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Biochemical Characterization of Chymotrypsins from the Hepatopancreas of Japanese Sea Bass (*Lateolabrax japonicus*)

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Two chymotrypsins (chymotrypsins A and B) have been purified to homogeneity from the hepatopancreas of Japanese sea bass (*Lateolabrax japonicus*) by ammonium sulfate fractionation and chromatographies on DEAE-Sepharose and Phenyl-Sepharose. Two-dimensional electrophoresis (2-DE) analysis revealed that the molecular masses of chymotrypsins A and B were approximately 27.0 and 27.5 kDa, respectively. Their respective isoelectric points were 8.0 and 7.0. Purified chymotrypsins also revealed a single band on native-PAGE, whereas their mobilities were quite different. Optimum temperature and pH of chymotrypsins A and B were 45 °C and 8.0, respectively. Both enzymes were strongly inhibited by chymostatin, phenylmethanesulfonyl fluoride (PMSF), and Pefabloc SC, but slightly inhibited by metalloproteinase inhibitor of 1,10-phenanthroline and EDTA. Using Suc-Leu-Leu-Val-Tyr-MCA as substrate, apparent K_m values of chymotrypsins A and B were 0.8 and 1.1 μM and k_{cat} values were 2.7 and 2.0 s^{-1}, respectively. The N-terminal amino acid sequences of chymotrypsins A and B were determined to the 21st and 18th residues, respectively, and were identical. These sequences exhibited high identities to chymotrypsins from other animals. The digestive effect of the two chymotrypsins on myofibrillar proteins indicated their effectiveness in the degradation of food proteins.

KEYWORDS: Purification; chymotrypsin; Japanese sea bass; hepatopancreas; Western blot

INTRODUCTION

As an important enzyme group, serine proteinases attract a growing interest because of their vital biological functions and thus have been extensively studied (*1*). Fish viscera, accounting for 5% of the total fishery byproduct, are a rich potential source of various enzymes, especially serine proteinases (*2*, *3*). Industrial applications of serine proteinases in detergent, food, pharmaceutical, leather, and silk industries have also been studied (*4*). Until now, a wealth of information about serine proteinases has been accumulated, and investigation is still expanding with the purpose of obtaining a deeper insight into their mechanism of biological and, especially, physiological functions (*5*, *6*).

Chymotrypsin (EC 3.4.21.1), a member of the large family of serine proteinases, specifically cleaves the peptide bonds on the carboxyl side of phenylalanine, tyrosine, and tryptophan residues and plays an important role in protein digestion. Chymotrypsins have been isolated and characterized from several species of fish, including common carp (*Cyprinus carpio*) (*7*), cod (*Gadus morhua* L.) (*8*), rainbow trout (*Oncorhynchus mykiss*) (*9*), anchovy (*Engraulis japonica*) (*10*), grass carp (*Ctenopharyngodon idellus*) (*11*), and Monterey sardine (*Sardinops sagax caeruleus*) (*12*), and, more recently, we have purified two chymotrypsins from the hepatopancreas of crucian carp (*Carassius auratus*) (*13*).

Japanese sea bass (*Lateolabrax japonicus*) is one of the most economically important marine fish cultured in China. In 2008, the yield of sea bass reached 100,000 tonnes, accounting for 12.8% in the total marine cultured fish of China (*14*). Japanese sea bass is a kind of predatory fish that feeds on other fish and shrimps in the wild. Investigation of the major digestive proteinases in this kind of fish is beneficial to understand its function in food digestion. Although chymotrypsins have been purified from different species of fish, much information concerning the characteristics of chymotrypsin and especially its digestive effect on native proteins is needed. In the present study, we describe the purification and biochemical characterization of two chymotrypsins from the hepatopancreas of Japanese sea bass.

MATERIALS AND METHODS

Materials. Cultured Japanese sea bass (*L. japonicus*) with body weights of about 1500*g* were purchased alive from the fish market of Jimei, Xiamen, China. Live fish were obtained from October to June, subdued in ice water, and sacrificed instantly. Hepatopancreases were collected and washed for experimental use immediately. The purification experiments were repeated three times. DEAE-Sepharose, Phenyl-Sepharose, and Protein A Sepharose were purchased from Amersham Biosciences

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(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). Phenylmethanesulfonyl fluoride (PMSF), benzamidine, 1,10-phenanthroline, and EDTA were products of Sigma (St. Louis, MO). Chymostatin, pepstatin, and Pefabloc SC were from Roche (Mannhem, Germany). *trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) was from Amresco (Solon, OH). Protein marker for 2D gel electrophoresis was from Fermentas (Vilnius, Lithuania). Prestained protein marker for Western blot was from New England Biolabs (Richmond, CA). Rabbit anti-crucian carp chymotrypsin B polyclonal antibody was prepared in our laboratory as described (*13*), and immunoglobulin G (IgG) was purified by Protein A Sepharose affinity column. Secondary antibody of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (HRP-IgG) and substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB) were from Pierce (Rockford, IL). Other reagents were of analytical grade.

