temperature to 37 °C and further incubation for 45 min. Finally, the gels were stained with CBB.

2.6. Immunoblotting

Immunoblotting was carried out, as described by Towbin, Staehelin, and Gordon (1979). Briefly, after SDS–PAGE, trypsins on acrylamide gels were electrophoretically transferred onto nitrocellulose membrane. 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 membrane was incubated with anti-common carp trypsin A immunoglobulin G (IgG) at room temperature for 2 h and washed with TBST (TBS, 0.05% Tween 20). After incubation for 1 h with goat anti-rat IgG coupled to horseradish peroxidase, the membrane was further washed extensively with TBST. Immunodetection was carried out using DAB as substrate.

2.7. pH and temperature profiles

The effect of pH on the two trypsins was determined across a pH range of 3.0–11.0 at 37 °C, using 0.1 M of the following buffers: sodium citrate buffer (pH 3.0–5.0), sodium phosphate buffer (pH 6.0–7.5), Tris–HCl buffer (pH 8.0–9.0) and Na_2CO_3–$NaHCO_3$ buffer (pH 9.0–11.0). To determine the temperature dependence of the two trypsins, the activities were assayed at temperatures of 20–60 °C at pH 8.0, using 0.1 M Tris–HCl buffer.

2.8. pH and thermal stability

The effect of pH on the stability of the two trypsins was evaluated by incubating the enzymes with buffers of different pH values ranging from 3.0 to 11.0, as described above. After incubation for 30 min at room temperature, residual activity was measured. Thermal stability of the two trypsins was examined by maintaining them at different temperatures from 20–60 °C in 0.1 M Tris–HCl buffer (pH 8.0) for 30 min and then placed in an ice bath for 5 min before the remaining activity was determined.

2.9. Effect of proteinase inhibitors

To examine the effect of different proteinase inhibitors on the two trypsins, purified enzymes were preincubated with various inhibitors at different final concentrations at room temperature for 15 min and the residual activity was measured. Control test was performed without any addition of inhibitor.

2.10. Substrate specificity

To characterise the substrate specificity of the two trypsins, various synthetic fluorogenic peptide substrates were incubated with purified trypsins and the amount of AMC released from the substrate was determined as described above.

2.11. Effects of metal ions

The two trypsins were preincubated with $CaCl_2$, $MgCl_2$, $CuCl_2$, KCl, $Zn(CH_3COOH)_2$, $FeSO_4$, $Al(NO_3)_3$, $BaCl_2$ and $CoCl_2$ at final concentrations of 1 mM and 5 mM in 0.1 M Tris–HCl buffer (pH 8.0) for 30 min at room temperature; residual activity was measured. Control test was performed without any addition of metal ion.

2.12. Kinetic studies

Trypsins prepared at concentration of 15 μg/ml were allowed to react with different concentrations of Boc-Phe-Ser-Arg-MCA at 37 °C for 5 min to determine their

enzymatic activities. Kinetic parameters, including v_{max} and K_m, were evaluated based on Lineweaver–Burk plots. The turnover number (K_{cat}) was calculated from the following equation: $K_{cat} = v_{max}/[E]$, where $[E]$ is the enzyme concentration and v_{max} is the maximal velocity.

2.13. Determination of the N-terminal amino acid sequence

Highly purified trypsin B from SP-Sepharose chromatography was applied to SDS–PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. After brief staining with CBB, the protein band corresponding to 21.5 kDa was cut out and submitted to amino acid sequencing using a protein sequencer (model 492, Applied Biosystems).

3. Results and discussion

3.1. Purification of trypsins

Two trypsins were purified from the pyloric caeca of mandarin fish by ammonium sulfate fractionation and column chromatography, using DEAE-Sephacel, Sephacryl S-200 HR, Q-Sepharose or SP-Sepharose. Anionic trypsin A and cationic trypsin B were purified from Q-Sepharose and SP-Sepharose, with yields of 8.5% and 13.1%, respectively (Table 1). Ammonium sulfate precipitation (30–70%) was an effective step for the purification of trypsins from the crude extract. After ion exchange chromatography on DEAE-Sephacel column, crude enzymes were divided into an adsorbed portion (trypsin A) and an unadsorbed portion (trypsin B) (Fig. 1a). After gel filtration on Sephacryl S-200, trypsin A was applied to Q-Sepharose while trypsin B was loaded on SP-Sepharose for further purification.

