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Isolation of cathepsin B from the muscle of silver carp (*Hypophthalmichthys molitrix*) and comparison of cathepsins B and L actions on surimi gel softening

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Abstract

Cathepsin B from silver carp muscle was purified to 263-fold by acid treatment, ammonium sulfate fractionation, followed by a series of chromatographic separations. The molecular mass of the purified enzyme was 29 kDa as determined by SDS-PAGE and immunoblotting. The purified enzyme was activated by dithiothreitol and cysteine while it was substantially inhibited by E-64, suggesting the purified enzyme belongs to the cysteine proteinase family. Optimal pH and temperature were 5.5 and 35 °C, respectively. The enzyme catalyzed the hydrolysis of *Z*-Arg-Arg-MCA with a parameter of K_m (90 μM) and K_{cat} (20.3 s^{-1}), but hardly hydrolyzed Arg-MCA. Analysis of surimi gel strength and microstructure showed that cathepsins B and L were capable of destroying the network structure of silver carp surimi gels, consequently causing gel softening. Cathepsin L might play an important role in the modori effect.

Keywords: Cathepsin B; Silver carp; Purification; Surimi; Gel softening

1. Introduction

Cathepsin B (EC 3.4.22.1) is a typical cysteine proteinase found in lysosomes and, together with cathepsin L and cathepsin H, is considered to have major biological roles in the degradation of both intracellular and extracellular proteins during catabolism (Aranishi, Ogata, Hara, Osatomi, & Ishihara, 1997). The enzyme is synthesized as a latent precursor, which is subsequently converted into the mature single- and/or two-chain forms by biological reactions such as acidification (Mach, Mort, & Glossl, 1994), action of other proteolytic enzymes (Kawabata, Nishimura, Higaki, & Kato, 1993), and auto-activation by cathepsin B itself (Mach et al., 1993). In some fish species, cathepsin B has been isolated from the muscle in the pure state (Jiang, Lee, & Chen, 1994; Sherekar, Gore, & Ninjoor, 1988; Yamashita & Konagaya, 1990). Since this enzyme has the ability to degrade surimi proteins at weak acidic or neutral pH and at temperature from 50 °C to 60 °C, similar to the condition of modori, cathepsin B might take part in the gel softening effect observed during surimi gelation (An, Weerasinghe, Seymour, & Morrissey, 1994; Aoki & Ueno, 1997; Jiang, Lee, Tsao, & Lee, 1997; Ramos-Martinez, Morales, Ramirez, Garcia, & Montejano, 1999; Yamashita & Konagaya, 1990).

Silver carp (*Hypophthalmichthys molitrix*), which is one of the fresh-water fish with the highest annual harvest both in China and India, is well accepted for human consumption due to its attractive white color. It has been processed in many forms including fillet and surimi products. The fillet production can cause a waste of meat because the edible portion has a high amount of fish-bone (Ramirez, Santos, Morales, Morrisey, & Vazquez, 2000). In contrast, surimi products have been considered to be promising due to its more efficiently utilization of silver carp resources.

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However, silver carp surimi exhibited considerable gel softening (modori) in the course of heating (Luo, Kuwahara, Kaneniwa, Murata, & Yokoyama, 2001), resulting in a decrease in textural quality, eventually having a negative effect on acceptance and price of the surimi products (Toyohara & Shimizu, 1988). It has been shown that the endogenous heat-stable cathepsins B and L in some fish muscle lysosomes could be involved in gel softening during surimi gelation (An et al., 1994; Ho, Chen, & Jiang, 2000; Jiang et al., 1997). In our previous work, we have elucidated the involvement of purified cathepsin L in degradation of silver carp surimi protein (Liu, Yin, Zhang, Li, & Ma, 2006), but little information of cathepsin B in silver carp was available. Accordingly, the objectives of this work were to purify and characterize endogenous heat-stable cathepsin B from the muscle of silver carp, and to compare the role of the purified cathepsins B and L in gel softening of silver carp surimi.

2. Materials and methods

2.1. Materials

Cultured live silver carp (*Hypophthalmichthys molitrix,* 900–1200 g/fish) were obtained from a local fishery market near the campus of China Agricultural University in autumn. The fish were sacrificed after purchasing, then iced and immediately transported to our lab for experiments.

2.2. Chemicals

DEAE-Sephacel, Sephacryl S-100, SP Sepharose Fast Flow and Blue Sepharose Fast Flow were purchased from Amersham Biosciences (Uppsala, Sweden). Benzyloxycarbonyl-arginylarginine-4-methyl-7-coumarylamide (*Z*-Arg-Arg-MCA), benzyloxycarbonyl-L-phenylalany-L-arginyl-4-methyl-7-coumarylamide (*Z*-Phe-Arg-MCA), L-arginine-4-methyl-7-coumarylamide (Arg-MCA), and molecular weight marker (SDS-7) were purchased from Sigma Chemical Co. (St Louis, USA). Anti-human cathepsin B was purchased from Merck Bioscience (Darmstadt, Germany). All other chemicals were of reagent grade.