Determination of Enzyme Activity. The proteolytic activity of chymotrypsin was measured using Suc-Leu-Leu-Val-Tyr-MCA as substrate, whereas the activity of trypsin was measured using Boc-Phe-Ser-Arg-MCA as substrate according to the method as described (*13*, *15*). 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, followed by the addition of 1.5 mL of stopping agent (methyl alcohol/isopropyl alcohol/distilled water = 35:30:35, v/v) to terminate the reaction. 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 an emission wavelength of 450 nm. One unit of chymotrypsin activity was defined as the amount of the enzyme to release 1 nmol of AMC/min. One unit of trypsin activity was defined as the amount of the enzyme to release 1 μmol of AMC/min. A proteolytic activity assay was performed in duplicate, and variation between duplicate samples was always < 5%. The mean values were used.

Purification of Chymotrypsins. All procedures were performed under 4 °C. Japanese sea bass hepatopancreas (50 g) was homogenized in 6-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 15000*g* for 30 min and fractionated with ammonium sulfate from 25 to 70% saturation. After centrifugation at 15000*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 had been previously equilibrated with the dialysis buffer at flow rate of 1 mL/min, and 5 mL fractions were collected. Unadsorbed fractions with chymotrypsin activity (designated chymotrypsin A) were collected for further experiment as described below. After the column had been washed 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, and fractions revealing chymotrypsin activity (designated chymotrypsin B) were pooled.

The two pooled chymotrypsin active fractions from the DEAE-Sepharose column were individually added $(NH_4)_2SO_4$ to a final concentration of 1 M and subsequently applied to a Phenyl-Sepharose column (1.5 × 8 cm), which had been previously 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 decreasing 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. Then, 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, and fractions of 1.5 mL/tube were collected. Finally, the column was eluted with 50% (v/v) ethylene glycol in 20 mM Tris-HCl buffer (pH 7.5) until the absorbance at 280 nm reached baseline. Active fractions were pooled for further study.

Protein Concentration Determination. Protein concentration was determined by measuring the absorbance at 280 nm of the sample solution from column chromatographies or according to the method of Lowry et al. (*16*), using bovine serum albumin as standard.

Native-PAGE and Western Blot. Native-PAGE was performed similar to SDS-PAGE according to the method of Laemmli (*17*), except samples were not heated and no SDS or reducing agent was added to samples and electrophoretic buffer. The electrophoresis was carried out at 4 °C. Finally, the gel (8%) was stained with Coomassie brilliant blue R-250 (CBB).

Western blot was performed as described by Towbin et al. (*18*). Briefly, chymotrypsins A and B were electrophoresed in 15% gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk for 1 h and incubated with rabbit anti-crucian carp chymotrypsin B IgG at room temperature for 2 h followed by washing with Tris-buffered saline Tween-20 (TBST) (20 mM Tris-HCl (pH 7.5) containing 0.145 M NaCl and 0.05% Tween-20). After incubation with secondary antibody for 1 h, the nitrocellulose membrane was rinsed extensively with TBST. Immunodetection was carried out using DAB as substrate.

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

SDS-PAGE in the second dimension was performed on a MiniVE Complete electrophoresis apparatus (Amersham Biosciences) using 15% gels. After electrophoresis, the gels were stained with CBB for purity and isoelectric point check.

pH and Temperature Profiles. For the optimal pH assay, the activity of the two chymotrypsins was determined at the temperature of 37 °C at a pH range of 4.0−11.0 using 0.1 M of the following buffers: citric acid−NaOH buffer (pH 4.0−6.0), phosphate buffer (pH 6.5−7.5), Tris-HCl buffer (pH 8.0−9.0), Na_2CO_3−$NaHCO_3$ buffer (pH 9.5−10.5), and $NaHCO_3$−NaOH buffer (pH 11.0). For the temperature profile study, the activity was assayed at a temperature range between 20 and 65 °C using 0.1 M Tris-HCl buffer (pH 7.5).

pH and Thermal Stability. The effect of pH on the stability chymotrypsin was evaluated by measuring the residual enzymatic activity at pH 7.5 (0.1 M Tris-HCl buffer) for 10 min after incubation of the enzyme at room temperature for 30 min at various pH values, using different buffers from pH 4.0 to 11.0 as described above. To investigate thermal stability, chymotrypsins in 0.1 M Tris-HCl buffer (pH 7.5) were incubated at different temperatures from 30 to 50 °C for 0, 15, 30, 45, and 60 min, followed by immediate cooling in ice water. Residual activity was determined at 37 °C.

Effect of Proteinase Inhibitors. To examine the effect of different proteinase inhibitors (chymostatin, PMSF, Pefabloc SC, benzamidine, 1,10-phenanthroline, EDTA, pepstatin, and E-64) on the two chymotrypsins, purified enzymes were preincubated with various inhibitors individually 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 test was performed without the addition of any chemical.

Digestive Effect of Chymotrypsins on Myofibrillar Proteins of Shrimp. Myofibrils were prepared from Pacific white shrimp (*Litopenaeus vannamei*) muscle according to the method of Cao et al. (*19*). The degrading effect of chymotrypsins on the myofibrillar proteins was investigated in 50 mM phosphate buffer (pH 7.5). Enzymatic reactions were performed as follows: A ratio of 0.2 U of chymotrypsin activity/μg of protein was selected for all tests. Digestion reactions were performed at 20 °C, the samples were mixed extensively, and reactions were stopped at different time intervals of 0, 1, 10, 30, 60, 120, 240, and 360 min by boiling for 5 min followed by the addition of sample buffer and further heated at 95 °C for 10 min for SDS-PAGE. In the control test, the enzyme was replaced by 20 mM Tris-HCl buffer (pH 7.5).

