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Purification and characterisation of cathepsin L2 from dorsal muscle of silver carp (*Hypophthalmichthys molitrix*)

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ABSTRACT

Cathepsin L2 was purified to homogeneity from silver carp muscle using an array of chromatography methods. The enzyme showed affinity to con A-sepharose. Although it appeared to be 78 kDa on non-reducing SDS–PAGE and gel–substrate-activity SDS–PAGE, it completely degraded into 31 kDa and 26 kDa sub-units, as well as some small polypeptides on reducing SDS–PAGE. The optimum pH and temperature of cathepsin L2 for hydrolysis of Z-Phe-Arg-MCA were pH 4.5–5.5 and 45 °C, respectively. It was stable at pH 5.5 and below 40 °C, but almost inactivated at pH 7.0 and 60 °C. Substrate specificity analysis indicated that it could hydrolyse Z-Phe-Arg-MCA but not Z-Arg-Arg-MCA or L-Arg-MCA. Cathepsin L2 was efficiently activated by Cys, DTT and β-ME, but was completely inhibited by E-64. $P_2O_7^{4-}$ and Cl^- have inhibitory effects on its activity. Cathepsin L2 showed a high K_m value of 9.5 μmol/l, but extremely low K_{cat} and K_{cat}/K_m values of 0.8 s^{-1} and 84.2 s^{-1} mM^{-1}, respectively. Except for under optimum conditions (pH 5.0, 35 °C), silver carp cathepsin L2 could also hydrolyse myosin heavy chain at softening temperatures ranging from 50 to 60 °C and at surimi pH of 6.5.

1. Introduction

Silver carp (*Hypophthalmichthys molitrix*) is reported to be the most abundant fresh water fishery resource in China with annual harvests of 4.9 million metric tons (Agricultural Bureau of China, 2002). The surimi products processed from white muscle of silver carp have a vast range of commercial prospects. However, silver carp surimi exhibits considerable gel softening (modori) in the course of heating, especially at 50 °C (Luo, Kuwahara, Kaneniwa, Murata, & Yokoyama, 2001), which leads to a poor textural quality and consequently limits the further utilisation of this enormous fishery resource.

Cathepsin L (EC3.4.22.15), a lysosomal cysteine proteinase, has been shown to be particularly powerful in degrading myofibrillar components in post-mortem autolysis (Aoki & Ueno, 1997). In recent years, the endogenous heat-stable cathepsin L in fish muscle has also been revealed to be implicated in the modori phenomenon of several fish species, especially at 50–60 °C (Ho, Chen, & Jiang, 2000; Hu, Morioka, & Itoh, 2007; Ogata, Aranishi, Hara, Osatomi, & Ishihara, 1998; Visessanguan, Menino, Kim, & An, 2001). In our previous work, it was also found that the cathepsin L activity was hardly removed, and it showed the highest residual ratio of 25.61% in the washed silver carp surimi when compared with cathepsin B and H activities (Li, Zhang, Liu, & Ma, 2004). Thus, it was suggested that the residual cathepsin L activity had the possibility to degrade surimi protein during the manufacturing of silver carp surimi products. Therefore, it is important to elucidate the relationship between the cathepsin L activity and silver carp modori for developing the processing technologies further and improving the textural quality of silver carp surimi product.

Therefore, we purified the cathepsin L from dorsal muscle of silver carp. During the purification, two active peaks with Z-Phe-Phe-MCA hydrolysis activity were isolated after cation-exchange chromatography on a SP-sepharose FF column, and these were named as cathepsin L1 and L2, respectively, according to their eluted sequence (Liu, Yin, Zhang, Li, & Ma, 2006). Cathepsin L1 has been characterised as an unglycosylated cysteine proteinase of 30 kDa and it could markedly hydrolyse surimi protein mainly at 65 °C and destroy the network structure of silver carp surimi gel (Liu et al., 2006).

Here, the apparent cathepsin L2 activity was considered to be probably an isoenzymic form of cathepsin L1, since multiple isoforms of cathepsin L have been detected in ordinary muscle of some fishes, especially some with higher molecular weight such as those in mackerel (58 kDa; Aoki, Nakano, & Ueno, 1997; Lee, Chen, & Jiang, 1993), salmon muscle (50 kDa; Yamashita & Konagaya, 1990) and pacific whiting (52.4 kDa; Benjakul, Seymour, Morrissey, & An, 1996; Seymour, Morrissey, Peters, & An, 1994). It was also shown that the cathepsin L-like of 58 kDa could

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hydrolyse myosin, the major textural protein in mackerel surimi at pH 6.0 (Jiang, Lee, & Chen, 1996) and had been involved in the mackerel surimi gel softening at pH 5.5–7.0 and at 40–55 °C (Ho et al., 2000). Additionally, the active isoform of pacific whiting cathepsin L of 54.2 kDa was tested to be a complex, consisting of two forms of cathepsin L of 37 kDa or 30 kDa and the 15 kDa endogenous cysteine inhibitor (Benjakul et al., 1996). A cathepsin B-like of 60 kDa from mackerel was identified as a precursor of cathepsin B that similarly to cathepsin L, also belonged to the family of papain-like cysteine proteases (Aoki, Yokono, & Ueno, 2002). Otherwise, the amino acid sequence of a precursor deduced from cDNA cloned of carp cathepsin L suggested a higher calculated molecular weight (about 43 kDa) than the purified cathepsin L of 28 kDa (Tsunemoto, Osatomi, Nozaki, Hara, & Ishihara, 2004).

Actually, in animals, except for the mature form of lower molecular weight (Ishidoh et al., 1998), cathepsins could still exist in the form of precursors (Mason, Gal, & Gottesman, 1987) and enzyme–endogenous inhibitor complexes (An, Peters, Seymour, & Morrissey, 1995). Proper acidification could help disassociation of the inhibitors, cystatin or α-cysteine, from the loose combination of the complex (Godiksen & Nielsen, 2007) and accelerate the conversion of the precursor to a mature form both with an increase in activity (McDonald & Emerick, 1995).

Silver carp cathepsin L1 had been presumed to be in an active mature form (Liu et al., 2006). However, the properties of cathepsin L2 from silver carp muscle and whether it also had been involved in the gel softening were still unclear. In this study, the further purification and some biochemical characteristics of cathepsin L2 were investigated. Moreover, its proteolytic actions on the main components of silver carp surimi protein–myosin were examined. Also, the possible form of cathepsin L2 and the difference from cathepsin L1 were discussed.