2.3. Assay of enzyme and protein

Enzyme activity was determined as described previously (Liu et al., 2006). Briefly, *Z*-Arg-Arg-MCA and *Z*-Phe-Arg-MCA were used as substrates for cathepsin B and cathepsin L, respectively. The enzyme and substrate were mixed, and then allowed to stand at 40 °C for 10 min. Subsequently, 1 mL of stopping solution consisting of 0.1 M acetate and 0.1 M sodium chloroacetate buffer (pH 4.3) was added to the mixture for terminating the hydrolysis. A control tube was prepared in parallel to each test sample, but the stopping solution was added before the addition of enzyme solution. The relative fluorescence intensity of aminomethylcoumarin (AMC) liberated by hydrolysis was measured in a spectrofluorometer (LS55, Perkin Elmer, USA) with 380 nm of excitation wavelength and 460 nm of emission wavelength. One unit of enzyme activity was defined as the amount of activity that released 1 nmol of AMC per min. Three replications were taken for enzyme activity measurements. Protein concentration was determined according to the method of Lowry, Rosebrough, Farr, & Randall (1951) with bovine serum albumin (BSA) as standard.

2.4. Purification of cathepsin B1 from silver carp muscle

All stages were carried out at 4 °C, and the activities of cathepsin B were detected by measurement of *Z*-Arg-Arg-MCA hydrolysis. Silver carp muscle was minced, homogenized at 12,000 rpm for 5 min in a homogenizer (DS-1, SpecimenModel, Shanghai Co. Ltd., China) with 4 volumes (v/w) of 25 mM sodium acetate buffer containing 5 mM cysteine and 0.3 mM phenylmethylsulfonyl fluoride (PMSF), pH 5.0 (buffer A), and then centrifuged at 12,000 g for 20 min. The supernatant was collected as the crude extract and acidified to pH 3.0 with 1 M HCl. After incubating the acidified extract at 30 °C for 10 min, the aqueous portion was adjusted to pH 6.0 with 1 M NaOH, and then centrifuged at 12,000 g for 20 min. The supernatant was fractionated with ammonium sulfate at 80% saturation. The precipitate collected by centrifugation was dissolved in 20 mM phosphate buffer containing 5 mM cysteine, pH 6.0 (buffer B) and dialyzed against the same buffer. The resulting solution was concentrated to 10 mL by an Amicon Membrane YM-10, and then was applied to a DEAE-Sephacel column (2.6 × 20 cm) equilibrated with buffer B and eluted with a linear gradient of NaCl from 0 to 1 M in the same buffer, and the flow rate as 0.5 mL/min. Every fraction contained 2 mL eluate. After 500 min elution, the active eluate from the DEAE-Sephacel column was concentrated again and loaded to a Sephacryl S-100 column (2.6 × 90 cm) equilibrated with buffer B containing 0.2 M sodium chloride (buffer C), and the flow rate as 0.5 mL/min. Each fraction contained 2 mL eluate. After 1000 min elution, a peak around 400 min was collected and dialyzed against 50 mM sodium acetate buffer containing 5 mM cysteine, pH 4.5 (buffer D). The resulting dialyzate was subjected to a column of SP Sepharose Fast Flow (1.6 × 12 cm) equilibrated with the same buffer and eluted with a linear gradient of NaCl from 0 to 1 M in the same buffer, and the flow rate was 0.5 mL/min. Each fraction contained 1 mL eluate. After 350 min elution, the active eluate was collected and dialyzed against 50 mM sodium acetate buffer containing 5 mM cysteine, pH 5.5 (buffer E). The dialyzate was finally applied to a column of Blue Sepharose Fast Flow (1.0 × 8 cm) equilibrated with the same buffer and eluted with a linear gradient of NaCl from 0 to 1 M in the same buffer, and the flow rate was 0.15 mL/min. Each fraction contained 0.8 mL eluate. After 550 min elution, the resulting sample was collected, concentrated and stored at −80 °C as purified proteinase.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

SDS-PAGE with an 11% acrylamide gel was carried out in a Mini PROTEIN 3 Cell (Bio-Rad, USA) as described previously (Laemmli, 1970). After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250. Immunoblotting was carried out as described previously (Towbin, Staehelin, & Gordon, 1979). Briefly, the purified proteinase was subjected to SDS-PAGE followed by electrophoretic transfer to a nitrocellulose membrane. Nonspecific protein sites were blocked with 5% nonfat milk in Tris–HCl buffered saline (TBS = 20 mM Tris–HCl, pH 7.5, containing 0.145 M NaCl). The membrane was incubated with primary antibody at room temperature for 2 h and washed with TBST (TBS, 0.05% Tween-20). After 1 h incubation with horseradish peroxidase labeled secondary antibody, the membrane was extensively washed with TBST. The immunodetection was carried out using a detection kit from Bio-Rad (Richmond, CA).

2.6. pH and temperature profiles of purified enzyme

Purified proteinase was assayed in the pH range of 3.0–8.0 using McIlvaine's buffer (0.2 M sodium phosphate and 0.1 M citrate), containing 8 mM DTT at 40 °C for 10 min. The determination of temperature activity of purified proteinase was carried out in the temperature range from 20 °C to 80 °C using 0.4 M phosphate buffer, containing 8 mM DTT, pH 6.0. The residual activity using *Z*-Arg-Arg-MCA as a substrate was determined as described above.