As shown in Fig. 1b, two peaks (peak I and peak II) were detected in the binding fractions from step-wise elution by Q-Sepharose chromatography. Trypsin A was eluted from peak I at an NaCl concentration of 0.075 M. Three other enzymes were detected in peak II; zymography and SDS–PAGE revealed that these three enzymes hydrolysed casein effectively and their molecular masses were 21, 25 and 28 kDa, respectively (Fig. 1b, inset). However, serine proteinase inhibitors such as STI, LBTI and benzamidine exhibited almost no inhibitory effect on their activity. Furthermore, immunoblotting using anti-common carp trypsin A IgG did not cross-react with these three proteins (data not shown). These results suggested that the three enzymes in peak II are not trypsin-like proteinases.

A cationic enzyme (trypsin B), which was not adsorbed by DEAE-Sephacel, was purified to homogeneity by passing the fractions through a cationic-exchange column SP-Sepharose (Fig. 1c). Animal trypsins usually have cationic and anionic forms, such as bovine (Walsh, 1970), dog (Pinsky, Laforge, & Scheele, 1985) and rat (Fletcher, Alhadeff, Craik, & Largman, 1987). Molecular cloning study has identified the existence of these two forms of trypsin in Atlantic salmon (Male, Lorens, Smalas, & Torrissen, 1995). However, to our knowledge, cationic trypsin from freshwater fish has not been reported. Our present work for the first time confirmed the existence of cationic trypsin in freshwater mandarin fish. Interestingly, during purification, both cationic and anionic trypsins were fully activated when extracted, suggesting that rapid activation of mandarin fish trypsinogens was performed under conditions of dissection and extraction.

Table 1
Purification of trypsin A and trypsin B from the pyloric caeca of mandarin fish

Purification steps	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Relative purity	Yield (%)
Crude enzyme	3115	5325	1.7	1	100
Ammonium sulfate	804	4852	6.0	3.5	91.1
Trypsin A					
DEAE-Sephacel	17.9	2756	154	90.1	51.8
Sephacryl S-200	3.6	1257	349	204	23.6
Q-Sepharose	0.3	455	1517	893	8.5
Trypsin B					
DEAE-Sephacel	18.6	3677	196	114	69.1
Sephacryl S-200	4.5	1866	415	243	35
SP-Sepharose	0.7	697	996	586	13.1

[a] Trypsin activity was assayed at pH 8.0, 37 °C for 10 min using Boc-Phe-Ser-Arg-MCA as substrate.

3.2. Electrophoresis and zymography

Both trypsin A and trypsin B gave a single band on SDS–PAGE, native-PAGE and zymography, suggesting their high purity (Fig. 2a, b and c). The molecular masses of trypsin A and trypsin B were 21 and 21.5 kDa, respectively, as estimated by SDS–PAGE. Generally, trypsins have been reported to have molecular masses between 20 and 30 kDa (Gendry & Launay, 1992). The molecular masses of trypsins from mandarin fish were similar to trypsins from feline (21 kDa) (Steiner, Medinger, & Williams, 1997), tongol tuna (21 kDa) and yellow tuna (21 kDa) (Klomklao, Benjakul, & Visessanguan, 2004). However, the molecular masses are lower than trypsins from common carp (28 kDa and 28.5 kDa) (Cao et al., 2000), rainbow trout (25.7 kDa) (Kristjansson, 1991), Atlantic bonito (29 kDa) (Klomklao et al., 2007) and walleye pollock (24 kDa) (Kishimura et al., 2008). The differences of molecular mass in trypsins may be due to genetic variation among species. However, the possibility that these differences are caused by autolytic degradation should not be excluded.

3.3. Immunoblotting

Immunoblotting analysis using against common carp trypsin A polyclonal antibody revealed positive reaction