2. Materials and methods

2.1. Materials and chemicals

Live silver carp *H. molitrix* (900–1200 g per fish) was sacrificed instantly after purchasing. Then, the dorsal muscle was manually carved away from bones and filleted at 4 °C. After that, it was immediately frozen in liquid nitrogen and stored at −80 °C for further investigation.

DEAE sephacel, sephacryl S-100, SP-sepharose fast flow, con A-sepharose, media for chromatography were purchased from Amersham Biosciences (Uppsala, Sweden). Benzyloxycarbonyl-L-phenylalany-L-arginyl-4-methyl-7-coumarylamide (Z-Phe-Arg-MCA), Benzyloxycarbonyl-arginylarginine-4-methyl-7-coumarylamide (Z-Arg-Arg-MCA), L-arginyl-4-methyl-7-coumarylamid (L-Arg-MCA), 7-amino-4-methylcoumarin (AMC), *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), β-mercaptoethanol (β-ME), L-cysteine (L-Cys), DL-dithiothreitol (DTT), sodium dodecyl sulphate (SDS), SDS-7 molecular weight marker (14–66 kDa), Sigma Marker (6–205 kDa), α-methyl-D-mannoside and phenylmethanesulphonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) and ethyleneglycol bis (2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) were purchased from Amresco Inc. (Ohio, Solon). All other chemicals used were of analytical grade.

2.2. Assay of enzyme activity and protein concentration

Enzyme activity assays for cathepsin L2 with fluorescent synthetic peptide substrate Z-Phe-Arg-MCA was essentially carried out according to Barrett and Kirschke (Barrett & Kirschke, 1981) with a few modifications. The reaction temperature was 40 °C and the pre-warming time of enzyme solution was for 2 min. The intensity of fluorescence was measured in a spectrofluorometer (RF-5300 PC) with excitation and emission wavelength of 380 nm and 460 nm. One unit of enzyme activity was defined as the amount of activity that released 1 nmol of AMC per min at the assay condition.

Cathepsin B and cathepsin H activities were routinely assayed with Z-Arg-Arg-MCA and L-Arg-MCA respectively by the method from Barrett (1980).

The protein concentration was determined as described by Lowry, Rosebrough, Farr, and Randall (1951) with BSA as the standard.

2.3. Purification

The purification procedure for cathepsin L, including crude extracting, acidification treatment, $(NH4)_2SO_4$ fractionation, ultrafiltration, ordinal chromatography on DEAE-sephacel, sephacryl S-100, SP-sepharose FF, was carried out as described by Liu et al. (2006). The second active peak corresponding to 43–56 ms/cm eluted from SP-sepharose FF was collected, concentrated and dialysed against 20 mmol/l phosphate buffer containing 5 mmol/l L-cysteine, 0.2 mol/l NaCl, 1 mmol/l $CaCl_2$ and 1 mmol/l $MnCl_2$ (pH 6.0). Then, the dialysis sample was purified by a con A-sepharose affinity chromatography (1.0 × 8 cm) equilibrated with the same buffer. The flow rate was 0.15 ml/min. After washing of the unabsorbed protein with equilibration buffer, the affinity column was eluted at a linear gradient of α-methyl-D-mannoside from 0 to 1 mol/l in equilibration buffer. The resulting samples of purified enzyme were combined, concentrated and stored at −80 °C for the subsequent characterisation.

2.4. Active-site titration by E-64

Active-site titration of cathepsin L by E-64 was essentially performed as previously described by Barrett and Kirschke (1981).

2.5. Kinetic constants

Kinetic constants of cathepsin L2 was determined according to Visessanguan, Benjakul, and An (2003), with a final concentration of Z-Phe-Arg-MCA ranging from 4.95 to 60 μmol/l.

2.6. Polyacrylamide gel electrophoreses

Analytical polyacrylamide gel electrophoresis (native-PAGE) was performed as described by Wang and Fan (2000) at pH 8.0 using 8% gel.

SDS–PAGE was conducted by the Laemmli's method (Laemmli, 1970). The stacking and resolving gels were 4% and 11% (7.5% for the analysis of myosin degradation), respectively. The samples were reduced with 10% β-ME at 100 °C for 5 min, while, for the non-reducing SDS–PAGE, this treatment was cancelled. Then 10 μl of sample was loaded on each lane and run on a Mini PROTEIN 3 apparatus acquired from Bio-Rad (BioRad Laboratories Inc., Richmond, CA) with a constant voltage of 120 V. After electrophoresis, the gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 40% ethanol and 10% acetic acid, and destained with 45% ethanol and 5% acetic acid.

The gelatin–substrate-active SDS–PAGE was carried out with 0.2% gelatin in 8.0% resolving gel, and without the treatments of adding β-ME and heating for the enzyme samples. The activity staining was performed as described by García-Carreño, Dimes, and Haard (1993) with a slight modification. The activity zone was developed in McIlvaine's Buffer of pH 5.5, containing 1 mmol/l DTT, at 37 °C for 9–12 h.

The standard proteins were SDS-7 molecular weight marker. Analysis of gel images was performed by Quantity One software from Bio-Rad (BioRad Laboratories Inc., Richmond, CA).

2.7. Prepare of myosin

Crude myosin was isolated and prepared by the method described by Martone, Busconi, Folco, Trucco, and Sanchez (1986).

2.8. Proteolysis of surimi protein–myosin

The prepared myosin was re-suspended in 400 mmol/l phosphate buffer containing 2 mmol/l DTT (with pH range 5.0–6.5) and incubated with four activity units of purified enzyme at 35 °C, 50 °C, or 60 °C for different time intervals. The reaction mixture was boiled for 5 min and an equal volume of stopping agent (62.5 mmol/l tris–HCl buffer containing 2% SDS, 8 mol/l urea, 5% β-ME, 0.005% bromine phenol blue, 20% glycerol, pH 6.8) was added. Ten microlitres of sample for each lane was then applied on SDS–PAGE for further analysis. SigmaMarker (6–205 kDa) was used as standard proteins.

3. Results and discussion

3.1. Purification of cathepsin L2

In this typical purification experiment, silver carp athepsin L2 was purified 2130-fold with 0.341% recovery, and 0.32 mg of purified enzyme was finally obtained from 1000 g of silver carp dorsal muscle. The detailed figures are listed in Table 1. The elution profiles of Cation-exchange chromatography on SP-sepharose FF column and of affinity chromatography on con A-sepharose column are shown in Fig. 1a and b.