2.7. Effects of inhibitors and reductants on the activity of cathepsin B1

Purified enzymes in 0.4 M phosphate buffer (pH 6.0) were, respectively, incubated with *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), PMSF, Pepstatin A, ethylenediaminetetraaceticacid (EDTA), dithiothreitol (DTT), L-cysteine (L-Cys) and β-mercaptoethanol (β-Me) at 40 °C for 10 min. The remaining activity of the samples was measured as described by using *Z*-Arg-Arg-MCA as a substrate.

2.8. Substrate specificity and kinetic studies

Kinetic studies of the purified cathepsin B1 was carried out as reported previously (Visessanguan, Benjakul, & An, 2003) with a final concentration of *Z*-Arg-Arg-MCA varying from 5 to 80 μM. The hydrolytic activities on synthetic substrates *Z*-Phe-Arg-MCA and Arg-MCA were determined by the same method under the same conditions as those of the *Z*-Arg-Arg-MCA hydrolyzing activity. The released AMC was measured according to the method described above.

2.9. Effect of cathepsins on surimi protein

Cathepsin L and frozen surimi were all prepared according to our previous reports (Liu et al., 2006). Frozen surimi was thawed at 4 °C until the internal temperature reached −3 °C. The changes in surimi protein were detected according to the method of Nishimoro et al. (1987) with some modifications. Cathepsins B or L (5 units/g surimi) were added to thawed surimi under stirring. As a control sample, equal volumes of water were added to the thawed surimi. After 2 h incubation at 40 °C or 65 °C, 0.5 g surimi with or without cathepsins were dissolved in 3 mL of 20 mM Tris–HCl buffer, pH 8.0, containing 2.0% SDS, 8.0 M urea, 2.0% β-Me, and then heated at 100 °C for 3 min. Resulting samples were analyzed using SDS-PAGE.

2.10. Gel property measurement and microstructure observations

The surimi gel was prepared according to our previous method (Liu et al., 2006). The gel property was determined as described previously (Luo et al., 2001). The surimi gels were cut into 3-cm-high cylindrical specimens. The gel properties were measured by a rheometer (EZTest, Shimadazu, Japan) using a round plunger (diameter 5 mm) and expressed as breaking force (*g*), deformation (cm), and gel strength (*g* × cm). The speed of the round plunger was maintained in a down direction at a rate of 6 cm/min, the distance of compression was 5 cm. Six replications were used for gel property measurements. The microstructure of the gel was observed using a scanning electron microscope (SEM) (S-570, Hitachi Co., Japan). It was carried out as described previously (Liu et al., 2006).

2.11. Statistical analysis

The results are expressed as mean ± SEM. Analysis of variance (ANOVA) was performed and mean comparison were run by Duncan's multiple-range test (SPSS 10.0 for windows, SPSS Inc, Chicago, IL). A significance level of $p < 0.05$ was chosen for all statistical analyses.

3. Results and discussion

3.1. Purification of cathepsin B1

Cathepsin B1 was purified by acid treatments, ammonium sulfate fractionation, and a series of chromatographic separations, which are shown in Figs. 1 and 2. A typical purification procedure is summarized in Table 1. About 7.0 mg pure cathepsin B1 was obtained from 1000 g of silver carp muscle. The purity of the final preparation increased about 263-fold as compared to that of crude extract. Purification recovery of carp hepatopancreas cathepsin B with a 17% yield has been reported (Aranishi, Hara, Osatomi, & Ishihara, 1997) while present yield is only 1.1%. The different purification recovery might be