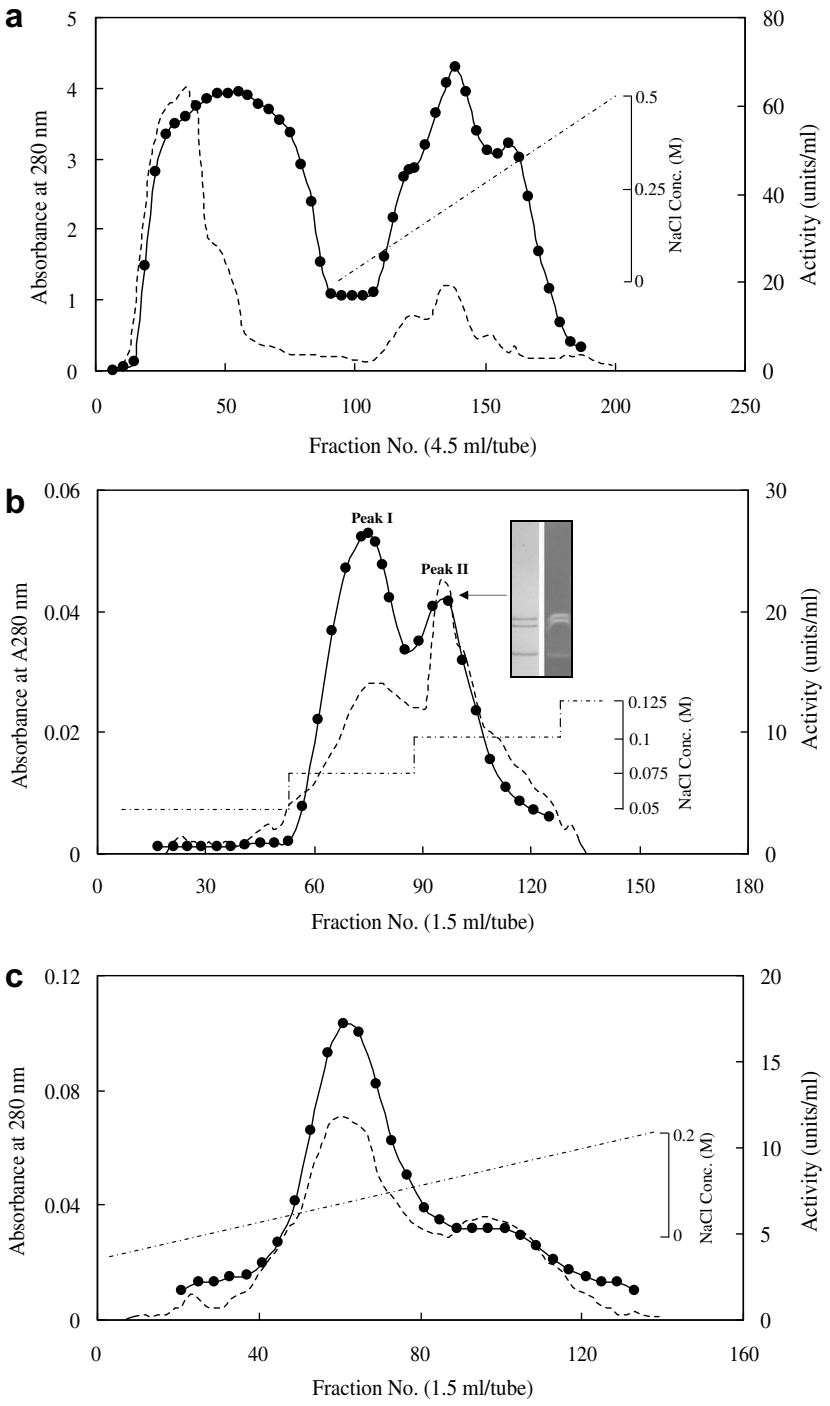

Fig. 1. Chromatographic purification of mandarin fish Trypsins. (a) DEAE-Sephacel chromatography. (b) Q-Sepharose chromatography. (c) SP-Sepharose chromatography. The SDS–PAGE and zymography in (b) showed the enzymes in Peak II eluted from Q-Sepharose. Absorbance at 280 nm (---); Boc-Phe-Ser-Arg-MCA hydrolysing activity (●).

bands (Fig. 3), suggesting the two enzymes are trypsins and have high homogeneity with trypsin from common carp hepatopancreas (Cao et al., 2000).