Amino Acid Sequence Analysis. Purified chymotrypsins were subjected to SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was briefly stained by

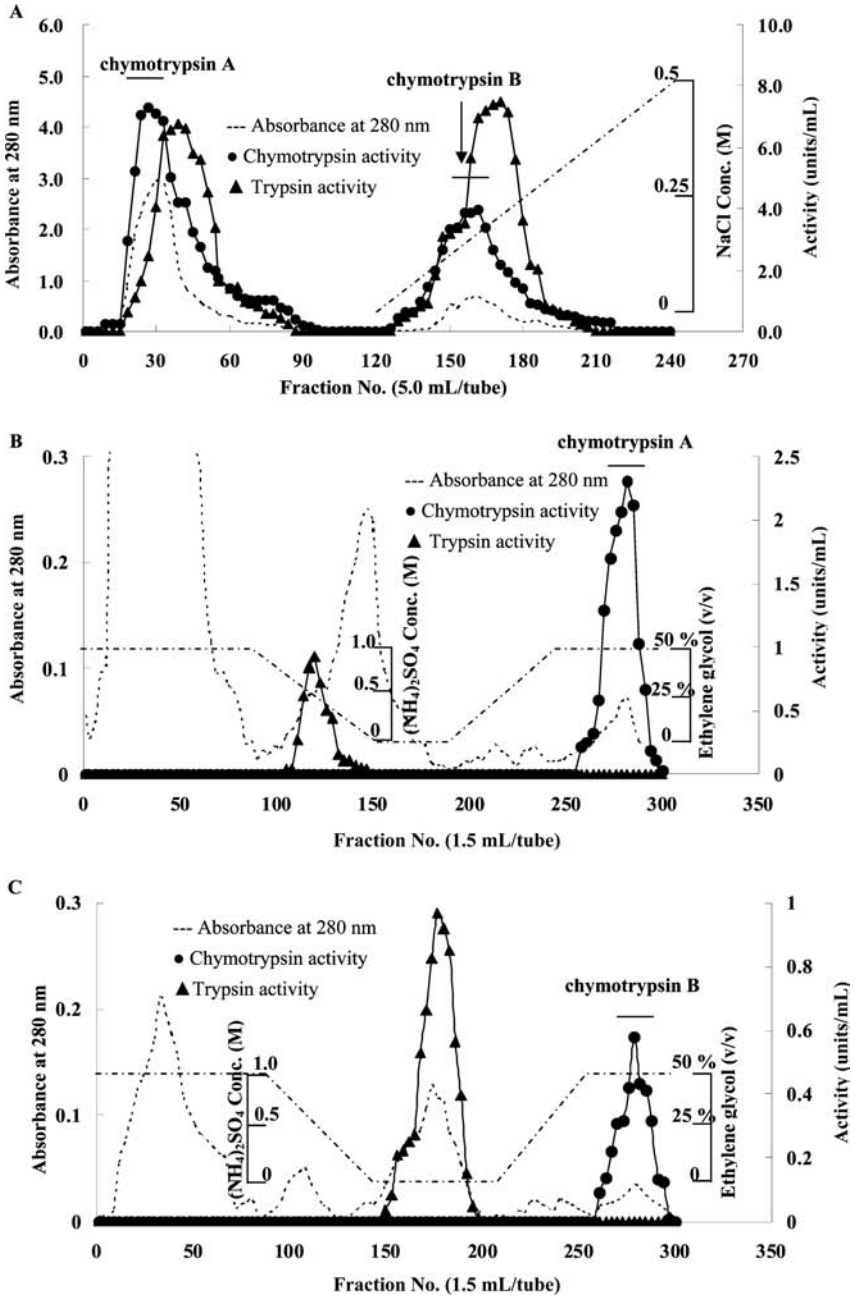

Figure 1. Chromatographic purification of Japanese sea bass chymotrypsins: (**A**) DEAE-Sepharose chromatography; (**B**) Phenyl-Sepharose chromatography for unadsorbed chymotrypsin from DEAE-Sepharose chromatography; (**C**) Phenyl-Sepharose chromatography for adsorbed chymotrypsin from DEAE-Sepharose chromatography. Fractions under the bars were pooled.

CBB, and the protein band was excised. The N-terminal amino acid sequences of chymotrypsins were determined individually using a protein sequencer (Shimadzu, PPSQ-33A).

Kinetic Parameter Study. Chymotrypsins prepared at a concentration of approximately 0.1 mg/mL were allowed to react with different final concentrations (0.1−2.0 μM) of Suc-Leu-Leu-Val-Tyr-MCA as substrate at 37 or 25 °C for 10 min. Kinetic parameters including V_{max} and K_m were evaluated on the basis of 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. All of the kinetic values were obtained from three independent determinations.