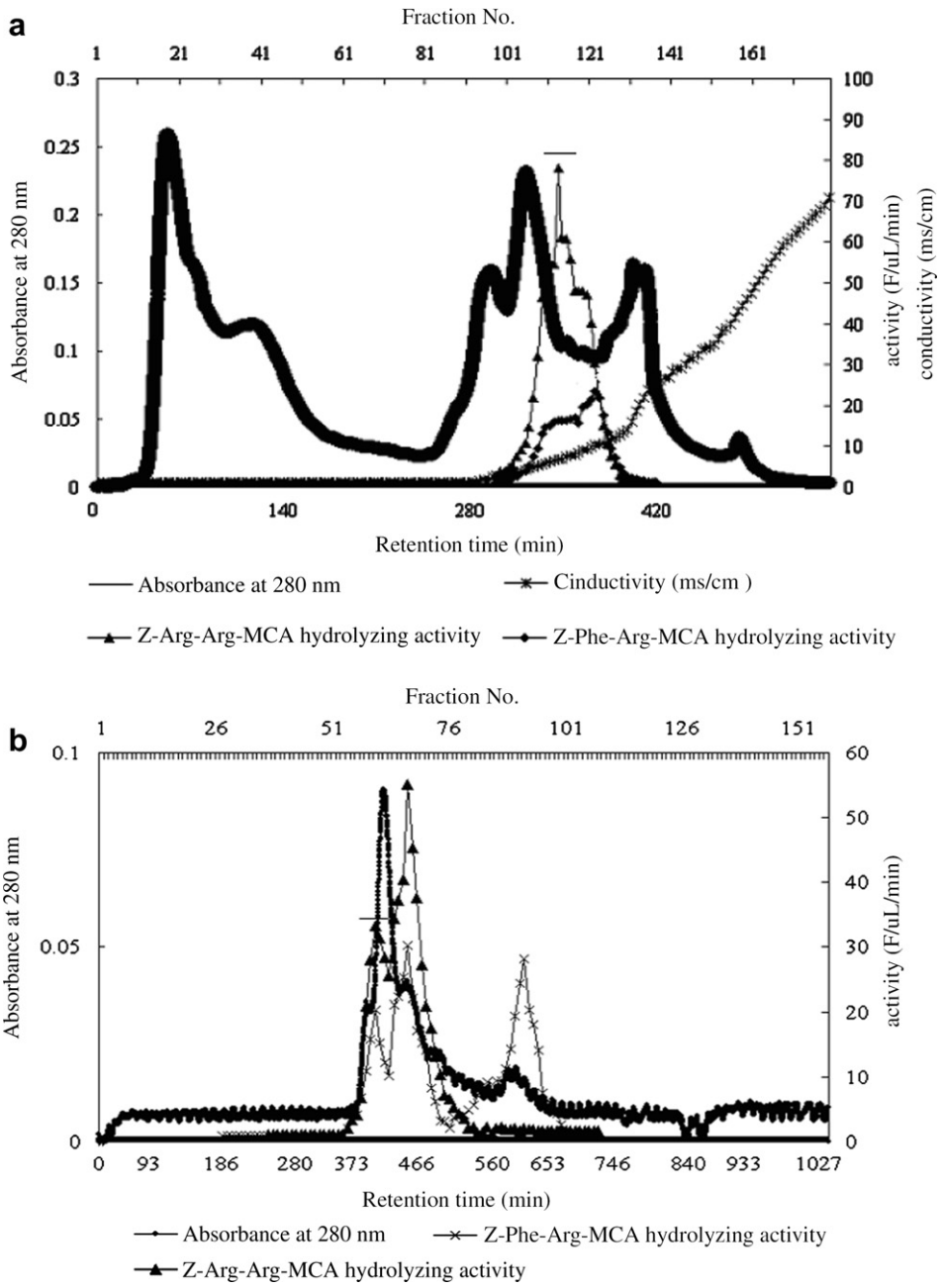

Fig. 1. (a) DEAE-Sephacel ionic exchange chromatography (2.6 × 20 cm) of the ammonium sulfate fraction. (b) Sephacryl S-100 gel filtration chromatography (2.6 × 90 cm) of the fraction obtained by preceding chromatography. The fractions indicated by a bar were collected for further purification.

due to different enzyme resources, for example, the levels of cathepsin B in the soluble extracts of skeletal muscle were significantly lower than that of viscera (Kominami, Tsukahara, Hara, & Katumuma, 1985). Another possibility is that different affinity chromatography was used in the present study (Blue-Sepharose affinity chromatography) from previous report showing that cathepsin B has a slightly stronger affinity toward semicarbazone-Sepharose under the respective experimental conditions (Rich, Brown, & Barrett, 1986). In addition, it is not excluded that the difference in purification recovery may be due to different experimental conditions. Moreover, we just chose a relatively narrow bar compared to the activity peak during collection, which might also cause the low yield finally.

As shown in Fig. 1b, cathepsins B and L were eluted at the retention time of 400 and 600 min, respectively, indicating that they were effectively separated by Sephacryl S-100 column chromatography, leading to a 78.2-fold increase in protein purity. Godiksen and Nielsen (2007) showed a new method to discriminate between cathepsin B and cathepsin L in crude extracts from fish muscle based on a simple acidification procedure. But our result indicated that acid treatment could improve the purification fold of cathepsin B, which was same to the results of Aranishi et al. (1997),