3.4. pH optimum and stability

The pH dependence of the two trypsins is shown in Fig. 4a. Both trypsin A and trypsin B exhibited maximal activity across a narrow pH range around 8.5. Trypsins generally belong to the alkaline proteinase group (Simpson & Haard, 1984). Under acid conditions, the charge distribution and conformation were changed and enzymes could not bind to substrate properly (Benjakul & Morrissey, 1997). The optimum pH of mandarin fish trypsins was in accordance with trypsins from Atlantic cod (Asgeirsson et al., 1989), common carp (Cao et al., 2000) and Tongol

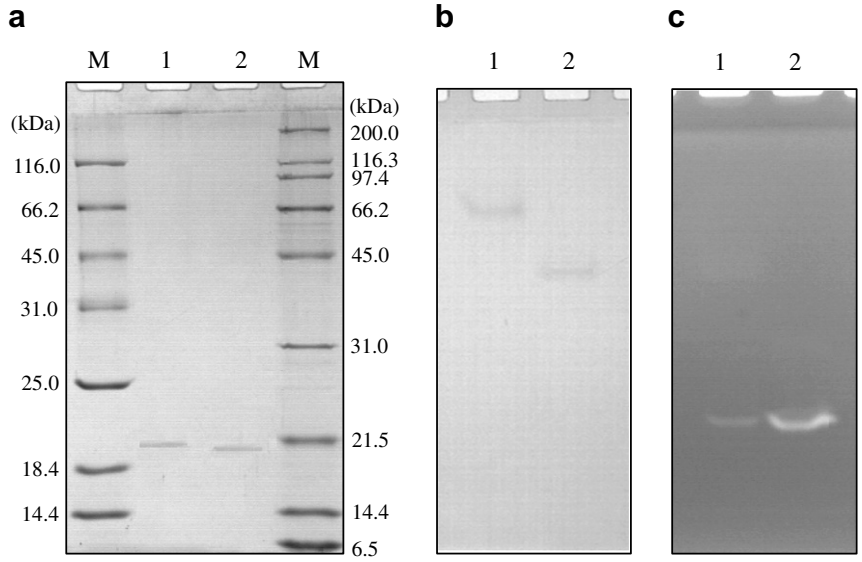

Fig. 2. SDS–PAGE (a), native-PAGE (b) and zymography (c) of mandarin fish trypsins. M, molecular marker; lane 1, trypsin B; lane 2, trypsin A. The gels were stained with Coomassie brilliant blue.

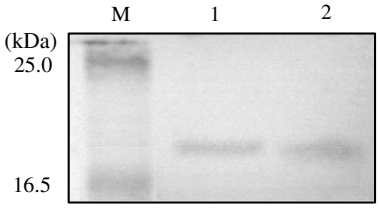

Fig. 3. Immunoblotting of mandarin fish trypsins. M, prestained protein marker; lane 1, trypsin B; lane 2, trypsin A.

tuna (Klomklao et al., 2006). For pH stability, trypsin A showed high stability in the pH range of 5–11, while trypsin B showed high stability in the range of 6–11. Unlike mammalian trypsins, inactivation of both trypsins was more pronounced after exposure below pH 4 (Fig. 4b). Instability at pH below 4 was also reported in trypsins from Tongol tuna (Klomklao et al., 2006) and walleye pollock (Kishimura et al., 2008).

3.5. Temperature optimum and stability

As shown in Fig. 5a, maximal activities of trypsin A and B were at 35 and 40 °C, respectively. The optimum temperatures of trypsins of mandarin fish were similar to trypsins from common carp (Cao et al., 2000) and anchovy (Martinez, Olsen, & Serra, 1988), whereas they are much lower than trypsins from many marine fish, including Greenland cod (Simpson & Haard, 1984), Atlantic bonito (Klomklao et al., 2007), true sardine and arabesque greenling (Kishimura et al., 2006). The differences in optimal temperatures may be associated with different living temperatures of fish. Approximately 59% and 46% of activities at the optimum temperature of trypsin A and B were detected at 20 °C, respectively, implying that the two enzymes have high