RESULTS AND DISCUSSION

In the present study, two chymotrypsins were highly purified from the hepatopancreas of Japanese sea bass by ammonium sulfate fractionation and column chromatographies on DEAE-Sepharose and Phenyl-Sepharose. As shown in **Figure 1A**, two chymotrypsin active peaks were detected in DEAE-Sepharose chromatography. The unadsorbed portion was named chymotrypsin A, and the adsorbed portion (named chymotrypsin B) was eluted when the NaCl concentration was 0.1 M.

After ionic column chromatography on DEAE-Sepharose, the two chymotrypsin active fractions were followed by trypsins (**Figure 1A**). These two active portions were pooled respectively and applied to Phenyl-Sepharose for further purification. As shown in **Figure 1B**, from fractions 0−90, which were washed by 20 mM Tris-HCl buffer (pH 7.5) containing 1 M $(NH_4)_2SO_4$, a large amount of contaminating proteins was removed. Fractions 91−150 were washed by a linear gradient of 1−0 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl buffer (pH 7.5), and trypsin was completely removed at this stage. Fractions 180−240 eluted with 20 mM Tris-HCl buffer (pH 7.5) containing 0−50% (v/v) ethylene glycol

Table 1. Purification of Chymotrypsins A and B from the Hepatopancreas of Japanese Sea Bass

purification step	total protein (mg)	total activity (units)	specific activity (units/mg)	purity (fold)	yield (%)
crude enzyme	6119.2	3117.5	0.5	1.0	100.0
ammonium sulfate (25−70%)	822.5	1657.6	2.0	4.0	53.2
DEAE-Sepharose					
chymotrypsin A	210.8	530.5	2.5	4.9	17.0
chymotrypsin B	21.6	163.7	7.6	14.9	5.3
Phenyl-Sepharose					
chymotrypsin A	1.5	308.9	205.9	404.2	9.9
chymotrypsin B	0.8	124.3	165.7	325.2	4.0

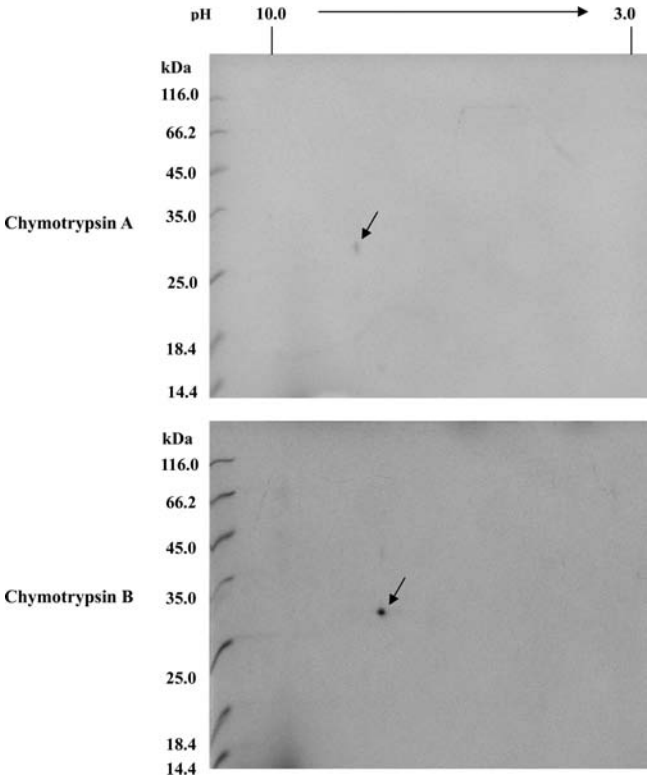

Figure 2. 2D gel electrophoresis of purified Japanese sea bass chymotrypsins. The gels were stained with CBB.

did not reveal any enzymatic activity. Chymotrypsin A was finally eluted by 20 mM Tris-HCl buffer (pH 7.5) containing 50% ethylene glycol. Chymotrypsin B, which was adsorbed by DEAE-Sepharose, was also purified by Phenyl-Sepharose in the presence of 20 mM Tris-HCl buffer (pH 7.5) containing 50% ethylene glycol (**Figure 1C**). The binding capacity of chymotrypsins to the hydrophobic interaction chromatography resin Phenyl-Sepharose was much stronger than that of trypsins, so it was effective to separate chymotrypsins from trypsins. Such strong binding to Phenyl-Sepharose was also reported in chymotrypsins from rainbow trout (*9*) and crucian carp (*13*). The purification of chymotrypsins is summarized in **Table 1**. As a result, starting from 50 g of hepatopancreas, 1.5 mg of chymotrypsin A and 0.8 mg of chymotrypsin B were purified to homogeneity with yields of 9.9 and 4.0%, respectively.

