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Existence of Cathepsin L and its Characterization in Red Bulleye Surimi

Yaqin Hu, Katsuji Morioka and Yoshiaki Itoh
Laboratory of Aquatic Product Utilization, Graduate School of Agriculture,
Kochi University, Nankoku, Kochi, 783-8502, Japan

Abstract: Cathepsin L could not be removed completely during conventional actomyosin extraction and still remained in the actomyosin of red bulleye surimi. Sepharose 6B gel filtration profile showed that the main peak of cathepsin L was separated from that of actomyosin suggesting the enzyme was non-binding to actomyosin. The fractions showing the main activity of cathepsin L were pooled and mentioned as L_{mix}. Optimal pH of cathepsin L in actomyosin and L_{mix} was 5.0 and the optimum temperature of L_{mix} was 45°C. Stability of L_{mix} was closely related to temperature and pH. At optimum temperature 45°C and optimum pH 5.0, activity of cathepsin L remained 76.8% after 120 min. At acidic pH 4.0, it remained 25% of its original activity after incubation at 45°C for 120 min. At neutral pH 7.0, incubated at 45°C, cathepsin L decreased the activity to 50% within 30 min and remained only 3% after 120 min. When incubated at low temperature 25°C, cathepsin L kept 83-85% of its original activity at pH 4.0, 5.0 and 7.0. Effect of NaCl concentration on cathepsin L was related to pH. At neutral pH 7.0, in 0.6 M NaCl solution, cathepsin L activity was about 85% that of pH 5.0, indicating that cathepsin L might contribution to the gel deterioration of red bulleye surimi even in neutral condition. Studies of substrate specificity and effects of activators and inhibitors confirmed L_{mix} to be a thiol-type cysteine protease.

Key words: Red bulleye surimi, cathepsin L, Sepharose 6B, cysteine protease, E-64, STI

INTRODUCTION

Cathepsins, such as cathepsin B, L, H, are usually characterized as members of the lysosomal cysteine protease (active site) family and the cathepsin family name has been synonymous with lysosomal proteolytic enzymes (Kirschke and Barrett, 1987). Cathepsin L is the predominant lysosomal endoproteinase of cysteine proteinases. Since cathepsin L was purified from human liver (Mason *et al*., 1985), it has been isolated from various fish and well characterized (Yamashita and Konagaya, 1990a; Lee *et al*., 1993; Aranishi *et al*., 1997; Visessanguan *et al*., 2003). It appears that cathepsin L from fishes has similar properties to those from mammals and chicken (Mason, 1986; Mason *et al*., 1984, 1985; Dofour *et al*., 1987). These enzymes exist in their processed form as disulfide-linked heavy and light chain subunits with molecular weights ranging from 20-35 kDa (Aranishi *et al*., 1997). It exhibits high hydrolytic activity toward the synthetic substrate Z-Phe-Arg-MCA. Cathepsin L is capable of hydrolyzing a broad range of proteins including myosin, actin, nebulin, cytosolic proteins, collagen and elastin (Kirschke and Barrett, 1987). Yamashita and Konagaya (1990b) reported that cathepsin L was the most probable protease responsible for the degradation of myofibrillar proteins in salmon muscle. This enzyme was also reported to be contributed to the degradation phenomena in pacific whiting surimi (An *et al*., 1994).

Currently, cathepsin L is known to have strong hydrolysis activity on a broad range of proteins from terrestrial animals (Etherington *et al*., 1990). In mackerel (Jiang *et al*., 1996), the myosin heavy chain was degraded by cathepsin L into 164, 155 KDa fragments. Consequently, the remained cathepsin L caused surimi gel softening (Jiang *et al*., 1997).