In Fig. 1a, the second active peak named cathepsin L2, was eluted at the conductivity range of about 43–56 ms/cm (about 0.5 mol/l NaCl), similar to the action of carp cathepsin L on S-sepharose column (Aranishi, Ogata, Hara, Osatomi, & Ishihara, 1997). It was notable that the cathepsin L2 activity was lower than that of cathepsin L1 initially, whereas it was significantly enhanced by about three times after storage in an acidic eluent buffer (pH 4.5) at 4 °C for 36 h (data not shown). In fact, we had also done the storage experiment for the collection of cathespin L1 from SP-sepharose FF column under the same conditions. To our surprise, its activity was not increased, but instead decreased by about 40% after 36 h (data not shown). The reason for this phenomenon still needs further investigation. However, Aoki et al. (2002) had reported that after leaving at 4 °C for 48 h, the precursor of mackerel cathepsin B could be converted to the mature form gradually. Additionally, distinct from cathepsin L1, the fraction of cathepsin L2 showed little cathepsin B activity. Therefore, cation-exchange chromatography on SP-sepharose FF column was efficient and successful for the separation of cathepsin L2 from cathepsin B activity.

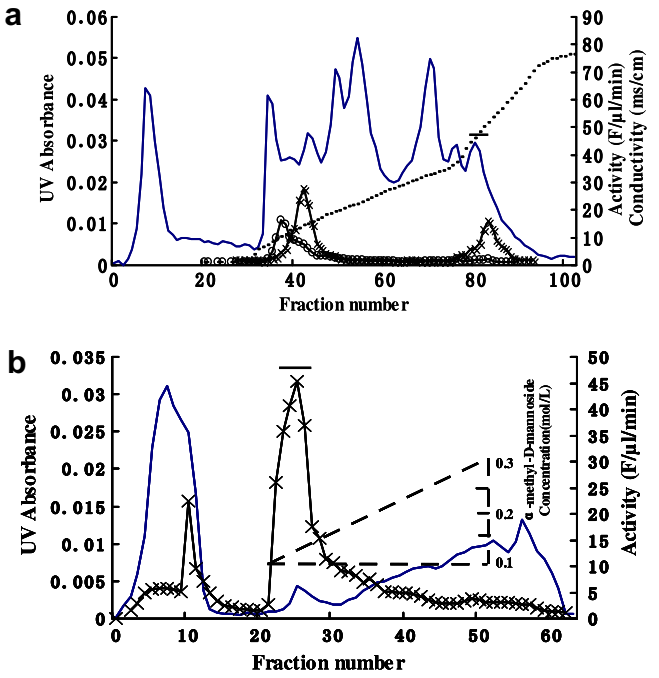

Fig. 1. Purification of silver carp cathepsin L2. (a) Sp-sepharose FF chromatography of the sephacryl S-100 fraction according to Liu et al. (2006). (b) Con A-sepharose chromatography. The fractions indicated by a bar were pooled. (–) Absorbance at 280 nm; (×) Z-Phe-Arg-MCA hydrolysing activity; (○) Z-Arg-Arg-MCA hydrolysing activity; and (- - -) conductivity or α-methyl-D-mannoside concentration.

After passing through the con A-sepharose column, cathepsin L2 activity was also resolved into two active peaks. However, most of the enzyme activities were eluted with 0.1–0.15 mol/l α-methyl-D-mannoside (Fig. 1b), consistent with the findings of Aranishi et al. (1997) about carp cathepsin L. This indicated that cathepsin L2 had been adsorbed by the con A-sepharose column, and had some carbohydrate side-chains. The natural lysosomal enzymes were initially synthesised as glycoprotein and it was proposed that glycosylation as a recognition marker was essential for lysosomal enzymes in keeping with their function of trafficking and targeting (von Figura & Hasilik, 1986). According to Barrett and Kirschke (1981), in a typical experiment, 30% of the applied activity of cathespin L was not adsorbed by con A-sepharose (because it had lost its carbohydrate by autolysis), and 65% was adsorbed. It was obvious that cathepsin L2 was absolutely coincident with this trait. However, cathepsin L1 had no carbohydrate since none of the applied activity was absorbed by con A-sepharose (Liu et al., 2006), which might be due to the limited proteolysis that occurred during the long time of purification.

In the final of purification process, the significant increases in specific activity of 176 units/mg and purity of 2130-fold was

Table 1
Purification of cathepsin L2 from silver carp dorsal muscle

Procedure	Total protein (mg)	Total act. (units)	Sp act. (units/mg)	Purification (fold)	Yield (%)
Crude extract	133750	16391	0.123	1.00	100
Acidification treatment	36560	6970	0.191	1.55	42.5
$(NH_4)_2SO_4$ fractionation	4029	1300	0.323	2.63	7.93
Ultrafiltration	3120	1220	0.391	3.18	7.45
DEAE-sephacel	347	356	1.03	8.35	2.17
Sephacryl S-100	43.7	65.0	1.50	12.1	0.40
SP-sepharose FF	3.28	55.0	16.8	203	0.336
Con A-sepharose	0.32	56.0	176	2130	0.341

Data of total protein, total activity, specific activity, purification fold and yield before the procedure of SP-sepharose FF were quoted from the results of our laboratory described by Liu et al. (2006).

obtained when compared to the crude extract. Therefore, con A-sepharose was highly effective for the removal of trace impurities.

3.2. Homogeneity and molecular weight of cathepsin L2

A single protein band was detected in the final preparation on native-PAGE (Fig. 2a), which corresponded to the active band on gelatin–substrate-active-native-PAGE (Fig. 2b). In the presence of 2-mercaptoethanol, on reducing SDS–PAGE, two major protein bands with the molecular weight about 31 kDa and 26 kDa were detected, together with some small polypeptides of about 20 kDa and 15 kDa (Fig. 3a). However, on a non-reducing SDS–PAGE, except for the above bands (formed possibly due to the instability of the cathepsin L2 to SDS), two bands of about 78 kDa and 66 kDa were also observed (Fig. 3b). This was in agreement with the analysis on gelatin–substrate-active SDS–PAGE under non-reducing conditions (Fig. 3c). Moreover, no gelatinolytic activity was detected when the substrate gels were incubated with E-64 (data not shown), indicating that substrate digestion was due to cathepsin L2. These results suggested that the purified cathepsin L2 was a high molecular weight proteinase of about 78 kDa, but it was unstable to SDS and reducing conditions, which dissociated parts of the proteinase into 66 kDa, 31 kDa and 26 kDa fragments. Indeed, the presence of polypeptides of 20 kDa and 15 kDa confirmed this speculative explanation.