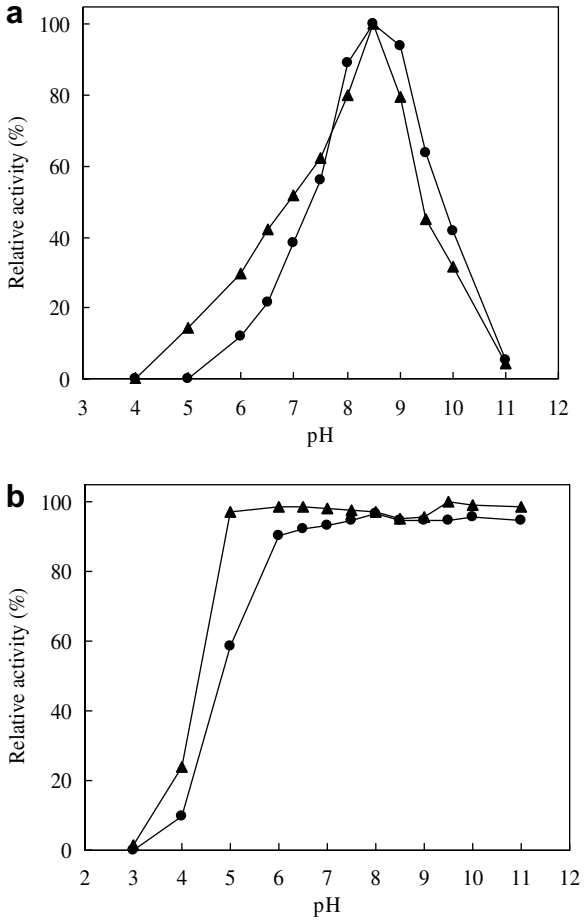

Fig. 4. pH profiles (a) and pH stability (b) of mandarin fish trypsins. Trypsin A (●) and trypsin B (▲).

activity at low temperatures, which may be beneficial for potential applications.For thermal stability study, both

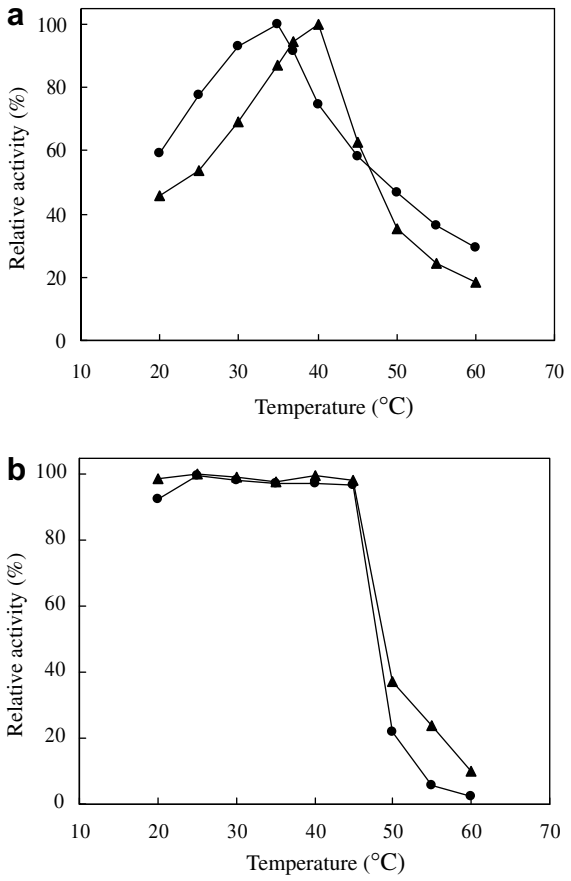

Fig. 5. Temperature profiles (a) and thermal stability (b) of mandarin fish trypsins. Trypsin A (●) and trypsin B (▲).

trypsins were stable below 45 °C, but their activities decreased sharply above 50 °C (Fig. 5b). The thermal stability of mandarin fish trypsins also resembles trypsins from common carp hepatopancreas (Cao et al., 2000). It seems that trypsins from freshwater fish were more thermally sensitive than those from marine fish, including true sardine and arabesque greenling (Kishimura et al., 2006), tongol tuna (Klomklao et al., 2006) and walleye pollock (Kishimura et al., 2008), suggesting freshwater fish trypsins are structurally less stable.

3.6. Effect of proteinase inhibitors and substrate specificity

The effect of proteinase inhibitors on the two trypsins was shown in Table 2. Activities were strongly inhibited by serine proteinase inhibitors such as PMSF, LBTI, STI, pefabloc SC and benzamidine. Metalloproteinase inhibitors of EDTA, 1,10-phenanthroline and aspartic proteinase inhibitor pepstatin showed hardly any inhibitory effect. These results strongly suggested that the two purified enzymes are serine proteinases, most likely trypsins.