In recent years, red bulleye (*Priacanthus macracanthus*) surimi enlarged its application for the production of kamaboko in Japan. In pre-experiment, we found the gel strength of red bulleye kamaboko was rather low. Electrophoretogram showed the myosin heavy chain was degraded to some extent when the kamaboko was heated at 40-60°C, indicating the effect of endogenous enzymes. Thus, it would be much important to elucidate the existence and characteristics of the endogenous enzymes in red bulleye so that to eliminate the degradation in red bulleye surimi-based productions.

Herein, this research was undertaken to investigate the existence of cathepsin L in natural actomyosin of red bulleye surimi. Some characteristics such as optimal pH and temperature, heat stability, substrate specificity, effect of activators and inhibitors as well as the effect of NaCl concentration were also evaluated.

Corresponding Author: Yaqin Hu, Laboratory of Aquatic Product Utilization, Graduate School of Agriculture, Kochi University, Nankoku, Kochi, 783-8502, Japan Tel/Fax: 81-88-864-5155

MATERIALS AND METHODS

Materials: Red bulleye surimi (*Priacanthus macracanthus*) obtained from a local producer of kamaboko products in Kochi (Japan) and was transported to Laboratory in ice. The surimi was immediately used for actomyosin extraction or stored at -80°C for future use.

Chemicals: leupeptin, Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, Arg-MCA and Boc-Gln-Ala-Arg-MCA were purchased from Peptide Institute Inc. (Osaka, Japan). Dithiothreitol (DTT), L-trans-epoxy-succinyl-leucyl-amido-(4-guanidino) butane (E-64), soy bean trypsin inhibitor (STI) and idoacetic acid were purchased from Sigma (St. Louis, MO). Other chemicals were of analytical grade.

Natural actomyosin (AM) extraction: Actomyosin (AM) was prepared as the method of Takashi and Arai (1970). Each of the procedures was performed at 4°C unless otherwise indicated. About 10 g surimi was homogenized with 4 volumes of I = 0.05 phosphate buffer (buffer A, pH 7.5) and centrifuged at 3,000×g for 10 min, using a non-bubbling homogenizer (Diameter 3.6 cm, Nihonseiki Kaisha Ltd., Tokyo Japan). Resulting supernatant was discarded and the precipitate was collected. The obtained precipitate was bleached 2 more times. The residue was resuspended in 3 volumes of buffer A containing 0.6 M NaCl (buffer B, pH 7.5) and stood for 20 h. The suspension was centrifuged at 10,000×g for 15 min. The supernatant was diluted with 10 volumes of cold deionized water. The sample was centrifuged at 3,000×g for 10 min. Resulting precipitate was dispersed in 50 mM phosphate buffer containing 0.6 M NaCl (buffer C, pH 7.0) and was considered as AM sample, which was ready for gel filtration.

Determination of protein concentration: Protein concentration was assayed by Biuret method (Robinson and Hodgen, 1940) with bovine albumin as standard.

Sepharose 6B gel filtration of actomyosin: AM suspended in buffer C was loaded to Excell SD450 column (2.6×40 cm) which was packed with Sepharose 6B (Pharmacia Fine Chemicals). The column was eluted with buffer C at 0.5 mL min^{-1}. Fractions (10 mL) were collected by a fraction-collector (Gilson 202, France). Cathepsin L activity was monitored for each fraction.

Assay of enzyme activity: Enzyme activity was measured by the method of Barrett (1980) and Barret and Kirschke (1981) with a slight modification. The substrate stock solution (10 mM Z-Phe-Arg-MCA) was diluted to 0.1 mM with cold deionized water before use. The reaction mixture comprised of 0.25 mL 0.4 M sodium acetate buffer (pH 5.5), containing 4 mM EDTA, 20 mM freshly made cysteine and 0.45 mL buffered enzyme solution. After preheating at 25°C for 2 min, reaction was immediately initiated by the addition of 0.25 mL of 0.1 mM substrate solution. Reaction mixture was incubated at 25°C for 30 min, the substrate hydrolytic reaction was terminated by adding 1.5 mL stop reagent (0.1 M sodium acetate buffer containing 0.1 M sodium monochloroacetate). The fluorescence intensity of the liberated 7-amino-4-methyl-coumarin (AMC) was measured with a fluorescence spectrophotometer (Hitachi 650-10S) at an excitation wavelength of 370 nm and emission wavelength of 460 nm. One unit was expressed as 1 nmol AMC in 30 min at 25°C.