Animal chymotrypsins usually have different isoforms. Our present results showed that there are two isoforms of chymotrypsin in the hepatopancreas of Japanese sea bass, which were named chymotrypsins A and B. In previous studies, similar

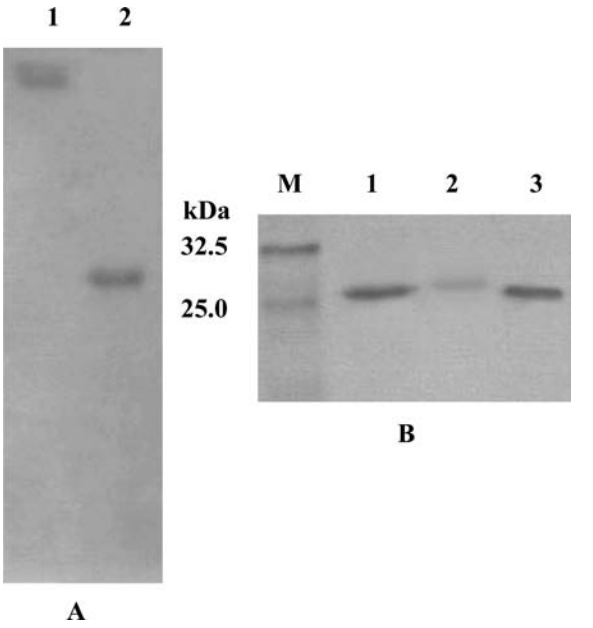

Figure 3. Native-PAGE and immunoblotting of Japanese sea bass chymotrypsins A and B: (**A**) native-PAGE of chymotrypsins followed by CBB staining; (**B**) immunoblotting of chymotrypsins. Lanes: M, prestained protein marker; 1, chymotrypsin A; 2, chymotrypsin B; 3, chymotrypsin B of crucian carp.

isoforms of chymotrypsin have been purified from rainbow trout (*O. mykiss*) (*9*), grass carp (*C. idellus*) (*11*), Monterey sardine (*S. sagax caeruleus*) (*12*), crucian carp (*C. auratus*) (*13*), and Atlantic cod (*G. morhua*) (*20*). As shown in **Figure 1B**,**C** and **Table 1**, the unadsorbed chymotrypsin (chymotrypsin A) was higher than the adsorbed chymotrypsin (chymotrypsin B) both in activity and in purification yield.

As shown in **Figure 2**, only a single spot was observed for both chymotrypsins A and B on 2D-PAGEs. Their molecular masses were 27.0 and 27.5 kDa, which were similar to those of mammalian chymotrypsins (22−30 kDa) and were closer to fish chymotrypsins from cod (*8*) (27 kDa), grass carp (*11*) (28 and 27 kDa), and crucian carp (*13*) (28 and 27 kDa). The 2D-PAGEs gave estimated isoelectric point (p*I*) values of approximately 8.0 and 7.0 for chymotrypsins A and B, respectively. The p*I* values of other species of fish, such as Atlantic cod (6.2, 5.8) (*20*), rainbow trout (5.0) (*9*), grass carp (5.6−5.8, 6.8−7.0) (*11*), and Monterey sardine (4.3, 5.8) (*12*, *21*), have been reported. These p*I* values were determined by analytical electrofocusing in thin-layer polyacrylamide flat gel containing ampholytes in the pH 3.5−9.5 range (*12*), whereas ours were determined by 2D-PAGE, which made the estimation of p*I* and molecular mass simultaneously. It is noteworthy that the p*I* value of most species of fish chymotrypsins reported are in the range of acidic pH, whereas that of chymotrypsin A from Japanese sea bass was 8.0, which made it a unique proteinase.

Native-PAGE of the two chymotrypsins also revealed a single band. As shown in **Figure 3A**, compared with chymotrypsin B, chymotrypsin A hardly moved. On the basis of their p*I* values, it is reasonable to obtain the result that chymotrypsin A with a p*I* of 8.0 hardly moved under the electrophoretic buffer condition (pH 8.3), whereas chymotrypsin B with a p*I* of 7.0 was negatively charged, causing its significant mobility. Western blot analysis using anti-crucian carp chymotrypsin B polyclonal antibody revealed positive reaction to chymotrypsin A and only weak cross-reaction to chymotrypsin B (**Figure 3B**), further suggesting that these two enzymes are chymotrypsins and have different degrees of homology.

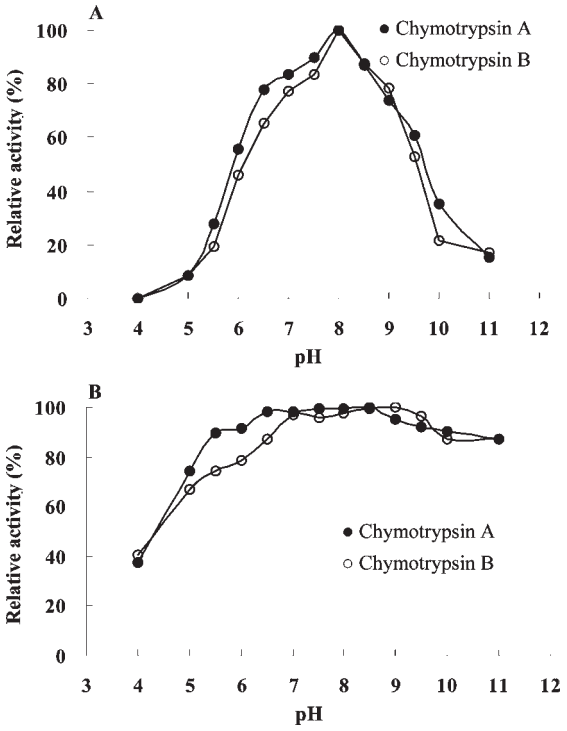

Figure 4. Effect of pH on chymotrypsins A and B: (**A**) pH profiles; (**B**) pH stability.