To characterise the substrate specificity of the two enzymes, various fluorescent MCA-substrates were chosen to react with them. As shown in Table 3, trypsin A and B hydrolysed Boc-Phe-Ser-Arg-MCA most effectively. Other

Table 2
Effects of various proteinase inhibitors on Trypsin A and Trypsin B activity

Inhibitors	Concentration	Relative activity (%)	
		Trypsin A	Trypsin B
None		100	100
STI	0.1 mg/ml	6.7	2.1
PMSF	10 mM	8.8	2
LBTI	0.1 mg/ml	2.9	3.7
Pefabloc SC.	5 mM	0	0
Benzamidine	10 mM	0	0
Pepstatin	0.01 mM	96.6	92.7
o-Phenanthroline	5 mM	99	101.2
EDTA	5 mM	99.9	98

Purified mandarin fish trypsins were preincubated with different inhibitors individually at room temperature for 15 min and the residual activity was determined. Control tests were performed under identical conditions in the absence of inhibitors.

Table 3
Substrate specificity of trypsin A and trypsin B on MCA-substrates

Substrates (10 μM)	Relative activity (%)[a]	
	Trypsin A	Trypsin B
Boc-Phe-Ser-Arg-MCA	100	100
Boc-Glu-Arg-Arg-MCA	99.7	52.8
Boc-Val-Pro-Arg-MCA	89.1	85.7
Boc-Leu-Lys-Arg-MCA	83.8	73.8
Boc-Val-Leu-Lys-MCA	81.7	39.8
Boc-Leu-Arg-Arg-MCA	64.8	40
Boc-Phe-Leu-Lys-MCA	31.3	28.7
Boc-Glu-Lys-Lys-MCA	25.2	8.8
Z-Phe-Arg-MCA	57.3	97.8
Suc-Leu-Leu-Val-Tyr-MCA	0	0
Arg-MCA	0	0

[a] Proteolytic activity was assayed at pH 8.0, 37 °C for 10 min.

substrates containing arginine or lysine residues at P_1 site were also cleaved to some degree and arginine residue was favoured to lysine residue. Chymotrypsin substrate (Suc-Leu-Leu-Val-Tyr-MCA) and aminopeptidase substrate (Arg-MCA) were not affected. The substrate specificities of these enzymes were also similar to trypsins from common carp hepatopancreas (Cao et al., 2000), while somewhat different from trypsin-type serine proteinases from the skeletal muscle of common carp (Osatomi, Sasai, Cao, Hara, & Ishihara, 1997) and crucian carp (Guo et al., 2007), further confirming that the two enzymes are trypsins.

3.7. Kinetic studies

Kinetic constants, K_m and K_{cat}, of the two trypsins for hydrolysis of Boc-Phe-Ser-Arg-MCA were determined from a Lineweaver–Burk plot (Table 4). K_m values of trypsin A and B were 2.18 μM and 1.88 μM, respectively. K_{cat} values of trypsin A and B were 81.6 S^{-1} and 111.3 S^{-1}, respectively. The K_m value of trypsin B was lower than

Table 4
Kinetic properties of mandarin fish trypsins for the hydrolysis of Boc-Phe-Ser-Arg-MCA

Enzyme	K_m (μM)[a]	K_{cat} (S^{-1}) [a]	K_{cat}/K_m (S^{-1} μM^{-1})
Trypsin A	2.18	81.6	37.4
Trypsin B	1.88	111.3	59.2

[a] The K_m and K_{cat} values were determined at pH 8.0 and 37 °C. The final enzyme concentration for the assay was 15 μg/ml.

that of trypsin A, suggesting it has higher affinity to Boc-Phe-Ser-Arg-MCA than trypsin A. For turnover number (K_{cat}), trypsin B had a higher value than trypsin A, and the catalytic efficiency (K_{cat}/K_m) value for trypsin B was also higher (1.6-folds) than that of trypsin A. This result suggests that trypsin B would be more efficient in hydrolysing native protein substrates physiologically.

3.8. Effects of metal ions

As shown in Table 5, both trypsins were slightly activated by Ca^{2+} at concentrations of 1 and 5 mM. Similar results regarding the effect of calcium on trypsins were identified in common carp (Cao et al., 2000), tuna (Klomklao et al., 2004), true sardine and arabesque greenling (Kishimura et al., 2006). It is known that calcium ions promote the formation of active trypsin from trypsinogen and stabilise trypsin against autolysis (Sipos & Markel, 1970). Bode and Schwager (1975) reported that calcium not only protected trypsin against self-digestion, but also slightly increased its proteolytic activity. Enzymatic activity of the two trypsins was also enhanced by Mg^{2+} to some extent while it was strongly inhibited by Fe^{2+} and partially inhibited by Cu^{2+}, Al^{3+}, Zn^{2+}, Ba^{2+} and Co^{2+} at 1 mM. However, when the concentration of Cu^{2+}, Al^{3+}, Zn^{2+}, Ba^{2+}, Co^{2+} reached 5 mM, strong inhibition was observed. A similar result was also reported in trypsin from spotted goatfish (Souza, Amaral, Espirito Santo, Carvalho, & Ramilson, 2007). However, the activity of trypsin from spotted goatfish was slightly suppressed by Ca^{2+} and Mg^{2+} also.