Effect of pH on the activity of cathepsin L: Activities of cathepsin L in AM before gel filtration and in each of the fractions after gel filtration were monitored. When performing activity reaction, 0.4 M sodium acetic buffer containing 4 mM EDTA (pH 4.0, 5.0, 5.5, 6.0, 6.5 and 7.0) was added as a reaction buffer.

Effects of NaCl concentration on cathepsin L activity: Partially purified cathepsin L (L_{mix}, suspended in buffer C, pH 7.0) was adjusted to pH 5.0 with 7% acetic acid. NaCl concentrations in L_{mix} samples (pH 5.0 and pH 7.0) were adjusted to 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 M by either 3 M NaCl or deionized water.

Effect of temperature on the activity of L_{mix}: AM and L_{mix} samples were adjusted to pH 5.0 with 7% acetic acid. The pH 7.0 and pH 5.0 samples were first incubated at various temperatures for 2 min. The activity measurement was thus initiated by adding with specific substrates followed by incubating at 0, 25, 35, 45, 50, 55, 60, 70, 80°C for 30 min before terminated by stop reagent. The activity of cathepsin L was monitored.

Heat stability of L_{mix}: L_{mix} was adjusted to pH 4.0, pH 5.0 and pH 7.0 with 7% acetic acid and was thus incubated at 25 or 45°C for 0, 5, 10, 30, 60, 90, 120 min. The remaining activity was monitored at 25°C, 30 min as the method of assay of enzyme activity.

Substrate specificity of L_{mix}: The hydrolytic activity of L_{mix} on synthetic substrates including Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and Arg-MCA were determined according to the method of assay of enzyme activity. But instead of 0.25 mL of 0.1 mM substrate solution, 0.25 mL of substrate solutions were added to the final concentration of 0.1, 0.5, 1.0, 1.5 and 2.0 mM.

Effect of activators and inhibitors: The partially purified cathepsin L from red bulleye surimi was incubated for 15 min at 25°C with an equal volume of 0.2 mM E-64, 2 mM

Table 1: Effect of inhibitors and activators on activity of cathepsin L in AM and L_{mix}

Inhibitor/activator	concentration	AM (%)	L_{mix} (%)
Control	-	100	100
E-64	0.1 mM	0	0
Leupeptin	0.1 mM	0	0
IDA	1 mM	2.32	2.60
STI	0.1 g L^{-1}	109.59	102.60
EDTA[a]	2 mM	139.73	115.58
DTT	2 mM	205.48	285.71
EDTA+DTT	2 mM each	219.18	298.70

EDTA: Ethylenediaminetetra acetic acid

iodoacetic acid, 0.2 g L^{-1} soybean trypsin inhibitor, 0.2 mM leupeptin, 4 mM DTT and 4 mM EDTA to give the final concentrations listed in Table 1. Sample that incubated with an equal volume of deionized water was used as control. The remaining activity was analyzed.

RESULTS AND DISCUSSION

Gel filtration profile of cathepsin L: AM dispersed in buffer C was loaded on Sepharose 6B column. The column was eluted with buffer C at 0.5 mL min^{-1}. Profiles of AM and cathepsin L were shown in Fig. 1.

Cathepsin L still remained in actomyosin after 3 bleaching followed by one dilution-precipitation during actomyosin extraction. This was consistent with the fact that cathepsin L remained in pacific whiting surimi after several times of bleaching (An *et al*., 1994).

