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Purification and Characterization of Cathepsin L from the Muscle of Silver Carp (*Hypophthalmichthys molitrix*)

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Cathepsin L in silver carp musle was purified to 48.4-fold by acid-heat treatment and ammonium sulfate fractionation, followed by a series of chromatographic separations. The molecular mass of the purified enzyme was 30 kDa determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The purified enzyme was activated by dithiothreitol and cysteine while it was substantially inhibited by E-64 and insensitive to PMSF and pepstatin A, suggesting that the purified enzyme belongs to a family of cysteine proteinase. Consistent with this conclusion, Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, and Fe^{2+} could strongly inhibit the activity of this enzyme. The optimal pH and temperature were 5.0 and 55 °C, respectively. The enzyme catalyzed the hydrolysis of Z-Phe-Arg-MCA with a parameter of K_m (8.27 μM) and K_{cat} (28.7 s^{-1}) but hardly hydrolyzed Z-Arg-Arg-MCA, Arg-MCA, and Boc-Val-Leu-Lys-MCA. The microstructure analysis by scanning electron microscopy showed that this proteinase is capable of destroying the network structure of silver carp surimi gels. The enzyme exhibited a higher hydrolytic activity on surimi protein at 65 °C than at 40 °C.

KEYWORDS: Cathepsin L; silver carp; purification; surimi; microstructure analysis

INTRODUCTION

In China, silver carp (*Hypophthalmichthys molitrix*) is one of the main fresh-water fishes with a production of 4.9 million tons in 2001, which was about 31% of the annual harvest of total culture fresh-water fish (*1*). However, large postharvest losses became a major problem due to the limited distribution sphere and storage period. As a result, its commercial value as a fresh fish is greatly compromised. Therefore, surimi products are considered promising products for the future to utilize silver carp sources effectively. However, silver carp surimi exhibited considerable gel softening (modori) in the course of heating, especially at 50 °C (*2*). Gel softening resulted in a decrease of the textural quality of surimi products, which eventually had a negative effect on the quality and price of the surimi product (*3*).

It has been shown that the endogenous heat-stable cathepsin L (EC 3.4.22.15) in some fish muscle lysosomes could be assumed as being involved in causing gel softening effect during the gelation of surimi (*4*−*8*). Cathepsin L, which is a typical cysteine proteinase found in lysosomes, has been reported to have major biological roles in the degradation of both intracellular and extracellular catabolism together with cathepsin B and cathepsin H (*9*). In some fish species, such as chum salmon (*10*), spotted mackerel (*11*), and arrowtooth flounder (*12*), cathepsin L has been purified from muscles. The optimal pH and temperatures of cathepsin L were in the ranges of 5.0−6.5 and 40−55 °C, respectively, similarly to the condition of modori, implying that this enzyme might take part in the gel softening effect during the gelation of surimi (*7*, *8*, *12*).

Both cathepsins S and L hydrolyze similar substrates (*13*), but they are different from each other in that cathepsin S is the only member of lysosomal cathepsins that can retain proteolytic activity after prolonged exposure to neutral pH (*14*). Therefore, cathepsin S might be responsible for autolysis of silver carp muscle. To clearly differentiate between cathepsin L and cathepsin S, it is necessary to clarify the biochemical characteristics of the two pure proteinases.

A scanning electron microscope (SEM) was employed to observe the texture and internal structure of meat products, such as beef, sausages, and chicken (*15*). However, it seemed to be limited on the insight of the surimi gel network structure (*16*−*18*). Little information about cathepsin L in silver carp was available. In our previous work, we elucidated the involvement of endogenous cathepsins L in the degradation of silver carp myofibrillar proteins (unpublished data). The objectives of this work were to purify and characterize the endogenous heat-stable cathepsin L from the muscle of silver carp and to investigate how the purified cathepsin L could affect the changes in silver carp surimi protein by SEM and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

MATERIALS AND METHODS

Materials. Cultured live silver carp (*H. molitrix*, 900−1200 g/fish) was obtained from a local fishery market near the campus of China Agricultural University in autumn. The fish was instantly sacrificed after purchasing and then iced for about 2 h before the experiment.

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Chemicals. DEAE-Sephacel, Sephacryl S-100, SP Sepharose fast flow, and Con A Sepharose 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), *t*-butyloxycarbonyl-Val-Leu-Lys-4-methyl-7-coumarylamide (Boc-Val-Leu-Lys-MCA), *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), β-mercaptoethanol (β-Me), SDS, L-cysteine (L-Cys), bovine serum albumin (BSA), α-methyl-D-mannoside, polyoxyethylene-23-lauryl ether (Brij35), hydroxy-methyl-amino methane (Tris) and molecular weight marker (SDS-7) were purchased from Sigma Chemical Co. (St. Louis, MO). dl-dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Bio. Basic. Inc. (Markham, Canada). All other chemicals were of reagent grade.