Both of the chymotrypsins exhibited optimum pH 8.0 for hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA (**Figure 4A**). This optimum pH was in agreement with chymotrypsins from other species of fish such as Atlantic cod (*20*), crucian carp (*13*), anchovy (*10*), and Monterey sardine (*12*, *21*). For pH stability, both chymotrypsins A and B showed high stability over a pH range of 7.0−11.0 (**Figure 4B**). However, they were unstable at pH below 5.0, and approximately 40% activity was detected at pH 4.0. This agrees with the report that fish chymotrypsins are stable only to neutral and alkaline pH (*22*, *23*). Similar results were also observed in chymotrypsins from common carp (*7*), Atlantic cod (*20*), rainbow trout (*9*), Monterey sardine (*21*), and crucian carp (*13*).

The temperature profiles of the two chymotrypsins are shown in **Figure 5A**. The maximal activities of chymotrypsins A and B were both at 45 °C. The optimum temperatures of chymotrypsins of Japanese sea bass were the same as that of anchovy chymotrypsin (*10*) (45 °C), but different from those of chymotrypsins A (40 °C) and B (50 °C) from crucian carp (*13*). The difference in optimal temperatures might be related to the fish habitat environments (*6*). A thermal stability profile revealed that enzymatic activity of the two enzymes decreased sharply at relatively higher temperature (≥50 °C) and remained stable at relatively lower temperature (≤30 °C) (**Figure 5B**,**C**). These results were in accordance with chymotrypsins from cod (*8*), rainbow trout (*9*), anchovy (*10*), crucian carp (*13*), Atlantic cod (*20*), Monterey sardine (*21*), and dogfish (*24*). The half-life of the two chymotrypsins was about 20 min at 50 °C (**Figure 5B**, **C**). A lower thermal stability is beneficial as these enzymes can be inactivated more readily with less heat treatment in industrial applications.

The effects of various proteinase inhibitors on the two enzymes are summarized in **Table 2**. Activity was strongly inhibited by chymostatin and serine proteinase inhibitors such as PMSF and Pefabloc SC, and partially inhibited by benzamidine. On the other hand, the asparatic proteinase inhibitor pepstatin and the

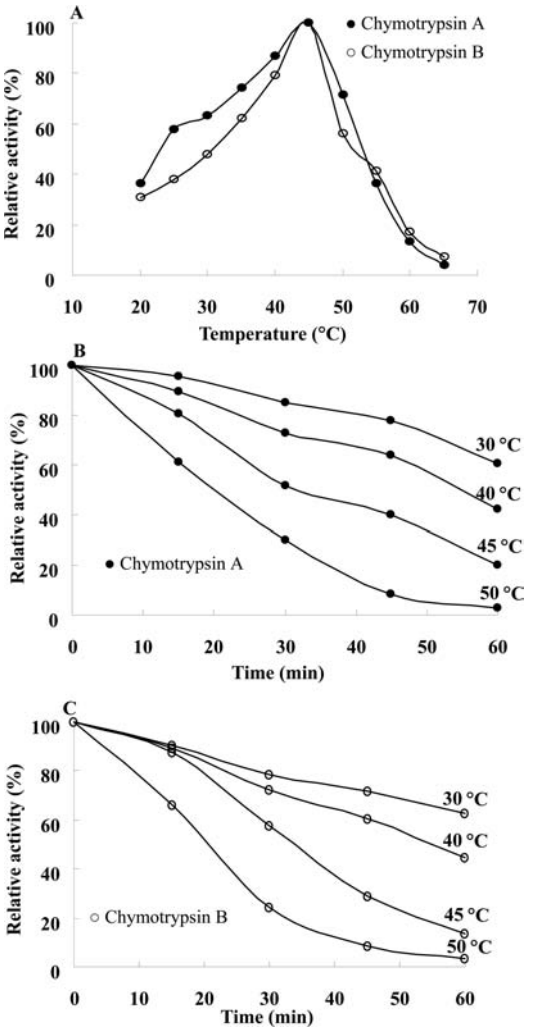

Figure 5. Effect of temperature on chymotrypsins A and B: (**A**) temperature profiles; (**B**) thermal stability of chymotrypsin A; (**C**) thermal stability of chymotrypsin B.

Table 2. Effects of Various Proteinase Inhibitors on the Activity of Chymotrypsins A and B[a]

		relative activity (%)	
inhibitor	concentration (mM)	chymotrypsin A	chymotrypsin B
none		100	100
chymostatin	0.1	0.5	0
PMSF	1	0	0
Pefabloc SC	1	23.4	41.8
	5	4.4	16.4
benzamidine	5	72.7	77.5
1,10-phenanthroline	10	87.4	91
EDTA	10	94.1	93.5
pepstatin	0.001	95.9	103
E-64	0.01	101.7	100.3

[a] Purified Japanese sea bass chymotrypsins were preincubated with different inhibitors individually at room temperature for 30 min ,and the residual activity was determined. Control tests were performed under identical conditions in the absence of inhibitors.

cysteine proteinase inhibitor E-64 scarcely showed any inhibitory effect. Metalloproteinase inhibitors EDTA and 1,10-phenanthroline slightly inhibited the activity. Combined with the substrate specificity result that these two enzymes specifically cleaved the chymotrypsin substrate Suc-Leu-Leu-Val-Tyr-MCA but did not reveal any activity to trypsin substrates such as Boc-Phe-Ser-Arg-MCA