Multiple forms of cathepsin L have been reported in some mammalian and fish tissues including: (1) an enzyme–endogenous inhibitor (cystatin or α-cysteine) complex form (An et al., 1995; Coetzer, Dennehy, Pike, & Dennison, 1995), which was liable to being dissociated by acidification with a remarkable increase of enzyme activity (Benjakul et al., 1996; Godiksen & Nielsen, 2007), (2) a precursor form (Mason et al., 1987; Aoki & Ueno, 1997) and (3) a mature form of the enzyme (Ishidoh et al., 1998; Kominami, Tsukahara, Hara, & Katunuma, 1988).

Both of the two former forms had higher molecular weight. Therefore, there may be two possibilities for the structure of silver carp cathepsin L2. One was that it might be the enzyme–endogenous inhibitor complex with a loose association. The other was that it might be the precursor of mature cathepsin L. It was reported that the precursor form was initially synthesised and gradually converted to the high-activity mature form with the occurrence of active intermediates, in vitro, by intramolecular self-processes at acidic pH range from 3.0–5.5 (Kawada et al., 2000; McDonald & Emerick, 1995). These hypotheses also might be the explanation for the remarkable increase of cathepsin L2 activity in an acidic environment (pH 4.5) after a period of storage (data not shown).

On the other hand, Seymour et al. (1994) applied acidification to the two forms of cathepsin L purified from muscle of pacific whiting (*Merluccius productus*) simultaneously. The results had suggested the form with an increase in activity after low-pH treatment should be the enzyme–endogenous inhibitor complex (its activity subsequently decayed after entirely acidification), whereas the other one without any increase in activity might be the mature form. Moreover, they also presumed that it might be because acidification removed the light chain involved in stabilization of the active enzyme (Mason, 1986; Seymour et al., 1994), that both forms of cathepsin L were very unstable after the entire acidification process even in a non-acidic condition at 4 °C. During our storage of silver carp cathepsin L2 and L1, a similar phenomenon had been observed. This reinforced our presumption that the cathepsin L2 might be the enzyme–endogenous inhibitor complex or precursor, while the cathepsin L1 of 30 kDa (Liu et al., 2006) might be the mature form. In particular, during the storage in an acid environment, cathepsin L2 might autolyze into the form of the carbohydrate-free cathepsin L1 with an increase in activity.

Additionally, it has been reported that some procathepsins L (Mason et al., 1987; McDonald & Emerick, 1995) and the complexes of cathepsin L-cystatin (Benjakul et al., 1996; Coetzer et al., 1995) exhibited proteolytical activity. In this study, the analysis on non-reducing gelatin–substrate-active SDS–PAGE (Fig. 3c) also gave similar results.

The bands of 31 kDa and 26 kDa might be the final mature forms of cathepsin L2, corresponding to a single chain form (monomer) and heavy chain form, respectively. The heavy chain form of the active site was generally linked by a disulphide with a light chain and thus was in a dimer form as reported for carp hepatopancreas cathepsin L (Aranishi et al., 1997). Coetzer et al. (1995) pointed out that cathepsin L existed in vivo in a single-chain form, and the two-chain form (dimer) of cathepsin L might in fact be an artifact due to the occurrence of limited proteolysis during the more lengthy and sophisticated isolation procedures. Therefore, in this study, the appearance of a band of 26 kDa might be really attributed to the limited proteolysis, since Tsunemoto et al. (2004) had demonstrated that unlike in mammals, it might be impossible for fish cathepsin Ls to be processed into a two-chain form *in vivo* due to the difference of amino acid residues in the cleavage site. Moreover, silver carp cathepsin L1 had given a single band on reducing SDS–PAGE (Liu et al., 2006).

Additionally, the monomer form of silver carp cathepsin L2 had a similar molecular weight to cathepisn L1 of silver carp (about 30 kDa; Liu et al., 2006), and that of carp (*Cyprinus carpio*; about 28–30 kDa; Aranishi et al., 1997; Tsunemoto et al., 2004), mackerel (*Scomber australasicus*; 30 kDa; Lee et al., 1993), salmon (30 kDa; Yamashita & Konagaya, 1990) and Pacific whiting (*M. productus*; 28.8 kDa; Seymour et al., 1994), but a little higher than arrowtooth flounder (*Atheresthes stomias*; 27 kDa; Visessanguan et al., 2003).

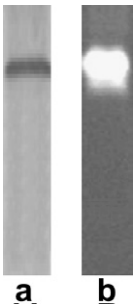

Fig. 2. Native-PAGE (a) and gelatin–substrate-active-naive-PAGE (b) of purified cathepsin L2.

3.3. Effects of pH and temperature on cathepsin L2

The purified cathepsin L2 exhibited a broad optimum pH ranging from pH 4.5 to 5.5 on Z-Phe-Arg-MCA as shown in Fig. 4a. The activity decreased sharply when the pH was increased to 6.5. The enzyme was insensitive to acidic conditions and it was quite stable at pH 3.0–5.5 (Fig. 4b). However, the enzyme was inactive at neutral pH of 7.0.

From the temperature dependence (Fig. 5a) and thermal stability profiles (Fig. 5b), it was found that cathepsin L2 had a maximum hydrolysis ability at 45 °C, and also kept some hydrolytic activities at 50–60 °C, which is approximated to the softening temperature of 50 °C for silver carp surimi (Luo et al., 2001). Therefore, this proteinase might have some potential relationship with the weakening of surimi gel strength at that temperature.