Assay of Enzyme and Protein. The activity of the enzyme was determined according to a previous method with slight modification (*19*). Z-Arg-Arg-MCA and Z-Phe-Arg-MCA were used as substrates for cathepsin B and cathepsin L, respectively; both of the solutions were diluted to 500 μL by 0.1% Brij35. Either 250 μL of 0.4 M phosphate buffer (pH 6.0) for cathepsin B or the same volume of 0.4 M acetate buffer (pH 5.5) for cathepsin L was added to their enzyme solutions, respectively. The resulting solutions were preheated for 2 min followed by the addition of 250 μL of substrate with a concentration of 20 μM. The mixture was 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 for each test sample, but the stopping solution was added before the addition of the enzyme solution. The relative fluorescence intensity of aminomethylcoumarin (AMC) liberated by hydrolysis was measured in a spectrofluorometer (LS55, Perkin-Elmer, United States) with 380 nm of excitation wavelength and 460 nm of emission wavelength. One unit of the enzyme activity was defined as the amount of activity that released 1 nmol of AMC per min. The protein concentration was determined according to the method of Lowry et al. (*20*) with BSA as the standard.

Purification of Cathepsin L1 from Silver Carp Muscle. All stages were carried out at 4 °C, and the activities of cathepsin L1 were detected by measurement of Z-Phe-Arg-MCA hydrolysis. Silver carp muscle was minced, homogenized at 12000 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 PMSF, pH 5.0 (buffer A), and then centrifuged at 12000*g* for 20 min. The supernatant was collected as the crude extract and acidified to pH 3.0 with 1 M HCl. After the acidified extract was incubated at 30 °C for 10 min, the aqueous portion was adjusted to pH 6.0 with 1 M NaOH and then centrifuged at 12000*g* for 20 min. The supernatant was fractionated with ammonium sulfate at 80% saturation. The resulting precipitate collected by centrifugation was dissolved in 20 mM phosphate buffer containing 5 mM cysteine (buffer B) and dialyzed against the same buffer. After it was concentrated by an Amicon Membrane YM-10, the active eluate was applied to a DEAE-Sephacel column (2.6 cm × 20 cm) equilibrated with buffer B and eluted with a linear gradient of NaCl from 0 to 1 M in the same buffer. The active eluate from the DEAE-Sephacel column was concentrated again and loaded to a Sephacryl S-100 column (2.6 cm × 90 cm) equilibrated with buffer B containing 0.2 M sodium chloride (buffer C). A peak around 600 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 cm × 12 cm) equilibrated with the same buffer and eluted with a linear gradient of NaCl from 0 to 1 M in the same buffer. The first active peak eluted at the conductivity of 10−18 ms/cm was collected, which was dialyzed against 20 mM phosphate buffer containing 5 mM cysteine, 0.2 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$, pH 6.0 (buffer E). The produced dialyzate was finally applied to a Con A Sepharose column (1.0 cm × 8 cm) equilibrated with the same buffer. After it was washed with the equilibration buffer for elution of unabsorbed protein, the affinity column was eluted at a linear gradient of α-methyl-D-mannoside in buffer E. The resulting sample was collected, concentrated, and stored at −80 °C as purified proteinase.

SDS-PAGE. SDS-PAGE with the 10 and 11% acrylamide gel was carried out in a Mini PROTEIN 3 Cell (Bio-Rad, United States) as described previously (*21*). After electrophoretic running, gels were stained with 0.125% Coomassie Brilliant Blue R-250 in 25% ethanol and 10% acetic acid and destained with 25% ethanol and 10% acetic acid.

pH and Temperature Profiles of Purified Enzyme. The 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 the optimal temperature of purified proteinase was carried out in the temperature range from 20 to 80 °C using 0.4 M sodium acetate buffer containing 8 mM DTT, pH 5.5. The heat stability of purified proteinase was measured by incubating the samples at various temperatures from 20 to 80 °C for 60 min. The residual activity using Z-Phe-Arg-MCA as a substrate was determined as the above description.

Effects of Inhibitors and Reductants on the Activity of Cathepsin L1. Purified enzymes in 0.4 M sodium acetate buffer (pH 5.5) were respectively incubated with E-64, PMSF, pepstatin A, EDTA, DTT, L-Cys, and β-Me at 40 °C for 10 min. The remaining activity of the samples was measured as described by using Z-Phe-Arg-MCA as a substrate.

Effects of Metal Ions on the Activity of Cathepsin L1. The effects of metal ions on proteinase activity were determined by a modified method of Lee et al. (*11*). Briefly, the purified enzyme in 20 mM sodium phosphate buffer containing 2 mM DTT, pH 6.0, was incubated with 1 mM various metals (K^+, Mg^+, Ca^+, Mn^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, Co^{2+}, and Ni^{2+}) at 40 °C for 10 min. The remaining activity of the samples was also measured by using Z-Phe-Arg-MCA as a substrate.

Kinetic Studies. Kinetic studies of the purified cathepsin L1 were carried out as reported previously (*12*) with a final concentration of Z-Phe-Arg-MCA varying from 5 to 80 μM. The concentration of purified cathepsin L1 was determined by E-64 titration (*19*).

Substrate Specificity. The hydrolytic activities on synthetic substrates including Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, Arg-MCA, and Boc-Val-Leu-Lys-MCA were determined by the same method under the same conditions as those of the Z-Phe-Arg-MCA hydrolyzing activity. The Z-Phe-Arg-MCA hydrolyzing activity was measured according to the method described above.