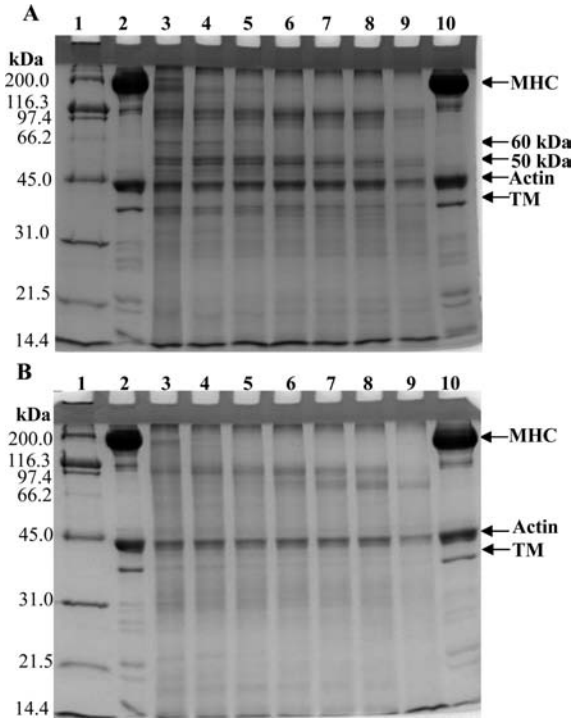

Figure 6. Effect of chymotrypsins on myofibrillar proteins of Pacific white shrimp at 20 °C. Proteinases used were chymotrypsin A (**A**) and chymotrypsin B (**B**). Lanes: 1, protein marker; 2−9, incubated for 0, 1, 10, 30, 60, 120, 240, and 360 min, respectively; 10, control. MHC, myosin heavy chain; TM, tropomyosin.

(**Figure 1**), the present result provided strong evidence that the two purified enzymes were chymotrypsins.

In our preliminary investigations, we found that in the presence of 5 mM calcium, the enzymatic activity of crude chymotrypsins (after dialysis) increased up to 50%, whereas for purified chymotrypsins, only a < 10% increase in activity was observed (data not shown). The result presented in **Table 2** reveals that in the presence of 10 mM divalent ion chelator EDTA, not much loss of enzymatic activity of the two chymotrypsins was observed. Hence, it can be assumed that chymotrypsins occurring in the impure preparations revealed an activation effect by calcium ion. Similar to trypsins from common carp (*C. carpio*) (*15*), the stability of these two chymotrypsins was also not affected by calcium. Thus, in the present study, calcium was added only in buffers of the former purification stages until DEAE-Sepharose chromatography.

The digestive effect of chymotrypsins on muscular proteins of Pacific white shrimp was also investigated. Because of the existence of serine proteinase inhibitor in the sarcoplasmic fraction, the degrading effect of water-soluble protein by chymotrypsins A and B was not observed (data not shown). For myofibrillar proteins, myosin heavy chain (MHC), actin, and tropomyosin (TM) were effectively digested by the two chymotrypsins at 20 °C in 10 min (**Figure 6**). The digestion of MHC by chymotrypsin A resulted in degradation products with sizes of approximately 50 and 60 kDa (**Figure 6A**), which was different from the digestion pattern of chymotrypsin B (**Figure 6B**). As prolonged incubation did not increase digestion rate significantly, this result indicated that as digestive proteinase, chymotrypsin should work together with other digestive enzymes such as pepsin, trypsin, and aminopeptidases to effectively degrade food proteins for further utilization by the animal.

The N-terminal amino acid sequences of the two chymotrypsins were also analyzed. As shown in **Figure 7**, the N- terminal

	10 20
Japanese sea bass chymotrypsin A	IVNGEEAVPHSWPWQVSLQDY
chymotrypsin B	IVNGEEAVPHSWPWQVSL
Japanese flounder chymotrypsin 1	IVNGETAVSGSWPWQVSLQDG
chymotrypsin 2	IVNGEEALPHSWPWQVSLQDY
Atlantic cod chymotrypsin A	IVNGEEAVPHSWSWQVSLQDQ
chymotrypsin B	IVNGEEAVPHSWPWQVSLQDS
Zebrafish chymotrypsin B_1	IVNGEEAVPHSWPWQVSLQDS
Mozambique tilapia chymotrypsin	IVNGEEAVPHSWPWQVSLQDY
Cattle chymotrypsin	IVNGEEAVPGSWPWQVSLQDK
Human chymotrypsin B_1	IVNGEDAVPGSWPWQVSLQDK
chymotrypsin B_2	IVNGEDAVPGSWPWQVSLQDK

Figure 7. Alignment of N-terminal amino acid sequences of chymotrypsins from different animals. The sequences of Japanese sea bass chymotrypsins were compared with those of Japanese flounder (*26*), Atlantic cod (*28*), zebrafish, Mozambique tilapia (*25*), cattle, and human (*27*).