It is very interesting that the main peak of cathepsin L in red bulleye surimi was separated from that of actomyosin, indicating that cathepsin L was most possibly not bound with actomyosin. Our pre-experiment results showing that increasing of bleaching and dilution-precipitation cycles could not remove cathepsin L effectively (data not shown here). We postulated that cathepsin L might have some interaction with actomyosin. As a result, despite it was not bound with actomyosin, cathepsin L still remained in actomyosin after conventional washing and dewatering.

Effect of pH on the cathepsin L activity: Lysosomal cathepsins are commonly known to be much more active under acidic conditions (Ogata *et al*., 1998). In this study, cathepsin L displayed higher activity at acidic range than that at neutral one (Fig. 2 and 3). Optimum pH of cathepsin L in AM extracted from red bulleye surimi was pH 5.0 . In red bulleye surimi, cathepsin L in AM at pH 4.0 was 72.7% of that at pH 5.0. At pH 6.0, relative activity decreased to about 23.4% that of pH 5.0. At pH 7.0, it decreased to 21.2% that of pH 5.0.

After gel filtration, cathepsin L in fractions also showed optimum pH at 5.0. Cathepsin L in fraction of 200 mL elution buffer displayed the highest activity.

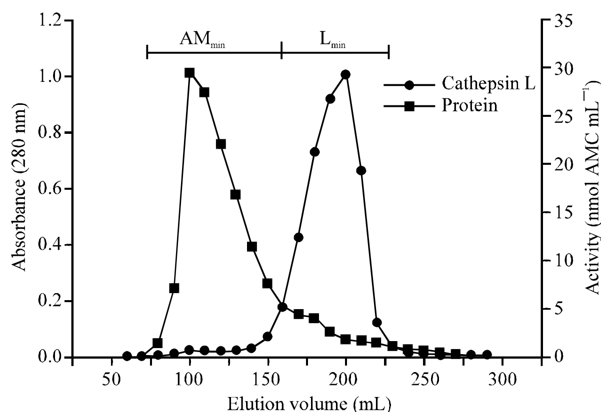

Fig. 1: Sepharose 6B gel filtration of actomyosin from red bulleye surimi AM extracted from red bulleye surimi was loaded on Sepharose 6B gel filtration column. The column was eluted with 0.6 M NaCl-50 mM phosphate buffer, 0.5 mL min^{-1}. Fractions were collected 10 mL $tube^{-1}$

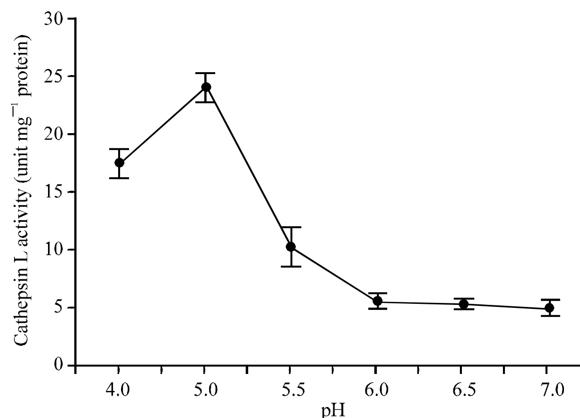

Fig. 2: The pH dependence of cathepsin L in actomyosin

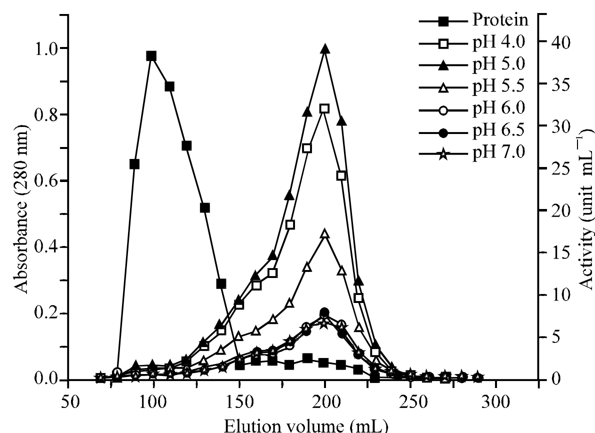

Fig. 3: The pH dependence of cathepsin L in actomyosin gel filtration fractions

The maximum activity at pH 4.0 of red bulleye surimi was 31.5% that of pH 5.0. At pH 6.0 and 7.0, the maximum activity rapidly decreased to 19.5 and 17.7% that of pH 5.0.