The certain differences of optimum temperature and optimum pH between cathepsin L2 and cathepsin L1 (55 °C, pH 5.0 according to Liu et al., 2006) to Z-Phe-Arg-MCA, showed that the two proteases

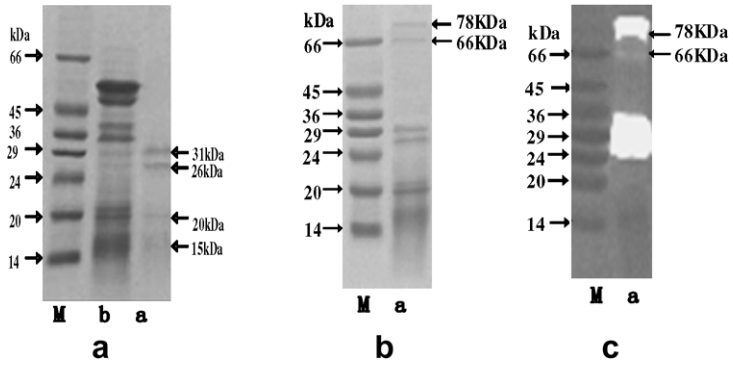

Fig. 3. Reducing SDS–PAGE (a), non-reducing SDS–PAGE (b) and non-reducing gelatin–substrate-active SDS–PAGE (c) of cathepsin L2; M, the standard proteins (SDS-7 molecular weight marker); a, purified cathepsin L2; b, cathepsin L2 activity from SP-sepharose FF.

were not entirely identical, and this might be due to the differences in structure, for example, whether being associated with a carbohydrate, light chain, endogenous inhibitors (cystatin and α-cysteine) and propeptide or not.

3.4. Effect of sulphydryl reagents, inhibitors and ions on cathepsin l2

As shown in Fig. 6, the enzyme was inactive in the absence of reductants, but was activated effectively by all the sulphur-containing reducing reagents. The property of thiol-dependence indicated that cathepsin L2 in silver carp belonged to a family of cysteine proteases.

The data on the effect of various potential inhibitors on the enzyme are presented in Table 2. The enzyme activity could be slightly suppressed by PMSF, EGTA and EDTA with the increase of the tested concentrations but remained higher than that of the control. The residual activities of cathepsin L2 were plotted against E-64 concentration from 0 to 50 μmol/l (Fig. 7). The presence of a thiol group in the active site was testified by complete loss of the enzyme activity by specific thiol-blocking agent E-64 at the final concentration of 50 μmol/l.

Sodium pyrophosphate ($P_2O_7^{4-}$) and NaCl (Cl^-) are often used as food additives in surimi products for preservation or improving the texture. When their final concentrations reached 25 mmol/l and 200 mmol/l, respectively, $P_2O_7^{4-}$ and Cl^-, either fully inhibition or partial inactivation of the enzyme (Table 2) was observed.

3.5. Substrate specificity

For further identification, substrate specificity was tested using Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and L-Arg-MCA, specific peptidyl-MCA substrates for cathepsins L, B and H, respectively. The purified enzyme only showed a strong hydrolytic activity towards Z-Phe-Arg-MCA, but did not towards Z-Arg-Arg-MCA or L-Arg-MCA. According to Barrett and Kirschke (1981), the inability to hydrolyse Z-Arg-Arg-MCA or L-Arg-MCA was a distinctive feature of cathepsin L differing from cathepsin B and H. Therefore, based on substrate specificity and the effects of activation and inhibition, we concluded that the purified protease from silver carp muscle had the characteristics of cathepsin L.

3.6. Kinetic constants

The values of kinetic constants K_m, K_{cat} and K_{cat}/K_m of cathepsin L2 are presented in Table 3. In spite of being slightly high, the K_m value (9.5 μmol/l) was comparable to those of cathepsins L from mammalian tissue of rat liver (7.0 μmol/l; Kirschke, Kembhavi, Bohley, & Barrett, 1982) or fish muscle of arrowtooth flounder (8.2 μmol/l; Visessanguan et al., 2003), mackerel (3.42 μmol/l; Lee, Chen, & Jiang, 1996), or chum salmon (1.7 μmol/l; Yamashita & Konagaya, 1990).

However, silver carp cathepsin L2 had an absolutely lower K_{cat} value of 0.8 s^{-1} and, therefore, it led to an extraordinarily lower catalytic coefficient K_{cat}/K_m of 84.2 s^{-1} mmol/l^{-1} than those of its counterparts from carp cathepsin L (9.5 s^{-1} and 278.1 s^{-1} mmol/l^{-1}; Aranishi et al., 1997), arrowtooth flounder (12.2 s^{-1} and 1488 s^{-1} mmol/l^{-1}; Visessanguan et al., 2003), salmon (15.8 s^{-1} and 9405 s^{-1} mmol/l^{-1}; Yamashita & Konagaya, 1990) and mackerel (43.3 s^{-1} and 12667 s^{-1} mmol/l^{-1}; Lee et al., 1996).

These discrepancies in the kinetic constants were partially due to the differences in pH and temperature conditions during the reaction (Copper, Martinez, & Hirschhorn, 1992) or in the conformation of cathepsin Ls from different fish species. Additionally, the obvious differences in kinetic constants between silver carp cathepsin L2 and L1 (K_{cat} 23.7 s^{-1}, K_{cat}/K_m 2870 s^{-1} mmol/l^{-1}; Liu et al., 2006), might be attributed to the multiple forms of cathepsin L in silver carp muscle. In a previous study, the mammalian cathepsin L-cystatin complex or procathepsin L displayed a relative lower

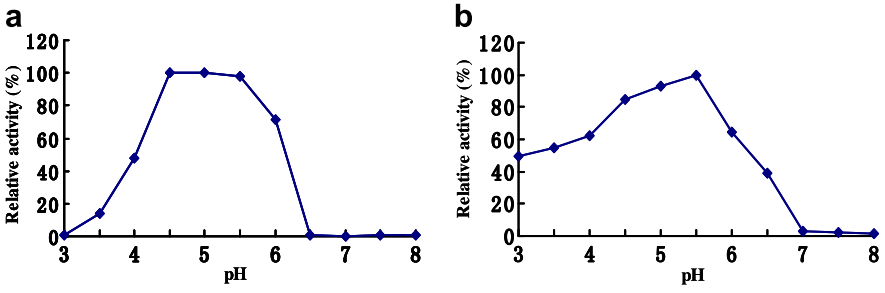

Fig. 4. Effect of pH on cathepsin L2. (a) pH dependency. Activity was assayed in McILvaine's buffer in a pH range of 3.0–8.0 with 2 mmol/l L-cysteine. (b) pH stability. cathepsin L2 was assayed after standing at 20 °C for 1 h in McIlvaine's buffer (pH 3.0–8.0) with 2 mmol/l L-cysteine. The relative activity against Z-Phe-Arg-MCA was expressed as a percentage of the activity measured or incubated at various pH to the maximum.