amino acid sequences of chymotrypsins A and B were determined to the 21st and 18th residues, respectively, and they were completely identical. The two Japanese sea bass chymotrypsins revealed identical sequence to chymotrypsin from Mozambique tilapia (*25*). Higher identities (81.0−95.2%) to Japanese flounder chymotrypsins 1 and 2 (*26*), cattle chymotrypsin B_1 (GenBank XM_608091), and human chymotrypsins B_1 and B_2 (*27*), which have the same sequence for compared to each other could also be observed. Furthermore, the first 18 amino acid residue sequences were identical to sequences of Atlantic cod chymotrypsin B (*28*) and zebrafish chymotrypsin B_1 (GenBank BC078367). It was found that N-terminal sequences of chymotrypsins showed extremely high identity to each other, especially the sequence from the 1st to the 20th residues. The Atlantic cod chymotrypsins A and B (*28*) share the same amino acid sequence until the 21st residue (**Figure 7**). Thus, it can be assumed that the two chymotrypsins may have differences starting from the 21st amino acid residue. The N-terminal sequence data further showed that the purified enzymes from the Japanese sea bass were members of the chymotrypsin family. The full-length sequences of these two chymotrypsins, however, should be determined by molecular cloning.

Kinetic constants, K_m and k_{cat}, of the two chymotrypsins for hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA were determined according to Lineweaver−Burk plots (**Table 3**). At 37 °C, the K_m value of chymotrypsin A was 0.8 μM and that of chymotrypsin B was 1.1 μM; the k_{cat} value of chymotrypsin A was 2.7 s^{-1} and that of chymotrypsin B was 2.0 s^{-1}. At 25 °C, the K_m value of chymotrypsin A was 0.7 μM and that of chymotrypsin B was 1.2 μM; the k_{cat} value of chymotrypsin A was 1.8 s^{-1} and that of chymotrypsin B was 1.3 s^{-1}. For turnover number (k_{cat}), chymotrypsin A had a higher value as well as a lower K_m value than did chymotrypsin B, and the catalytic efficiency (k_{cat}/K_m) value of chymotrypsin A was also higher (1.6- and 1.3-fold, respectively at 37 and 25 °C) than that of chymotrypsin B. Compared with the K_m, k_{cat}, and k_{cat}/K_m of bovine α-chymotrypsin (*13*), which were measured using the same substrate at the same temperature, both Japanese sea bass chymotrypsins revealed relatively higher catalytic efficiency (k_{cat}/K_m) (2.7- and 1.7-fold at 37 °C; 3.7- and 1.6-fold at 25 °C, respectively) (**Table 3**). All of the above results suggested that fish chymotrypsins are relatively more efficient in hydrolyzing not only synthetic fluorogenic peptide substrate in vitro but, most importantly, native protein substrates physiologically than mammalian ones.

In the present study, two chymotrypsins from the hepatopancreas of Japanese sea bass were purified to homogeneity. Both enzymes showed maximal activity at pH 8.0 and 45 °C. Although the molecular masses of the two chymotrypsins were quite similar

Table 3. Kinetic Properties of Japanese Sea Bass Chymotrypsins for the Hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA

temperature (°C)	source	enzyme	K_m[a] (μM)	k_{cat}[a] (s^{-1})	k_{cat}/K_m[a] (s^{-1} μM^{-1})	ratio[b]
37	Japanese sea bass	chymotrypsin A	0.8 ± 0.06	2.7 ± 0.09	3.0 ± 0.07	2.7
		chymotrypsin B	1.1 ± 0.03	2.0 ± 0.06	1.9 ± 0.10	1.7
	crucian carp	chymotrypsin A	1.4	2.7	1.9	1.7
		chymotrypsin B	0.5	3.4	6.8	6.2
	bovine	chymotrypsin	0.8	0.9	1.1	1.0
25	Japanese sea bass	chymotrypsin A	0.7 ± 0.02	1.8 ± 0.02	2.6 ± 0.05	3.7
		chymotrypsin B	1.2 ± 0.03	1.3 ± 0.04	1.1 ± 0.01	1.6
	bovine	chymotrypsin	1.3 ± 0.08	0.9 ± 0.04	0.7 ± 0.01	1.0

[a] Mean ± SD from three independent determinations. [b] Ratio: the catalytic efficiency (k_{cat}/K_m) divided by that of bovine chymotrypsin. The K_m and k_{cat} values were determined at pH 7.5.

(they have the same N-terminal amino acid sequences), several lines of evidence indicated that these two chymotrypsins were actually different: (1) The two chymotrypsins revealed different binding capacities to the ion-exchange column of DEAE-Sepharose. Chymotrypsin A came from the unadsorbed portion, whereas chymotrypsin B was from the adsorbed portion. (2) The isoelectronic points of the two chymotrypsins on 2D-PAGE were different, which was further confirmed by their mobilities on native-PAGE, suggesting their surface charges are different under the same electrophoresis conditions. (3) Their immunological reaction to anti-crucian carp chymotrypsin B suggested that these two enzymes have different degrees of homology. (4) The degrading effect of chymotrypsins on myofibrillar proteins of Pacific white shrimp also revealed some difference. Therefore, chymotrypsins A and B are not identical, and these differences may contribute to the genetic evolution of chymotrypsins. In addition, chymotrypsins exhibited higher catalytic efficiency at low temperature than bovine α-chymotrypsin. These characteristics suggested their potential application value in conditions of low processing temperature and higher enzymatic activity (*2*, *4*).

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