Preparation of Frozen Surimi. Preparation for silver carp surimi was as follows: silver carp was eviscerated, and the dorsal muscle freed from bones and skin was fetched to get fish mince ($3-6\ mm^3$). The minced muscle was washed three times [twice with distilled water and once with 0.15% (w/w) NaCl, each time for 10 min] at about 4 °C with a solution/fish mince ratio of 4 (v/w), and then, the mixture was dewatered by employing cheesecloth as a filtering material. The dewatered fish mince was mixed with the cryoprotectant sucrose (8%). The resultant mixture was packed into polyethylene bags (2 kg/bag), which were kept frozen at −40 °C for later use.

Effect of Cathepsin L1 on Surimi Protein. 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 (*22*) with some modifications. Cathepsin L1 (5 units/g surimi) was added to thawed surimi under stirring. As a control sample, an equal volume of water instead of cathepsin L1 was added to the thawed surimi. After 2 h of incubation at 40 or 65 °C, 0.5 g of surimi with or without cathepsin L1 was, respectively, dissolved in 3 mL of 20 mM Tris-HCl buffer, pH 8.0, containing 2.0% SDS, 8.0 M urea, and 2.0% β-Me and then heated at 100 °C for 3 min. The resulting samples were analyzed using SDS-PAGE.

Preparation of Surimi Gel in the Presence or Absence of Cathepsin L1. Frozen surimi was thawed at 4 °C until the internal temperature reached −3 °C. The thawed surimi was sliced and ground with a mortar and pestle followed by the addition of cathepsin L1 (5 units/g surimi). For a control sample, all procedures are the same as the above except that an equal volume of water was used instead of the enzyme. Ground surimi with or without cathepsin L1 was stuffed into polyvinylethylene chloride tubes with a diameter of 3.0 cm and then incubated in a water bath at 40 °C for 30 min, followed by 85 °C

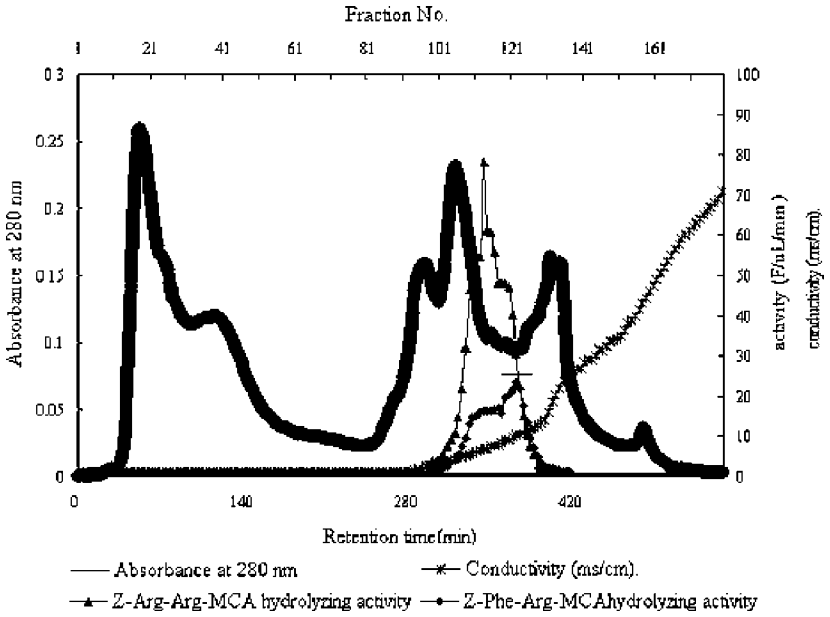

Figure 1. DEAE-Sephacel ionic exchange chromatography (2.6 cm × 20 cm) of the ammonium sulfate fraction. The flow rate was 0.5 mL/min, and 2 mL fractions were collected. The fractions indicated by a bar were pooled for further purification.

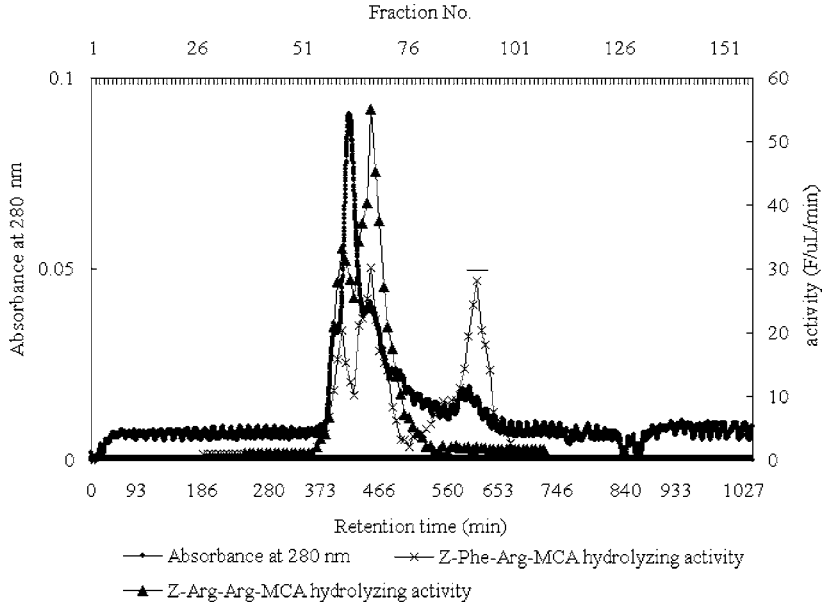

Figure 2. Sephacryl S-100 gel filtration chromatography (2.6 cm × 90 cm) of the fraction obtained by the preceding chromatography. The flow rate was 0.5 mL/min, and 2 mL fractions were collected. The fractions indicated by a bar were pooled for further purification.