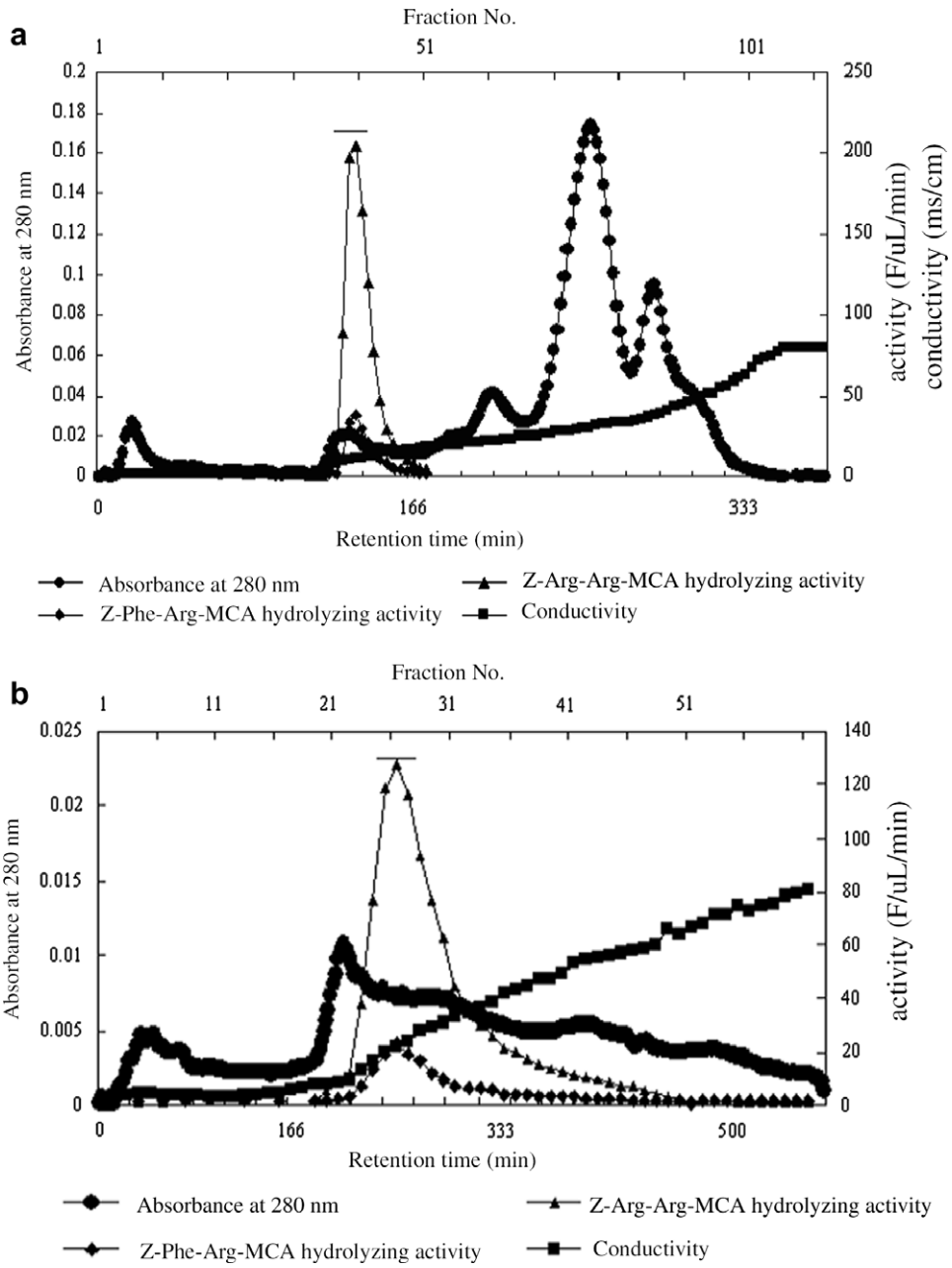

Fig. 2. (a) SP Sepharose Fast Flow cation exchange chromatography (1.6 × 12 cm) of the fraction obtained by preceding chromatography. (b) Blue Sepharose affinity chromatography (1.0 × 8 cm) of the fraction obtained by preceding chromatography. The fractions indicated by a bar were collected.

Table 1
Purification of cathepsin B1 from the muscle of silver carp

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification fold	Yield (%)
Crude extract	133746.7	21934.8	0.16	1.0	100.00
Acid treatment	36560.0	11761.0	0.32	2.0	53.62
$(NH4)_2SO_4$ 80%	4028.9	4768.5	1.18	7.2	21.74
Concentration	3123.5	4628.4	1.48	9.1	21.10
DEAE Sephacel	346.9	1725.4	4.97	30.3	7.87
Sephacryl S-100	43.7	558.5	12.82	78.2	2.55
SP Sepharose Fast Flow	8.6	286.3	33.12	203.3	1.31
Blue Sepharose Fast Flow	7.0	240.0	42.86	263.0	1.10

Yamashita and Konagaya (1990). The different results may come from the difference in fish species (Godiksen and Nielsen, 2007). Fig. 1b also showed that there were two peaks of cathepsin B obtained by Sephacryl S-100 column chromatography. The first cathepsin B peak eluted at 400 min was referred to cathepsin B1; another peak appearing at 470 min was assigned to cathepsin B2. Cathepsins B1 and B2 were named according to their eluted sequence by Sephacryl S-100 column chromatography. It has been reported that multiple forms of cathepsin B exist in fish muscle such as procathepsin B, the intermediate form, and the mature form. All three forms have the ability to hydrolyze *Z*-Arg-Arg-MCA (Aoki, Yokono, & Ueno, 2002). The precursor and intermediate forms could be converted into the mature forms by acidification (Mach et al., 1994) or action of other proteolytic enzymes (Kawabata et al., 1993) or auto-activation by cathepsin B itself (Mach et al., 1993). Therefore, it was possible that the two apparent forms of silver carp cathepsin B were derived from a latent precursor, but have different intermediate forms. We considered that an extensive investigation of cathepsin B2 is also necessary.

SDS-PAGE and immunoblotting analysis exhibited a single band with an apparent $M_r = 29$ kDa, indicating that cathepsin B1 was purified to homogeneity, which contained one kind of subunit (Fig. 3). Our result was nearly the same as that of cathepsin B from mackerel (28 kDa) (Jiang et al., 1994), carp (29 kDa) (Hara, Suzumatsu, & Ishihara, 1988), and salmon (28 kDa) (Yamashita & Konagaya, 1990). Cathepsin B existed as multiple forms including procathepsin B, intermediate, and mature form. The previous report indicated that mackerel procathepsin B (60 kDa) was converted to cathepsin B (23 kDa), by way of intermediate forms of 40 kDa and 38 kDa (Aoki et al., 2002). Our data showed that the molecular mass of purified cathepsin B1 was approximately 29 kDa, which was much lower than that of procathepsin B or intermediate enzyme forms. Accordingly, it is suggested that the purified cathepsin B1 might be the mature cathepsin B.