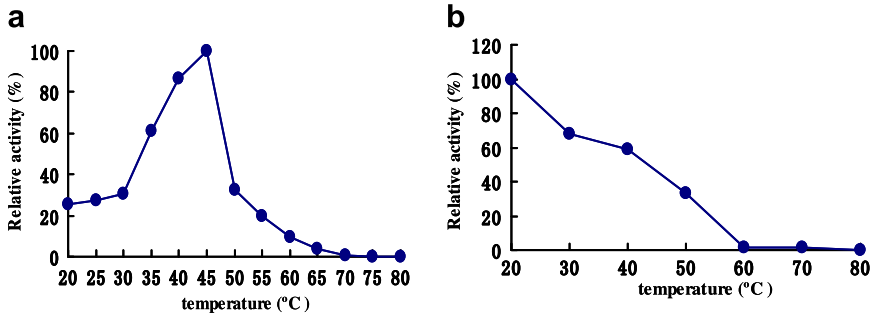

Fig. 5. Effect of temperature on cathepsin L2. (a) Temperature-dependency. Activity was assayed at temperatures between 20 °C and 80 °C. (b) Thermal stability. Cathepsin L2 was assayed after standing at 20–80 °C for 1 h. The relative activity against Z-Phe-Arg-MCA was expressed as a percentage of the activity measured or incubated at various temperatures to the maximum.

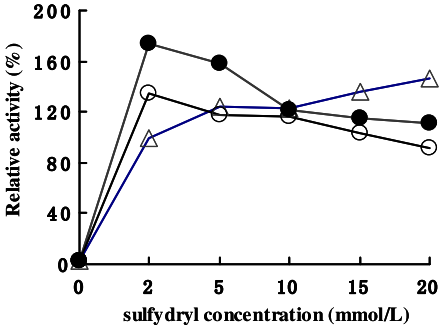

Fig. 6. Effect of sulphydryl agents on cathepsin L2. Various sulphydryls agents (0–20 mmol/l) were used in place of the usual 2 mmol/l cysteine in assay buffer. Activity determined in the presence of 2 mmol/l cysteine was taken as 100%. (○) Cys; (●) DTT; (△) β-Me.

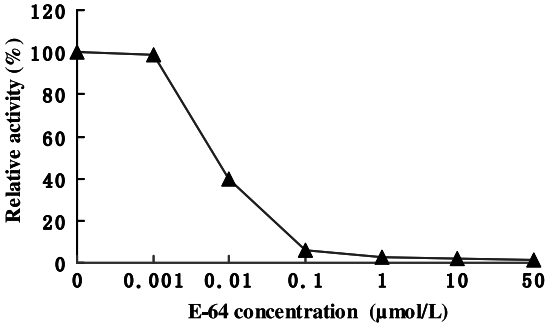

Fig. 7. Inhibitory effect of E-64 on cathepsin L2.

Table 2
Effect of inhibitors and salt ions on of cathepsin L2

Inhibitor	Concentration (mmol/l)	Relative activity (%)
Control		100
PMSF	0.1	116
	0.5	133
	2	100
EDTA	1	203
	2	155
	5	97
EGTA	1	146
	2	139
	5	131
Cl^-	50	93
	100	98
	200	76
$P_2O_7^{4-}$	25	0
	50	0

Z-Phe-Arg-MCA hydrolysis activity was measured after incubating with various inhibitors for 15 min at 30 °C or measured in presence of Cl^- and $P_2O_7^{4-}$ at 40 °C for 10 min.

Table 3
Specificity and kinetic constants for the hydrolysis of peptidyl-MCA substrates by cathepsin L2

Substrate	K_m (μmol/l)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mmol/l^{-1})
Z-Phe-Arg-MCA	9.5	0.8	84.2
Z-Arg-Arg-MCA	ND	ND	ND
L-Arg-MCA	ND	ND	ND

ND, non-detectable.

affinity to the fluorimetric substrate Z-Phe-Arg-MCA, and yielded an exceptionally lower catalytic coefficient as compared to its mature form (Coetzer et al., 1995; McDonald & Emerick, 1995).

3.7. Proteolysis of surimi protein–myosin

Considering the residual endopeptidase activity to Z-Phe-Arg-MCA at pH 6.0–6.5 and between 50 and 60 °C and the strong gelatinolytic activity, especially the 25.6% residual activity in silver carp surimi after washing (Li et al., 2004), the silver carp cathepsin L2 might be directly involved in the breakdown of the major textural protein–myosin in surimi and consequently lead to loss of surimi gel strength. Therefore, the degradation of silver carp myosin heavy chain (MHC) by cathepsin L2 was investigated (Fig. 8).

The analysis on SDS–PAGE showed that at pH 6.5, approaching to that of silver carp surimi (pH 6.5–7.5), and at softening temperature of 50–60 °C, silver carp cathepsin L2 hydrolysed the MHC effectively during an incubation of about 24 h, especially at 60 °C (Fig. 8a and b). Significantly, it was also observed that at acidic pH 5.0 the degradation of MHC by cathepsin L2 was dramatically accelerated even at a lower temperature of 35 °C as shown in Fig. 8c. These results were similar to the proteolytic actions of cathepsin Ls isolated from other fish species on the corresponding MHC (Ho et al., 2000; Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2003; Ogata et al., 1998).

Additionally, in despite of the certain difference of optimum temperature to Z-Phe-Arg-MCA, in aspect of hydrolysis of MHC in silver carp surimi, cathepsin L2 and cathepsin L1 displayed some similarity; both degraded the MHC more strongly near 60 °C (65 °C for cathepsin L1 and at the same time it destroyed the network structure of silver carp surimi gels; Liu et al., 2006). Therefore, when considering improvement of the textural quality of silver carp surimi products, the influence of cathepsin L2 activity also could not be ignored.