for 20 min. After the gels were cooked, the tubes were immediately removed, placed in cold water (0 °C), and cooled at 4 °C for 30 min. All gels were removed from the tubes and stored overnight at 4 °C in polystyrene bags prior to testing.

Gel Microstructure Observations. Samples (5 mm × 5 mm × 5 mm) were cut from the center of surimi gel and washed with 0.1 M sodium phosphate buffer (pH 7.0) four times. The samples were prefixed in 3% glutaraldehyde for 2 h and then washed with 0.1 M sodium phosphate buffer (pH 7.0) four times (10 min per each time). After the sample was fixed with 1% osmic acid for 1 h, the resultant sample was washed with 0.1 M sodium phosphate buffer (pH 7.0) four times (10 min per each time). The fixed sample was dehydrated by a series of alcohol solutions (30, 50, 70, 80, 90, 95, and 100%) and isoamyl acetate. The specimen was dried using a critical point dryer (HCP-2, Hitachi Co. Japan) and then sprayed with gold using an ion sputtering coater (IB-3, Giko. Engineering Co., Japan). The microstructure of the gel was observed using a SEM (S-570, Hitachi Co., Japan).

RESULTS AND DISCUSSION

Purification of Cathepsin L1. Cathepsin L1 was purified by acid and heat treatments, ammonium sulfate fractionation, and a series of chromatographic separations, which are shown in **Figures 1**−**4**. A typical purification procedure is summarized in **Table 1**. About 0.81 mg of pure cathepsin L1 was obtained from 1000 g of silver carp muscle. The purity of the final preparation was increased about 48.4-fold from the crude extract. SDS-PAGE analysis exhibited a single band with an apparent M_r = 30 kDa, indicating that cathepsin L1 was purified to homogeneity, containing one kind of subunit (**Figure 5**).

It was observed that the purity of cathepsin L1 was increased about 1.55-fold after acid and heat treatments, suggesting that the acid and heat treatments were suitable for the isolation of silver carp cathepsin L (**Table 1**). Coinciding with the present

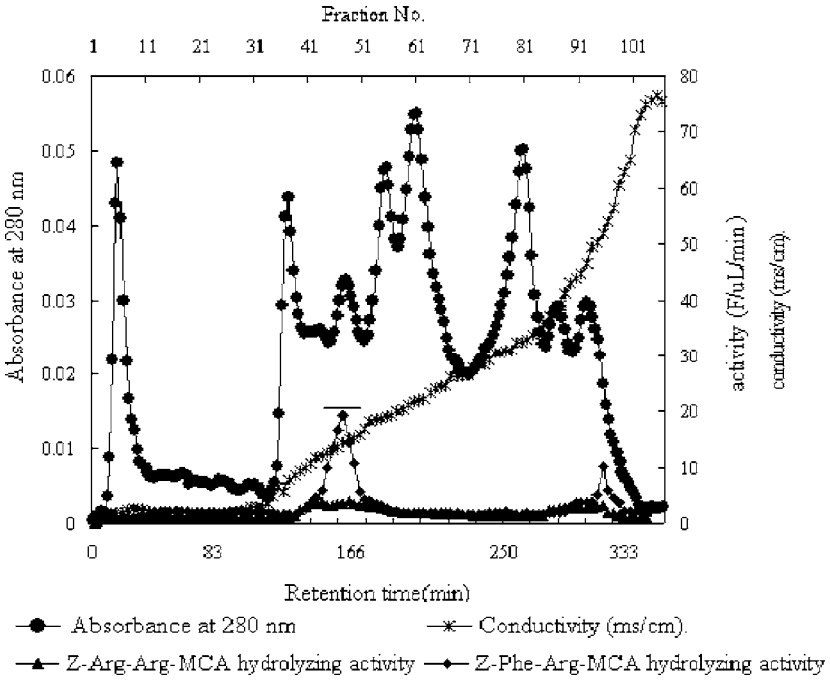

Figure 3. SP Sepharose fast flow cation exchange chromatography (1.6 cm × 12 cm) of the fraction obtained by the preceding chromatography. The flow rate was 0.5 mL/min, and 1 mL fractions were collected. The fractions indicated by a bar were pooled for further purification.