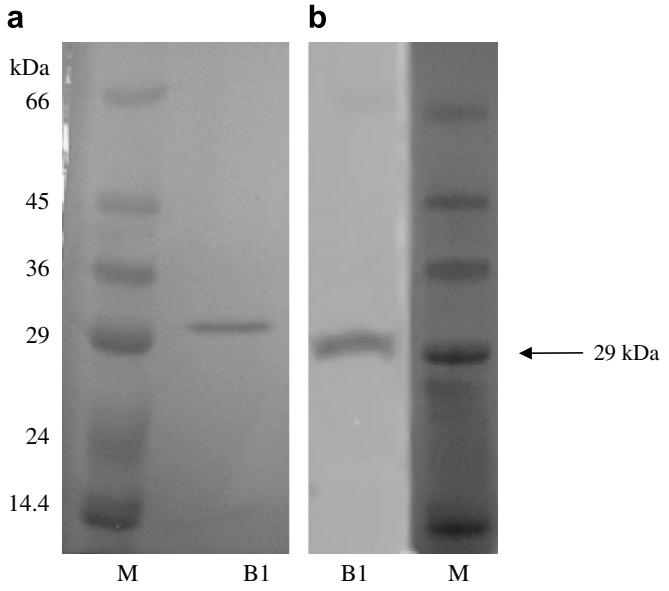

Fig. 3. SDS-PAGE (a) and immunoblotting (b) pattern of purified silver carp cathepsin B1. M, standard of molecular weight proteins; B, the purified cathepsin B1.

3.2. pH and temperature profiles of purified cathepsin B1

Fig. 4a showed that the enzyme was most active around pH 5.5 for the hydrolysis of *Z*-Arg-Arg-MCA at 40 °C, a result similar to that of mackerel (*S. japonicus*) cathepsin B (5.5) (Matsumiya, Mochizuki, & Otake, 1989), but was more acidic than that from mackerel (*S. australasicus*) (6.5) (Jiang et al., 1994), tilapia (6.0) (Sherekar et al., 1988), and carp muscle (6.0) (Hara et al., 1988).

It is recognized that setting is an indispensable processing step in the production of surimi, which is carried out below 40 °C. In present study, the maximal activity of cathepsin B1 was observed at 35 °C (Fig. 4b), being close to the temperature of setting. In addition, our previous work found that the myosin heavy chain (MHC) could be significantly hydrolyzed by silver carp cathepsin B at 35 °C (Liu, Yin, Zhang, Li, & Ma, 2008). The enzyme activities at different temperatures in this study suggested that cathepsin B might take part in the proteolysis of silver carp surimi protein at setting temperatures.

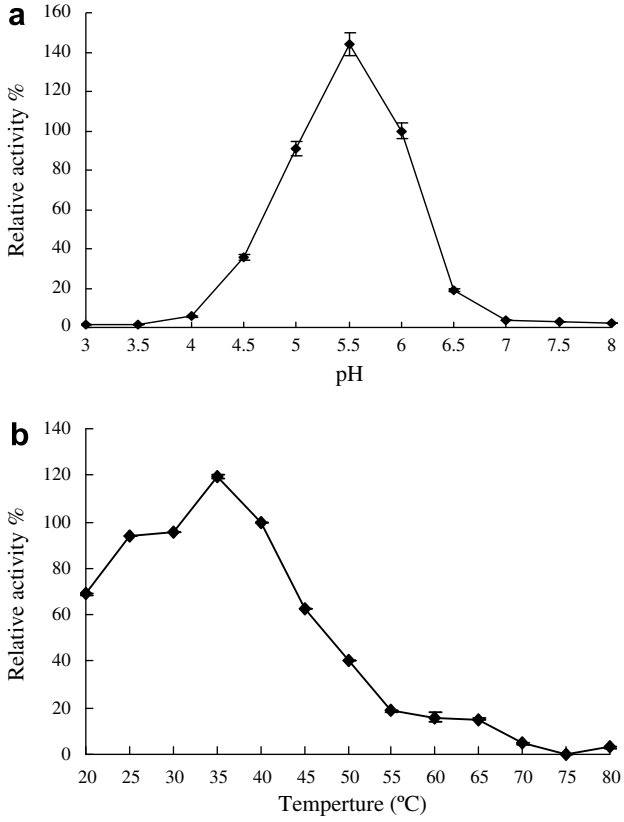

Fig. 4. Effect of pH (a) and temperature (b) on the activity of purified silver carp cathepsin B1. $n = 3$.

3.3. Effects of inhibitors and reductants on the activity of cathepsin B1

The effects of inhibitors and reductants on the activities of cathepsin B1 are presented in Table 2. The activity of cathepsin B1 was completely inhibited in the presence of thiol-blocking agent (E-64) while thiol-activating agents such as DTT, Cys and EDTA could enhance the activity. These results suggested that the purified proteinase was a thiol proteinase. However, the activity of cathepsin B1 was partially inhibited by Pepstatin A, an aspartic-proteinase inhibitor, implying the active center of cathepsin B1 might contain Asp-residue(s).