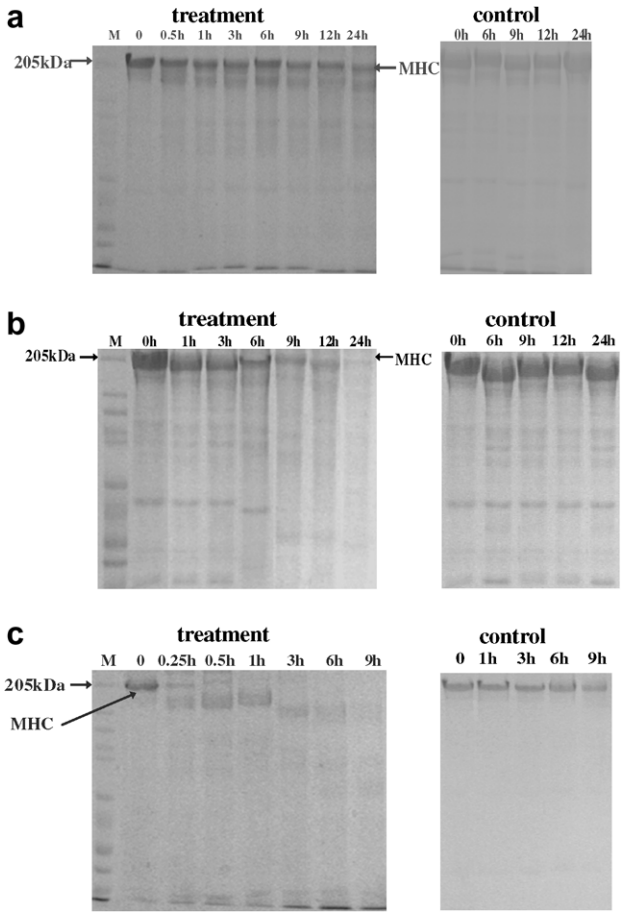

Fig. 8. SDS–PAGE of degradation of myosin heavy chain (MHC) by silver carp cathepsin L2. (a) At pH 6.5, 50 °C; (b) at pH 6.5, 60 °C; (c) at pH 5.0, 35 °C. M, the standard proteins.

4. Conclusion

According to this study, it was concluded that the purified cysteine protease from silver carp dorsal muscle could be characterised as cathepsin L2 with high molecular weight of about 78 kDa and instability to SDS and reducing conditions. By comparison of various characteristics, we considered that unlike cathepsin L1, cathepsin L2 might be the enzyme–endogenous inhibitor complex or precursor of the mature form. Despite that, cathepsin L2 still manifested considerable proteolytic activity on silver carp MHC, a major textural protein in surimi, at the pH 6.5 and 50–60 °C. Consequently, this study suggested that cathepsin L activity embodied in the form of L2 in surimi was also important in silver carp modori.

Upon storage of cathepsin L2, we observed cathepsin L activity increased markedly. It had been presumed that extended storage in the acid environment of pH 4.5, may have led to some limited hydrolysis and partial potential conversion of cathepsin L2 from the high-molecular weight form to the high-activity intermediate or the mature form. While the pH of surimi was much higher than 4.5, mouse procathepsin L was found to undergo conversion even at pH 6.0 (Mason & Massey, 1992). Moreover, cathepsin B-like from mackerel was also converted to cathepsin B at pH 6.2, near to that of postmortem fish muscle by adding a small amount of cathepsin B (Aoki et al., 2002). Besides, several other types of proteases such as metalo-protease (Hara, Kominami, & Katunuma, 1988), pepsin (Mort, Leduc, & Recklies, 1983) and cathepsin D (Wiederanders & Kirschke, 1989) had been demonstrated to be involved in the conversion of procathepsins *in vitro*. Whether during the processing of silver carp surimi, the supposed conversion could occur or whether there existed some factors that could inhibit or prompt it are still unknown. Consequently, the further investigation of cathepsin L2 based on this study would be necessary.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2008.04.072.

References

Agricultural Bureau of China (2002). Fisheries economic statistics. In *China Fisheries Yearbook* (p. 173). Beijing, China: Chinese Agricultural Press.

An, H., Peters, M. Y., Seymour, T. A., & Morrissey, M. T. (1995). Isolation and activation of cathepsin L-inhibitor complexes from Pacific whiting (*Merluccius productus*). *Journal of Agricultural Food Chemistry, 43*, 327–330.

Aoki, T., Nakano, T., & Ueno, R. (1997). Purification and some properties of a latent form cathepsin L from mackerel (*Scomber japonicus*) white muscle. *Fisheries Science, 63*(5), 824–829.

Aoki, T., & Ueno, R. (1997). Involvement of cathepsin B and L in the post-mortem autolysis of mackerel muscle. *Food Research International, 30*(8), 585–591.

Aoki, T., Yokono, M., & Ueno, R. (2002). A cathepsin B-like enzyme from mackerel white muscle is a precursor of cathepsin B. *Comparative Biochemistry Physiology, 133 B*, 307–316.

Aranishi, F., Ogata, H., Hara, K., Osatomi, K., & Ishihara, T. (1997). Purification and characterization of cathepsin L from hepatopancreas of carp *Cyprinus carpio*. *Comparative Biochemistry Physiology, 118B*(5), 531–537.

Barrett, A. J. (1980). Fluorimetric assays for cathepsin B and cathepsin H with methylcoumarylamide substrates. *Biochemical Journal, 187*, 909–912.

Barrett, A. J., & Kirschke, H. (1981). Cathepsin B, cathepsin H and cathepsin L. *Methods Enzymology, 80*(41), 535–561.

Benjakul, S., Seymour, T. A., Morrissey, M. T., & An, H. (1996). Proteinase in Pacific whiting surimi wash water: Identification and characterization. *Journal of Food Science, 61*(6), 1165–1170.

Coetzer, T. H. T., Dennehy, K. M., Pike, R. N., & Dennison, C. (1995). Baboon (*Papio ursinus*) cathepsin L: Purification, characterization and comparison with human and sheep cathepsin L. *Comparative Biochemistry Physiology, 112B*(3), 429–439.

Copper, Z., Martinez, G., & Hirschhorn, M. (1992). pH and temperature effects of the K_m values of muscle lactate dehydrogenase isozyme LDH-A4 from fishes of the family Sefaenidae (*Perciformes*). *Comparative Biochemistry Physiology, 103B*(3), 869–874.

García-Carreño, F. L., Dimes, L. E., & Haard, N. F. (1993). Sbustrate–gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Analytical Biochemistry, 214*, 65–69.

Godiksen, H., & Nielsen, H. H. (2007). New method to discriminate between cathepsin B and cathepsin L in crude extracts from fish muscle based on a simple acidification procedure. *International Journal of Food Science and Technology, 42*, 102–106.

Hara, K., Kominami, E., & Katunuma, N. (1988). Effect of proteinase inhibitors on intracellular processing of cathepsin B, H and L in rat macrophages. *FEBS Letters, 231*, 229–231.

Ho, M. L., Chen, G. H., & Jiang, S. T. (2000). Effect of mackerel cathepsins L and L-like, and calpain on the degradation of mackerel surimi. *Fisheries Science, 66*(3), 558–568.