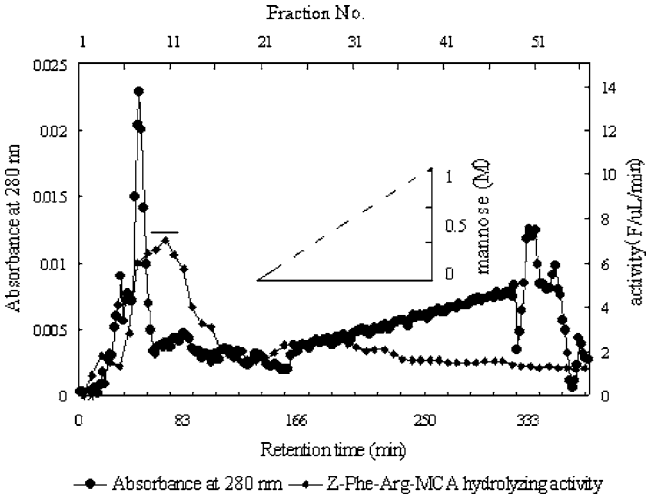

Figure 4. Con A Sepharose affinity chromatography (1.0 cm × 8 cm) of the fraction obtained by the preceding chromatography. The flow rate was 0.15 mL/min, and 0.8 mL fractions were collected. The fractions indicated by a bar were pooled.

Table 1. Purification of Cathepsin L1 from the Muscle of Silver Carp

step	total protein (mg)	total activity (units)	specific activity (units/mg)	purification fold	yield (%)
crude extract	133746.7	16391.5	0.12	1.0	100.00
acid-heat treatment	36560.0	6970.8	0.19	1.6	42.53
80% $(NH4)_2SO_4$	4028.9	1300.6	0.32	7.2	7.93
concentration	3123.5	1221.2	0.39	3.2	7.45
DEAE Sephacel	346.9	356.1	1.03	8.4	2.17
Sephacryl S-100	43.7	65.0	1.50	12.1	0.40
SP Sepharose fast flow	3.3	11.4	3.46	27.9	0.07
Con A Sepharose	0.8	4.9	6.00	48.4	0.03

result, similar observations have been made that acidification could isolate the enzyme from the enzyme-endogenous inhibitors (cystatin and α-cystatin) complex (*10*, *23*) or accelerate the conversion of procathepsin L into its mature form (*24*, *25*); heat

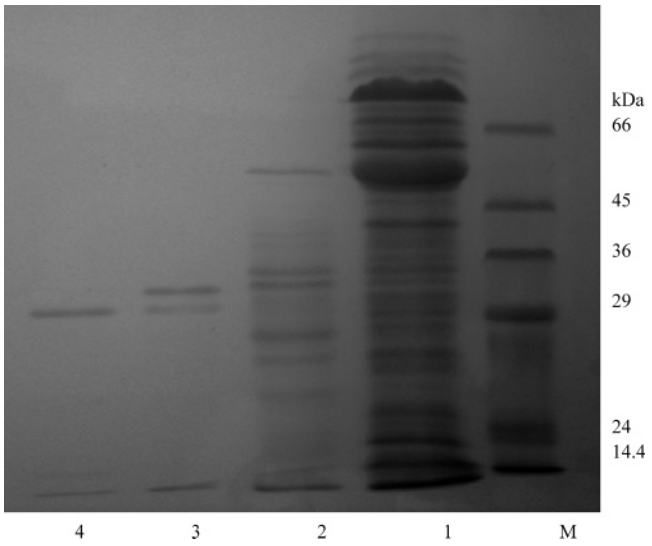

Figure 5. SDS-PAGE pattern of purified silver carp cathepsin L1. M, standard of molecular weight proteins; 1, pooled fraction from DEAE-Sephacel ionic exchange chromatography; 2, pooled fraction from Sephacryl S-100 gel filtration chromatography; 3, pooled fraction from SP Sepharose fast flow cation exchange chromatography; and 4, the finally preparation from Con A Sepharose affinity chromatography.

treatment was useful to rapidly remove heat sensitive proteins and inactivate other proteolytic enzyme (*26*). Thus, it seems that treatments with acid and heat could be employed as a simple way to improve the yield during the purification of cathepsin or related proteinases.

As **Figure 2** shows, cathepsins B and L were eluted at retention times of 450 and 600 min, respectively, indicating that they were effectively separated by Sephacryl S-100 column chromatography, leading to a 12.10-fold increase in protein purity. The hydrolyzing activities of peaks between 400 and 500 min against Z-Phe-Arg-MCA and Z-Arg-Arg-MCA are also shown in **Figure 2**. It has been reported that Z-Phe-Arg-MCA can be degraded by both cathepsins L and B while cathepsin L

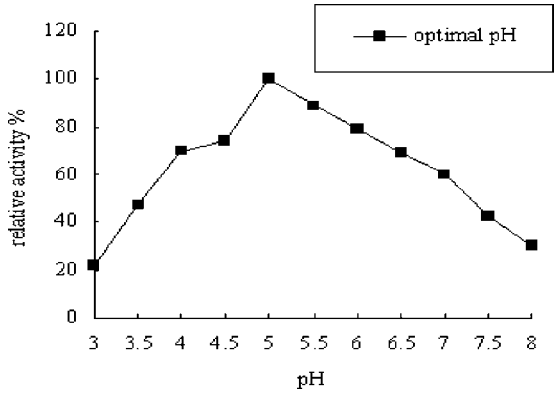

Figure 6. Effect of pH on the activity of purified silver carp cathepsin L1.