3.4. Substrate specificity and kinetic studies

Substrate specificity of the purified proteinase was examined using synthetic substrate for cathepsin B, cathepsin L and cathepsin H (Table 3). Results indicated that cathepsin B1 significantly hydrolyzed *Z*-Arg-Arg-MCA and *Z*-Phe-Arg-MCA, which was identical with the previous result made by Aranishi et al. (1997) showing that cathepsin B could hydrolyze the substrates that have an arginine residue at P1 position and a basic or aromatic residue at P2 position. Moreover, it has been reported that *Z*-Phe-Arg-MCA can be degraded by both cathepsins L and B while cathepsin L could hardly degrade *Z*-Arg-Arg-MCA that was the substrate of cathepsin B (Barrett & Kirschke, 1981). Since the pure cathepsin B1 was not able to catalyze the hydrolysis of Arg-MCA (a substrate for cathepsin H), it is not difficult to distinguish between silver carp cathepsins B and H by using the appropriate degradable MCA substrate.

Kinetic constants for hydrolysis of *Z*-Arg-Arg-MCA and *Z*-Phe-Arg-MCA were determined for the purified cathepsin B1 (Table 4). Cathepsin B1 had a stronger affinity to *Z*-Arg-Arg-MCA than *Z*-Phe-Arg-MCA based on their K_m, 90 μM for *Z*-Arg-Arg-MCA versus 150.6 μM for *Z*-Phe-Arg-MCA. In contrast, the K_{cat} value for *Z*-Arg-Arg-MCA was much lower than that for *Z*-Phe-Arg-MCA hydrolysis. The K_{cat}/K_m value consequently indicated that *Z*-Phe-Arg-MCA was the preferable substrate for assay of the enzyme activity, agreeing with the observations by other groups (Aranishi et al., 1997; Jiang et al., 1994; Yamashita & Konagaya, 1990). However, this substrate is unsuitable for the detection of crude solutions involving cathepsins B and L because *Z*-Phe-Arg-MCA is also preferentially degraded by cathepsin L (An, Margo, Thomas, & Michael, 1995; Barrett & Kirschke, 1981; Nishimoro et al., 1987; Yamashita & Konagaya, 1990). Therefore, we choose *Z*-Arg-Arg-MCA rather than *Z*-Phe-Arg-MCA as the substrate of cathepsin B in this study.

3.5. Effect of cathepsins B and L on surimi protein

Changes in surimi proteins were detected by SDS-PAGE analysis (Fig. 5). As showed in the electrophoretic profile, the degree of surimi protein degradation by cathepsins was much larger than that in controls, suggesting that surimi protein was susceptible to cathepsin hydrolysis. Electrophoresis result also indicated that cathepsin L induced a greater degree of proteolysis than did cathepsin B (Fig. 5). In agreement with the present result, previous data showed that cathepsin L had stronger ability to hydrolyze myofibrillar proteins than cathepsin B (Yamashita & Konagaya, 1991a; 1991b). Moreover, Fig. 5 indicated that the proteolytic rate at 65 °C was much faster than that at 40 °C. The previous report showed that silver carp surimi exhibited considerable gel softening (modori) in the course of heating, especially at 50–70 °C (Luo et al., 2001). Judging from the above results, we conclude that cathepsins B and L can participate in the silver carp gel softening, and cathepsin L might play a more important role in surimi protein degradation than cathepsin B.

3.6. Gel property measurement and microstructure observations

The internal structures of gels, with or without cathepsins, were observed with SEM at a magnification of 1500 times (Fig. 6). Surimi gel C (without enzyme) had a dense structure with little space due to protein aggregation. However, the network structure of surimi gels L (with cathepsin

Table 2
Effect of inhibitors and activators on the activity of cathepsin B1 from the muscle of silver carp

Inhibitors and activators	Concentration (mM)	Relative activity (%)
Control	–	100
E-64	1	0
PMSF	1	102
Pepstatin A	1 mg/mL	75
EDTA + DTT	1 + 2	200
DTT	2	124
Cys	2	104
β-Me	2	100

Table 3
The substrate specificity of cathepsin B1 from the muscle of silver carp

Substrate	Relative activity (%)
Z-Arg-Arg-MCA	100
Z-Phe-Arg-MCA	112
Arg-MCA	5

Table 4
The kinetic constants for the hydrolysis of peptide methylcoumarylamide substrates by purified cathepsin B1 from the muscle of silver carp

Substrate	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} $mmol^{-1}$ L)
Z-Arg-Arg-MCA	90.0 ± 2.4	20.3 ± 3.8	226
Z-Phe-Arg-MCA	150.6 ± 1.0	80.2 ± 0.7	533