Effects of NaCl concentration on cathepsin L activity: In kamaboko products, 2-3% NaCl (0.3-0.5 M) was necessary to form gel network. Jiang *et al.* (1996) reported that NaCl could affect the hydrolytic activity of cathepsin L on AM and the hydrolytic patterns. Therefore, the effect of NaCl concentration on cathepsin L activity was examined in this study.

After gel filtration, fractions that containing cathepsin L main activities were pooled to be L_{mix} (Fig. 1). Activity of L_{mix} under various NaCl concentrations was measured. Before activity assay, L_{mix} was adjusted to pH 5.0 with 7% acetic acid. NaCl concentration in L_{mix} (both pH 5.0 and pH 7.0 samples) was adjusted to 0.1, 0.2, 0.4, 0.6, 0.8 M, by 3 M NaCl solution or deionized water, respectively.

Activity of the partially purified cathepsin L (L_{mix}) at 1.0 M NaCl, pH 5.0 was used as 100%, results were shown in Fig. 4. In red bulleye surimi, concentration effect of NaCl on the activity of cathepsin L was closely related to pH. In acetic range, at pH 5.0, cathepsin L activity increased with the increasing of NaCl concentration above 0.2 M. On the contrary, in neutral range, at pH 7.0, the activity decreased in the range of 0.2-0.4 M of NaCl. Further increment of the NaCl concentration had no obvious effect on the activity of cathepsin L.

When NaCl was less than 0.36 M, activity of cathepsin L at pH 7.0 was higher than that at pH 5.0. Above 0.36 M NaCl, cathepsin L activity at pH 7.0 was much lower than that of pH 5.0. In 1.0 M NaCl, activity of cathepsin L at pH 7.0 was about 53% that of pH 5.0. In 0.6 M NaCl, activity of cathepsin L at pH 7.0 was much low, but still had about 85% that of pH 5.0. This result indicates that cathepsin L might be related to the gel deterioration of red bulleye surimi even in neutral condition.