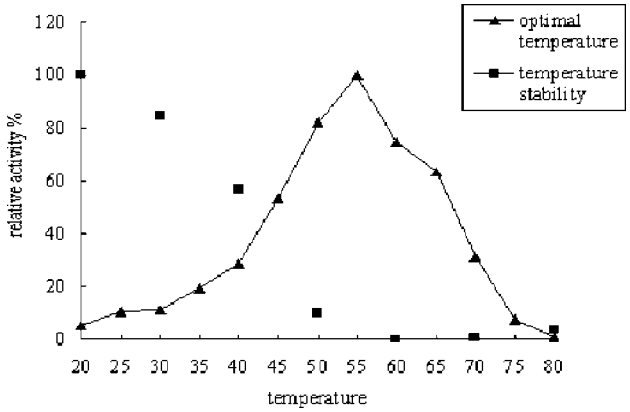

Figure 7. Effect of temperature on the activity of purified silver carp cathepsin L1.

could hardly degrade Z-Arg-Arg-MCA, which is the substrate of cathepsin B (*19*). Therefore, the peaks of the Z-Phe-Arg-MCA hydrolyzing activity that eluted from 400 to 500 min are possibly caused by cathepsin B. **Figure 3** shows that there are two peaks of cathepsin L obtained by cation-exchange chromatography. The first cathepsin L peak that eluted at the conductivity of 10−18 ms/cm refers to cathepsin L1; another peak appearing at the conductivity of 45−55 ms/cm is assigned to cathepsin L2. Cathepsins L1 and L2 are named according to their eluted sequence by cation-exchange chromatography. It has been reported that multiple isoenzymic forms of cathepsin L exist in the muscle of fish such as mackerel (*11*), salmon (*4*, *10*), and Pacific Whiting (*23*, *27*). Therefore, it is possible that the two apparent activities of silver carp cathepsin L are derived from multiple isoenzymic forms. We consider that an extensive investigation on cathepsin L2 is also necessary. From **Figure 4**, it can be seen that cathepsin L1 can not be absorbed by a Con A Sepharose column, suggesting that there are no carbohydrate side chains in this enzyme. Consistent with the present result, previous results have shown that about 10% of the purified proteinase was not glycosylated when the purified cathepsin L was chromatographed on Con A Sepharose (*28*). Further support comes from observations made by Kirschke et al. (*29*) and Lee et al. (*11*) showing that cathepsins L from rat liver and mackerel were not glycosylated.

The molecular mass of purified cathepsin L1 is approximately 30 kDa by SDS-PAGE, which is nearly the same as that of cathepsin L from mackerel (30 kDa) (*11*), carp (30 kDa) (*9*), and Pacific Whiting (28 kDa) (*27*). In the studies on cathepsin S, the molecular masses were 37 kDa from carp (*13*), 20 kDa from bovine (*14*), and 24 kDa from human spleen (*30*). Because the molecular mass of purified silver carp proteinase was significantly different from that of cathepsin S, this proteinase was assigned to cathepsin L rather than its S analogue.

Table 2. Effect of Inhibitors and Activator on the Activity of Cathepsin L1 from the Muscle of Silver Carp

inhibitors and activators	concentration (mM)	relative activity (%)
control[a]		100
E-64	0.1	0
PMSF	0.1	107
pepstatin A	1 mg/mL	101
EDTA (mM)	2	98
EDTA + DTT	1 + 2	172
DTT	2	100
Cys	5	114
β-Me	2	50

[a] Containing 2 mM DTT.

Table 3. Effect of Metal Ion on the Activity of Cathepsin L1 from the Muscle of Silver Carp

metal ion (1 mM)	relative activity (%)	metal ion (1 mM)	relative activity (%)
none	100	Cu^{2+}	0
K^{+}	99	Zn^{2+}	10
Mg^{2+}	66	Fe^{2+}	5
Ca^{2+}	51	Ni^{2+}	0
Mn^{2+}	65	Co^{2+}	0
Fe^{3+}	0		

Table 4. Kinetic Constants for the Hydrolysis of Peptide Methylcoumarylamide Substrates by Purified Cathepsin L1 from the Muscle of Silver Carp[a]

substrate	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} $mmol^{-1}$ L)
Z-Phe-Arg-MCA	8.25 ± 1.38	23.70 ± 0.90	2870
Z-Arg-Arg-MCA	ND	ND	ND

[a] ND, not detected.