Hu, Y., Morioka, K., & Itoh, Y. (2007). Hydrolysis of surimi paste from walleye pollock (*Theragra chalcogramma*) by cysteine proteinase cathepsin L and e.ect of the proteinase inhibitor (E-64) on gelation. *Food Chemistry, 104*(2), 702–708.

Ishidoh, K., Saido, T. C., Kawashima, S., Hirose, M., Watanabe, S., Sato, N., et al. (1998). Multiple processing of procathepsin L to cathepsin L in vivo. *Biochemical and Biophysical Research Communications, 252*(1), 202–207.

Jiang, S. T., Lee, J. J., & Chen, H. C. (1996). Proteolysis of actomyosin by cathepsins B, L, L-like and X from Mackerel (*Scomber australasicus*). *Journal of Agricultural Food Chemistry, 44*, 769–773.

Kawada, A., Hara, K., Kominami, E., Tezuka, T., Takahashi, M., & Takahara, H. (2000). Precursor of rat epidermal cathepsin L: Purification and immunohistochemical localization. *Journal of Dermatological Science, 23*, 36–45.

Kirschke, H., Kembhavi, A. A., Bohley, P., & Barrett, A. J. (1982). Action of rat liver cathepsin L on collagen and other substrates. *Biochemical Journal, 201*, 367–372.

Kominami, E., Tsukahara, T., Hara, K., & Katunuma, N. (1988). Biosyntheses and processing of lysosomal cysteine proteinases in rat macrophages. *FEBS Letters, 231*(1), 225–228.

Ladrat, C., Verrez-Bagnis, V., Noël, J., & Fleurence, J. (2003). In vitro proteolysis of myofibrillar and sarcoplasmic proteins of white muscle of sea bass (*Dicentrarchus labrax* L.): Effects of cathepsins B, D and L. *Food Chemistry, 81*, 517–525.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Lee, J. J., Chen, H. C., & Jiang, S. T. (1993). Purification and characterization of proteinases identified as cathepsins L and L-Like (58 kDa) proteinase from Mackerel (*Scomber australasicus*). *Bioscience, Biotechnology, and Biochemistry, 57*(9), 1470–1476.

Lee, J. J., Chen, H. C., & Jiang, S. T. (1996). Comparison of the kinetics of cathepsin B, L, L-like and X from the dorsal muscle of mackerel on the hydrolysis of methylcoumarylamide substrates. *Journal of Agricultural Food Chemistry, 47*, 774–778.

Liu, H., Yin, L. J., Zhang, N., Li, S. H., & Ma, C. W. (2006). Purification and characterization of cathepsin L from the muscle of silver carp (*Hypophthalmichthys molitrix*). *Journal of Agricultural Food Chemistry, 54*(25), 9584–9591.

Li, S. H., Zhang, N., Liu, H., & Ma, C. W. (2004). Preliminary study of the relationship between autolysis of silver carp (*Hypophthalmichthys molitrix*) myofibrillar proteins and endogenous cathepsin B, L and H. *Journal of China Agricultural University, 9*(5), 71–75.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Luo, Y. K., Kuwahara, R., Kaneniwa, M., Murata, Y., & Yokoyama, M. (2001). Comparison of gel properties of surimi from Alaska pollock and three freshwater fish species: Effects of thermal processing and protein concentration. *Journal of Food Science, 66*(4), 548–554.

Martone, C. B., Busconi, L., Folco, E. J., Trucco, R. E., & Sanchez, J. J. A. (1986). Simplified myosin preparation from marine fish species. *Journal of Food Science, 51*(6), 1554–1555.

Mason, R. W. (1986). Species variants of cathepsin L and their immunological identification. *Biochemical Journal, 240*, 285–288.

Mason, R. W., Gal, S., & Gottesman, M. M. (1987). The identification of the major excreted protein (MEP) from a transformed mouse fibroblast cell line as a catalytically active precursor form of cathepsin L. *Biochemical Journal, 248*, 449–454.

Mason, R. W., & Massey, S. D. (1992). Surface activation of procathepsin L. *Biochemical and Biophysical Research Communications, 189*(3), 1659–1666.

McDonald, J. K., & Emerick, J. M. C. (1995). Purification and characterization of procathepsin L, a self-processing zymogen of guinea pig spermatozoa that acts on a cathepsin D assay substrate. *Archives of Biochemistry and Biophysics, 323*(2), 409–422.

Mort, J. S., Leduc, M. S., & Recklies, A. D. (1983). Characterization of a latent cysteine proteinase from ascitic fluid as a high molecular weight form of cathepsin B. *Biochimica et Biophysica Acta, 755*(3), 369–375.

Ogata, H., Aranishi, F., Hara, K., Osatomi, K., & Ishihara, T. (1998). Proteolytic degradation of myofibrillar components by carp cathepsin L. *Journal of the Science of Food and Agriculture, 76*, 499–504.

Seymour, T. A., Morrissey, M. T., Peters, M. Y., & An, H. (1994). Purification and characterization of Pacific whiting proteases. *Journal of Agricultural Food Chemistry, 42*(11), 2421–2427.

Tsunemoto, K., Osatomi, K., Nozaki, Y., Hara, K., & Ishihara, T. (2004). Molecular characterization of cathepsin L from hepatopancreas of the carp *Cyprinus carpio*. *Comparative Biochemistry Physiology, 137B*, 107–114.

Visessanguan, W., Benjakul, S., & An, H. (2003). Purification and characterization of cathepsin L in arrowtooth flounder (*Atheresthes stomias*) muscle. *Comparative Biochemistry Physiology, 134B*, 477–487.

Visessanguan, W., Menino, A. R., Kim, S. M., & An, H. (2001). Cathepsin L: A predominant heat-activated proteinase in arrowtooth flounder muscle. *Journal of Agricultural Food Chemistry, 49*(5), 2633–2640.

von Figura, K., & Hasilik, A. (1986). Lysosomal enzymes and their receptors. *Annual Review of Biochemistry, 55*, 167–193.

Wang, J. Z., & Fan, M. (2000). Gel electrophoresis of protein under undenaturation condition. In *Manual of protein technology* (pp. 111–123). Beijing, China: Scientific Press.

Wiederanders, B., & Kirschke, H. (1989). The processing of a cathepsin L precursor *in vitro*. *Archives of Biochemistry and Biophysics, 272*(2), 516–521.

Yamashita, M., & Konagaya, S. (1990). Purification and characterization of cathepsin L from the white muscle of chum salmon, *Oncorhynchus keta*. *Comparative Biochemistry Physiology, 96B*(2), 247–252.