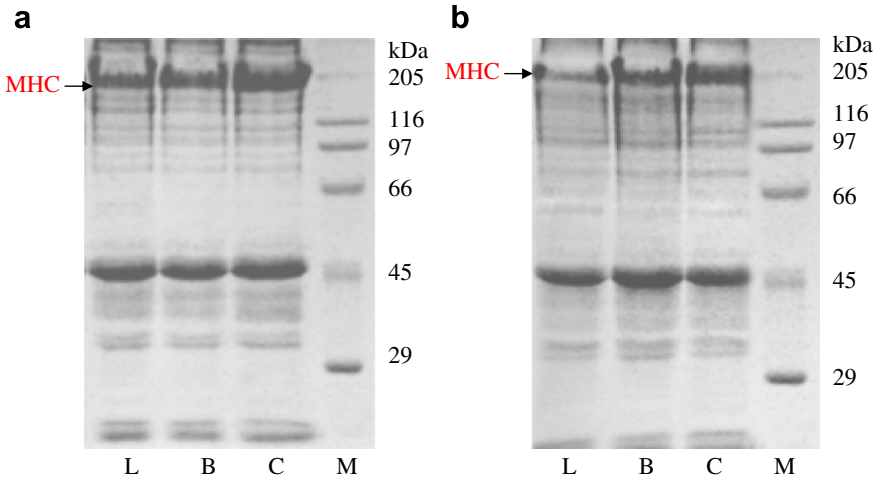

Fig. 5. Changes in SDS-PAGE patterns of silver carp surimi protein. L, incubated with purified cathepsin L; B, incubated with purified cathepsin B; C, incubated without enzyme; M, standard of molecular weights proteins; MHC, myosin heavy chain; a, incubated at 40 °C; b, incubated at 65 °C.

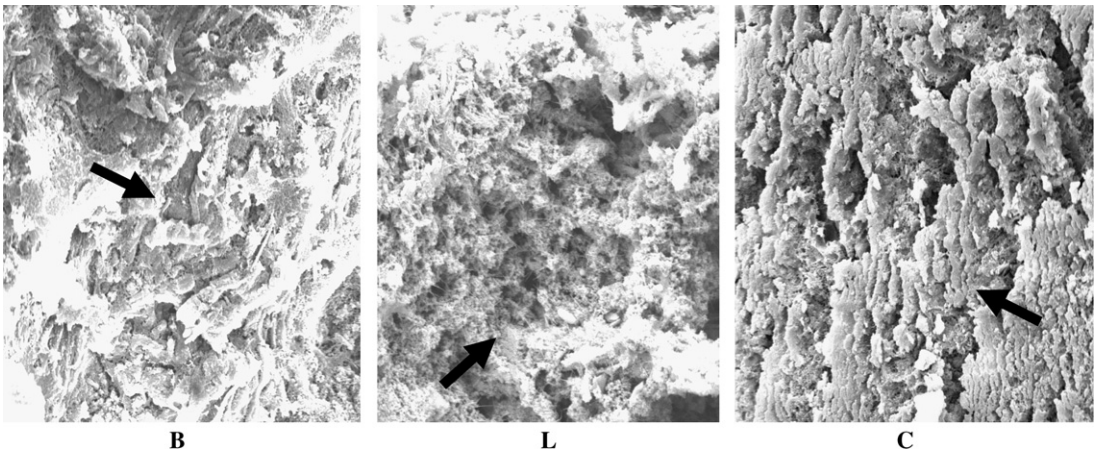

Fig. 6. Gel microstructure of silver carp surimi. B, observed with pure cathepsin B; L, observed with pure cathepsin L; C, observed without enzyme.

L) and B (with cathepsin B) were pronouncedly destroyed and a large number of pores were distributed on the gel surface. This indicates that the surimi proteins were hydrolyzed to a large extent by cathepsins. These results further support the above conclusion that cathepsins B and L have the ability to destroy network structure of the surimi gel, resulting in a quality decrease of silver carp surimi gel.

The gel properties of silver carp surimi gels, with or without cathepsins, are shown in Table 5. Gel strength, breaking force and deformation were significantly decreased in the groups treated by enzyme. Notably, the strength of the cathepsin L-added gel reduced by about 38% as compared to the control while the cathepsin B-added group decreased by 25%; breaking force and deformation decreased by 25% and 18% in L-added group respectively, compared to that of control. This also suggests cathepsin L has a greater capacity to hydrolyze the structure of surimi gel. Consistent with this result, Fig. 6 shows that gel L matrix had a lower uniformity of the protein dispersion than gel B did. Consequently, our results provided direct evidence that cathepsins B and L could induce the softening of fish gels. Since the degree of gel degradation was more remarkable with cathepsin L than cathepsin B, cathepsin L likely plays a more important role in gel softening.

Table 5
Effects of cathepsins B and L on the gel property of silver carp surimi gel ($n = 6$)

Treatment	Breaking force (g)	Deformation (cm)	Gel strength ($g \times$ cm)
Control	596.6 ± 24.0^{a}	0.97 ± 0.04^{a}	578.7 ± 27.0^{a}
Cathepsin B	512.8 ± 10.9^{b}	0.85 ± 0.02^{b}	435.7 ± 10.4^{b}
Cathepsin L	450.6 ± 14.8^{c}	0.80 ± 0.01^{b}	360.5 ± 12.2^{c}

Values in same column with different letters are significantly different ($p < 0.05$).

4. Conclusion

Cathepsin B from silver carp muscle was purified to 263-fold by a series of separations, and the molecular mass of

the purified enzyme was 29 kDa. Both of silver carp cathepsins B and L could take part in surimi gel softening of silver carp, but cathepsin L makes more contribution to the fish gel softening than cathepsin B.

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