Table 5. Substrate Specificity of Cathepsin L1 from the Muscle of Silver Carp

substrate	relative activity (%)
Z-Phe-Arg-MCA	100
Z-Arg-Arg-MCA	9
Arg-MCA	7
Boc-Val-Leu-Lys-MCA	1

It has been reported that multiple isoenzymic forms of cathepsin L exist in the muscle of fish including inactive procathepsin L, active free cathepsin L, and a complex form with its endogenous inhibitors (*10*, *23*). Cathepsin L is generally synthesized as procathepsin L, which can be further processed to the mature form by acid treatment (*25*). Yamashita and Konagaya reported that a cathepsin L−inhibitor complex was purified from chum salmon with a molecular mass of 50 kDa, which consisted of the 37 or 30 kDa form of cathepsin L and the 15 kDa endogenous cysteine protease inhibitor (*10*). Our data showed that the molecular mass of purified cathepsin L1 was approximately 30 kDa, which was much lower than that of the enzyme−inhibitor complex. Accordingly, it is suggested that the purified cathepsin L1 might be the active free cathepsin L.

pH and Temperature Profiles of Purified Cathepsin L1. **Figure 6** shows that the optimal pH is 5.0 for the hydrolysis of

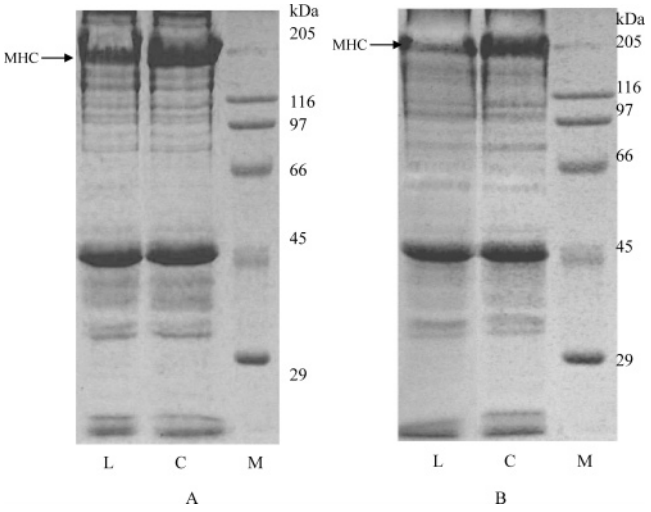

Figure 8. Changes in SDS-PAGE patterns of silver carp surimi protein. L, incubated with purified enzyme; C, incubated without enzyme; and M, standard of molecular weights proteins. (**A**) Incubated at 40 °C and (**B**) incubated at 65 °C.

Z-Phe-Arg-MCA by cathepsin L1, a result similar to that of cathepsin L isolated from other fish such as chum salmon (*4*), mackerel (*11*), arrowtooth flounder (*12*), and Pacific Whiting (*27*). In contrast, the optimal pH of carp cathepsin S is 7.0 using Z-Phe-Arg-MCA as the substrate (*13*). At pH 7.0−8.0, the cathepsin S still remained more than 95% of its maximum activity (*13*) whereas the activity of cathepsin L1 was greatly lost in the present study. Thus, a big difference in the optimal pH can be used to distinguish cathpsin S from other lysosomal cysteine proteinases (*14*).

The maximal activity of cathepsin L1 was observed at 55 °C (**Figure 7**). The stability of this enzyme was gradually decreased with an increase in the incubation temperature. At 40 °C, about 60% of its maximal activity still remained after 1 h of incubation (**Figure 7**). It was known that the setting was an indispensable processing in the production of surimi, which was carried out below 40 °C. Moreover, our previous work found that the myosin heavy chain (MHC) could be significantly hydrolyzed by silver carp cathepsin L at 35 °C (unpublished data). Accordingly, the heat-dependent stability in this study suggested that cathepsin L might play a key role on the proteolysis of silver carp surimi protein at the setting temperature.

Effects of Inhibitors, Reductants, and Metal Ions on the Activity of Cathepsin L1. The effects of inhibitors and reductants on the activities of cathepsin L1 are exhibited in **Table 2**. The activity of cathepsin L1 is 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 suggest that the purified proteinase belongs to a thiol proteinase. In accordance with this conclusion, neither serine- (PMSF) nor aspartic- (pepstatin A) proteinase inhibitors have an effect on the activity of cathepsin L1.

Table 3 indicates the effects of metal ions on the activities of purified cathepsin L1. The activity of this enzyme is inhibited strongly by Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+} and moderately by Mg^{2+}, Ca^{2+}, and Mn^{2+}. However, K^{+} did not affect its activity. The inhibition of the enzymatic activity by the Fe^{2+}, Cu^{2+}, and Zn^{2+} is presumably attributed to either binding to the sulfhydryl residue or catalyzing the oxidation of sulfhydryl residue (*11*). These results further support the above conclusion that the active center of cathepsin L1 contains SH group(s).

Kinetic Studies and Substrate Specificity. Kinetic constants for hydrolysis of Z-Phe-Arg-MCA were determined for silver carp cathepsin L1 (**Table 4**). The concentration of purified cathepsin L1 was 0.05 μM determined by E-64 titration. The K_m value of silver carp cathepsin L1 (8.25 μM) was slightly higher than that of chum salmon (1.7 μM) (*4*) and spotted mackerel (3.42 μM) (*11*) but was similar to that of arrowtooth flounder (8.2 μM) (*12*). The silver carp cathepsin L1 generally had a higher K_{cat} value (23.7 s^{-1}) than its counterparts from rat brain (5.5 s^{-1}) (*31*), pacific hake muscle (5.42 s^{-1}) (*32*), and carp hepatopancreas (9.5 s^{-1}) (*9*). These different kinetic constants may come from different experimental conditions such as pH and temperature (*33*). Another possibility, which was not excluded, was that the difference in kinetic constants might be due to different enzyme resources.