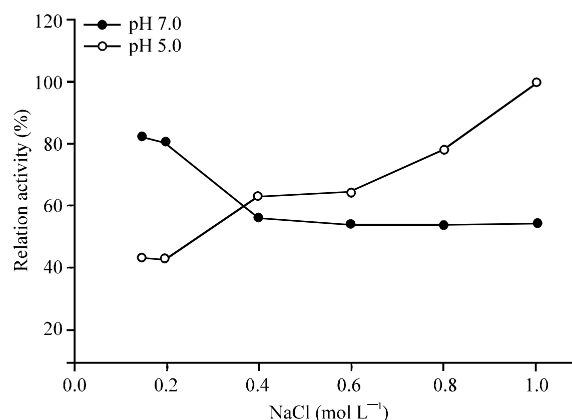

Fig. 4: Effect of NaCl concentration on the activity of cathepsin L in L_{mix}

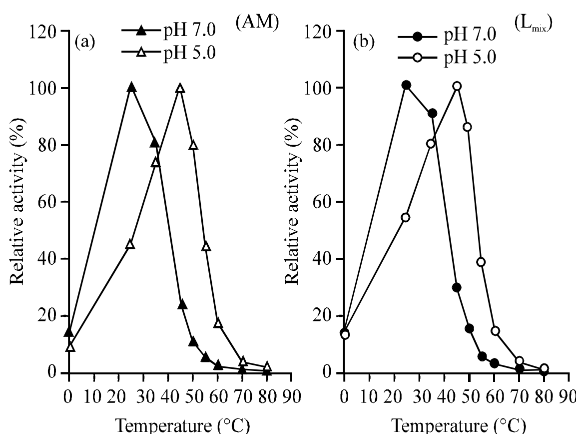

Fig. 5: Temperature profiles of cathepsin L in AM (a) and L_{mix} (b) AM and L_{mix} was adjusted to pH 7.0 and 5.0. The resulting samples were pre-incubated at different temperatures for 2 min before adding with substrates and incubated for exactly 30 min. The activity reaction was terminated by stop regent and fluorescence was read at excitation wavelength of 370 nm, emission wavelength of 460 nm

Effect of temperature on the activity of L_{mix}: Temperature profiles of cathepsin L in AM and L_{mix} closely related to the pH (Fig. 5).

At optimal pH of 5.0, cathepsin L in L_{mix} had optimum temperature of 45°C. This was consistent with that from other species with optimal temperature in the ranges of 45-50°C (Visessanguan *et al.*, 2003). Consequently, cathepsin L was concluded to be a predominant proteinase responsible for autolysis of arrowtooth flounder muscle.

On the other hand, in neutral condition, at pH 7.0, cathepsin L had optimum temperature of 25°C. Cathepsin L was in an enzyme-inhibitor complex form, dissociation from inhibitors made the enzyme more susceptible than in a complex (Seymour *et al.*, 1994; An *et al.*, 1995). This might explain why the optimal temperature of partially purified cathepsin L (L_{mix}) was 45°C, higher than that in AM which is 25°C.

Heat stability of L_{mix}: L_{mix} samples adjusted to different pH were incubated at 25 or 45°C for different time intervals. The remaining activities were monitored.

Incubated at optimum temperature 45°C, partially purified cathepsin L (L_{mix}) was sensitive to pH. At optimal pH 5.0, its activity decreased to 87.7% within 10 min. But after that, it was stable and after 120 min, it still had

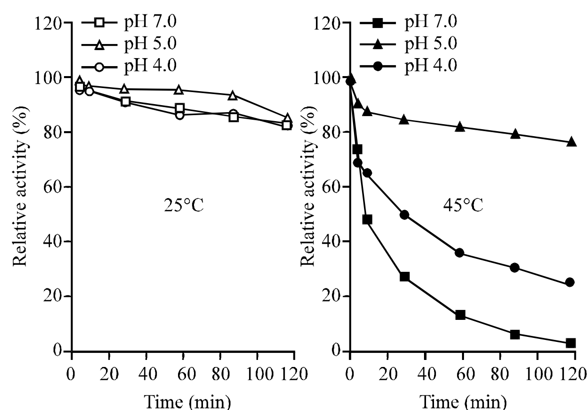

Fig. 6: Stability of partially purified cathepsin L (L_{mix}) L_{mix} was adjusted to objective pH and incubated at 25 or 45°C with different time intervals. The remaining activity was monitored at 25°C, 30 min.

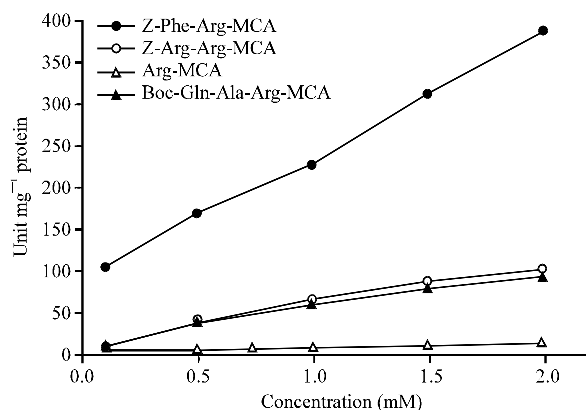

Fig. 7: Activity of cathepsin L in L_{mix} on various synthetic substrates

76.8% of its original activity. At pH 4.0, after incubation of 120 min, it still had 25% of its original activity. It was well known that cathepsin L is an acidic enzyme and cannot expose long time to neutral and alkaline conditions. In this study, at pH 7.0, activity of cathepsin L decreased with the incubation time. The activity decreased to 50% within 30 min. After 120 min, it remained about 3% of its original activity (Fig. 6).