Table 5
Effects of metal ions on enzyme activity

Metal ions	Concentration (mM)	Activity (%)[a]	
		Trypsin A	Trypsin B
None		100	100
KCl	1	100	100
	5	100	100
$CaCl_2$	1	111.8	110.7
	5	115.9	116.4
$MgCl_2$	1	105.2	110
	5	114.6	111.2
$CuCl_2$	1	90.5	84.6
	5	61.4	43.4
$Zn(CH_3COO)_2$	1	86.7	18.2
	5	5.2	8.4
$FeSO_4$	1	43.8	13.9
	5	4.5	5.7
$Al(NO_3)_3$	1	78.8	94.6
	5	47.8	61.1
$BaCl_2$	1	72.7	89.2
	5	26.9	71.4
$CoCl_2$	1	70.2	59
	5	26.9	31.3

[a] Purified trypsins were preincubated with metal ions at each concentration at room temperature for 15 min, and the residual activity was determined at 37 °C after 10 min. Control tests were performed under identical conditions in the absence of metal ions.

3.9. N-terminal amino acid sequence

The N-terminal amino acid sequence of trypsin B was analysed and a sequence of IVGGYECEAH was determined (Fig. 6). This sequence, although short, exhibits high similarities with trypsins from common carp (Cao et al., 2000), Japanese anchovy (Kishimura, Hayashi, Miyashita, & Noami, 2005), Atlantic salmon (Male et al., 1995), tongol tuna (Klomklao et al., 2006), dogfish (Titani, Ericsson, Neurath, & Walsh, 1973), Atlantic cod (Asgeirsson et al., 1989), cow (Walsh, 1970), pig (Hermodson, Ericsson, Neurath, & Walsh, 1973), dog (Pinsky et al., 1985) and rat (Craik et al., 1984). Though the N-terminal sequence of trypsin A was not successfully determined, our present data clearly demonstrated that the two enzymes from the pyloric caeca are most likely members of the trypsin family. In order to elucidate these two trypsins, determination of their complete sequences by, for example, cDNA cloning, is necessary.

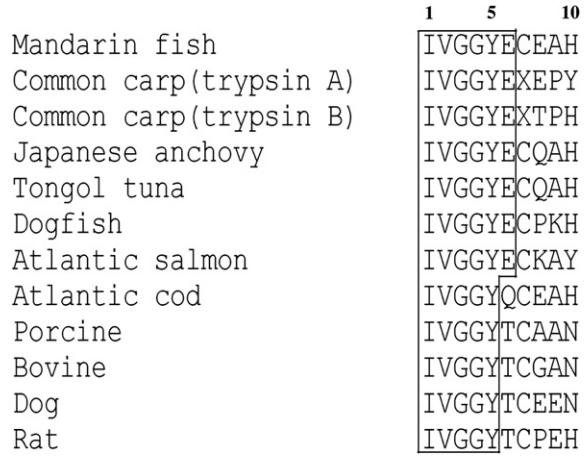

Fig. 6. Alignment of the N-terminal amino acid sequence of mandarin fish trypsin B with trypsins from other species. Common carp trypsin A and B (Cao et al., 2000), Japanese anchovy (Kishimura et al., 2005), Atlantic salmon (Male et al., 1995), Atlantic cod (Asgeirsson et al., 1989), tongol tuna (Klomklao et al., 2006), dogfish (Titani et al., 1973), bovine (Walsh, 1970), porcine (Hermodson et al., 1973), dog (Pinsky et al., 1985) and rat (Craik et al., 1984). Same amino acid residues are boxed.

4. Conclusion

One cationic and one anionic trypsin from the pyloric caeca of mandarin fish were purified and identified based on molecular weights, inhibitor sensitivity, substrate specificity, immunoblotting and N-terminal sequencing. The two trypsins showed maximum activity at pH 8.5 and 35–40 °C and have more than 45% of their maximum activity at 20 °C. These characteristics suggested their potential application where at high enzymatic activity low processing temperature is needed.

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