Substrate specificity of the purified proteinase was examined using synthetic substrate for cathepsin B, cathepsin H, and trypsin type proteases (**Table 5**). Results indicated that cathepsin

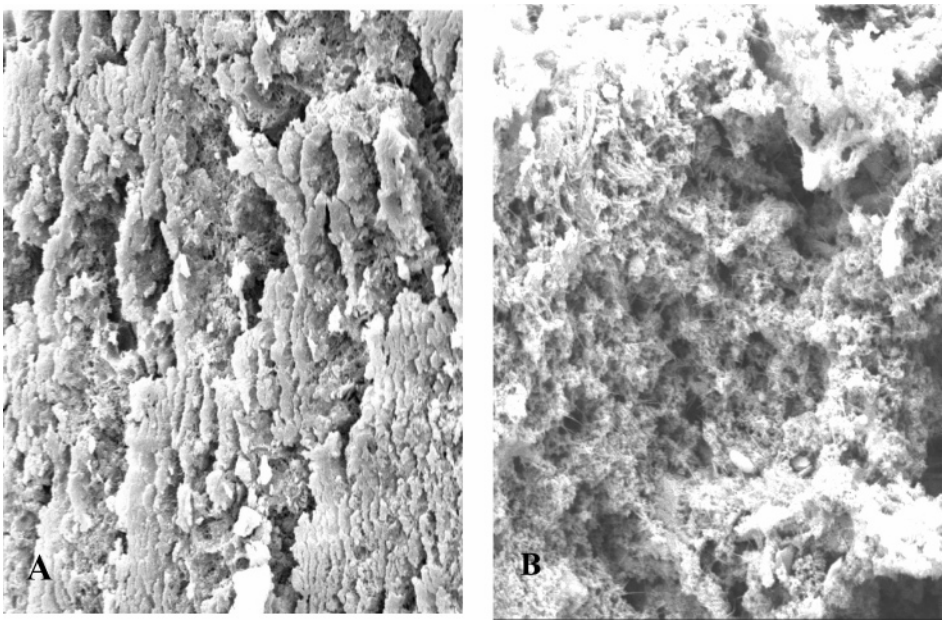

Figure 9. Gel microstructure of silver carp surimi. (**A**) Observed without enzyme and (**B**) observed with enzyme.

L1 significantly hydrolyzed Z-Phe-Arg-MCA, a specific substrate commonly used to assay the activity of cathepsin L. However, it hardly catalyzed the hydrolysis of either Z-Arg-Arg-MCA (a substrate for cathepsin B) or Arg-MCA (a substrate for cathepsin H) possibly because cathepsin L preferentially cleaves peptide bonds with hydrophobic amino acid residues in *P*2 and *P*3 (*34*). In addition, the N-terminal Boc-blocked substrate that was suitable for trypsin type proteinases was insusceptible to cathepsin L while its S analogue could slightly hydrolyze Boc-Val-Leu-Lys-MCA (*13*). Therefore, Z-Phe-Arg-MCA was concluded to be the best substrate for the assay of silver carp cathepins L (*9*).

Effects of Cathepsin L1 on the Surimi Gels Protein. The changes in surimi protein were detected by SDS-PAGE analysis. As the electrophoretic profile showed, the degradation degree of the MHC by cathepsin L1 was much larger than that in the control, suggesting that MHC was potentially susceptible to the hydrolysis by cathepsin L1 (**Figure 8**). It was also observed that the proteolytic rate at 65 °C was much greater than that at 40 °C (**Figure 8**). Myosin played an important role in the gelation of surimi (*35*), so its degradation could decrease the textural quality of surimi products, considerably affecting the strength and elasticity of the gel (*3*). A washing treatment had always been employed to improve the quality of surimi because it could remove certain modori-inducing proteinases occurring in the sarcoplasm of muscle. Our previous work indicated that the residual activity percentage of cathepsin L was about 25.61% after the washing treatment, suggesting that the washing treatment alone hardly removed the cathepsin L from silver carp (*36*), which is in agreement with observations by other groups (*5*, *8*). Thus, it is reasonable to believe that the residual activity of the cathepsin L still has an opportunity to degrade myosin during the processing of silver carp surimi products.

Effects of Cathepsin L1 on the Microstructure of Gels. The internal structures of gels with or without cathepsin L1 were observed with SEM at a magnification of 1500 times (**Figure 9**). Surimi gel A (without cathepsin L1) had a dense structure with little space due to protein aggregation. However, the network structure of surimi gel B was pronouncedly destroyed and a large number of pores were distributed on the gel surface upon addition of cathepsin L1, indicating that the surimi proteins were hydrolyzed to a large extent by cathepsin L1. It was also shown that the gel B matrix had a lower uniformity of the protein dispersion than gel A did (**Figure 9**). It has been known that fine-stranded gel was usually formed by an ordered association of molecules (*37*). The present results exhibited that the purified cathepsin L1 had the ability to destroy network structure of the surimi gel, resulting in a decrease of the quality of silver carp surimi gel during its gelation.

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