On the contrary, in low temperature, either at acidic or neutral condition, cathepsin L kept much stable. After incubated at 25°C for 120 min, it still remained 83-85% of its original activity despite the value was much low.

Visessanguan *et al.* (2003) reported the purified cathepsin L in Arrowtooth flounder lost 80% of its activity after incubated at optimum temperature of 55°C within 10 min. Compared to that in Arrowtooth flounder, cathepsin L appeared to be a highly heat stable enzyme in red bulleye surimi. Purified enzymes were known to be much more sensitive than crude ones (Seymour *et al.*, 1994). And the variation of fish species might be another reason to explain the difference.

Effect of activators and inhibitors: The partially purified cathepsin L from red bulleye surimi was incubated for 15 min at 25°C with an equal volume of activators and inhibitors. The remaining activity of cathepsin L was monitored as the method of assay of enzyme activity.

Partial or complete inhibition was observed in presence of thiol-blocking agents, E-64 and iodoacetic acid, confirming that the partially purified proteinase was a thiol proteinases (Table 1). Enzymatic activity was enhanced by thiol-activating agents, such as DTT and EDTA, used for specific activation of cysteine proteinase activity. Proteinase inhibitor for serine proteases, STI, did not show distinctive effects on the activity of cathepsin L in AM and L_{mix}.

Substrate specificity of L_{mix}: The hydrolytic activity on synthetic substrates including Boc-Gln-Ala-Arg-MCA, Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and Arg-MCA were determined. The enzymatic reaction was performed at 25°C by the addition of 0.250 mL of substrate solutions to the final concentration ranging from 0.10-2.0 mM (Fig. 7).

The partially purified proteinase could strongly hydrolyze Z-Phe-Arg-MCA, a specific substrate commonly used to assay cathepsin L activity. Arginine is a very efficient P1 residue for the hydrolysis of methylcoumarin substrates for cathepsins (Mason *et al.*, 1985). Cathepsin L has only endopeptidase activity and preferentially cleaves peptides bonds with hydrophobic amino acid residues in P2 and P3 (Kargel *et al.*, 1980, 1981). Purified cathepsin L hydrolyzed only Z-Phe-Arg-MCA suggesting that hydrophobic amino acid was necessary for substrate specificity of arrowtooth flounder proteinases (Visessanguan *et al.*, 2003).

The partially purified proteinase also showed some hydrolytic activities on Z-Arg-Arg-MCA, Z-Arg-MCA and Boc-Gln-Ala-Arg-MCA specific substrates for cathepsin B, H and trypsin. The hydrolytic activity was obvious especially when the substrates were in high concentrations. This was considered to be the contamination of cathepsin B, H and trypsin in L_{mix}.

In conclusion, cathepsin L could not be removed completely during conventional bleaching and still remained in actomyosin disregarding the fact that it was non-binding with actomyosin in red bulleye surimi. Partially purified cathepsin L had optima temperature and pH of 45°C, 5.0, respectively. It was highly heat-stable in mild condition (pH 7.0, 25°C) as well as optimal pH and temperature. Substrate specificity and the effect of activators and inhibitors confirmed the enzyme to be a thiol-type cysteine protease.

The actomyosin non-binding cathepsin L displayed high activity in neutral condition indicating its potential hydrolysis ability on actomyosin of red bulleye surimi during kamaboko processing. The degradation behavior of L_{mix} on red bulleye surimi and kamaboko are currently undertaken in our laboratory.

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