Proteolysis of Actomyosin by Cathepsins B, L, L-like, and X from Mackerel (*Scomber australasicus*)

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Cathepsins B and L and cathepsin L-like proteinase degraded myosin heavy chain of actomyosin (AM) into several fragments with molecular masses (MW) of 154, 146, 138, 67, and 36 kDa; 164 and 155 kDa; and 135, 128, and 69 kDa, respectively. In the presence of 0.6 M NaCl, however, the MHC was degraded by cathepsin L into 164, 155, 41, and 37 kDa fragments. The hydrolytic rate of these three proteinases on AM was faster at pH 5.0 than at pH 6.0. Actin seemed to be resistant against hydrolysis by cathepsins B, L, and L-like in the absence of 0.6 M NaCl. According to SDS−PAGE analysis, cathepsin X, a novel cysteine proteinase, did not degrade AM.

Keywords: *Mackerel; cathepsins; actomyosin; proteolysis; seafood*

INTRODUCTION

Functions of lysosomal proteinases, such as cathepsins B, D, H, and L, have been studied in aging processes of terrestrial animal muscle (Etherington et al., 1987, 1990; Mikami et al., 1987; Ouali et al.; 1987) and in muscle-softening of chum salmon caught during spawning migration (Yamashita and Konagaya, 1990a−c, 1991). From the studies on the hydrolysis of myofibrillar and cytoskeletal proteins by these proteinases, cathepsins B and L were considered to be more critical than cathepsin D, since the pH optimum for cathepsins B and L (pH 5.5−6.5) (Lee et al., 1993; Jiang et al., 1994a) was near that of postmortem muscle. Cathepsin H had no hydrolytic action on myofibrils. Some cathepsin L-like proteinases were also found to affect the surimi-based products of fish muscle (Masaki et al., 1993; Toyohara et al., 1993). Except for myofibrillar proteins, cathepsins B and L from terrestrial animal muscle also hydrolyzed collagen (Kirschke et al., 1982) and proteoglycan (Nguyen et al., 1990). Although cathepsins B (Matsumiya et al., 1989) and L (Yamashita and Konagaya, 1990b) from fish muscles have been purified, studies on the degradation of fish muscle proteins by these proteinases are still limited.

In our endeavors through a series of investigations of the participation of muscle cysteine proteinases in postmortem tenderness of fish muscle, cathepsins L and L-like (Lee et al., 1993) and B (Jiang et al., 1994a) and a novel cysteine proteinase (designated cathepsin X; Jiang et al., 1994b) have been purified from mackerel. This study was undertaken to compare the degradation profiles of myofibrillar proteins produced by these proteinases.

MATERIALS AND METHODS

Materials. Mackerel (*Scomber australasicus*), iced for about 4 h before experiment, was ocean-harvested north of Taiwan. Electrophoresis calibration kits were purchased from Pharmacia (Uppsala, Sweden). Acrylamide, *N*,*N*′-methylenebis(acrylamide), and dye reagent for the protein assays were obtained from Bio-Rad (Hercules, CA). Coomassie blue G-250 and dithiothreitol (DTT) were obtained from Merck (Darmstadt, Germany). Pepstatin A was the product of Sigma (St. Louis, MO). Z-Phe-Arg-MCA (4-methylcoumaryl-7-amide) and Z-Arg-Arg-MCA were purchased from Peptide Institute Inc. (Osaka, Japan).

Preparation of Cysteine Proteinases. The enzyme assays of cathepsins B, L, and L-like and a novel cysteine proteinase (designated cathepsin X) from the dorsal muscle of mackerel were performed according to our previous descriptions (Lee et al., 1993; Jiang et al., 1994a,b). One unit of enzyme activity was expressed as the amount of enzyme that can hydrolyze methylcoumarylamide substrate and release 1 μmol of aminomethylcoumarin within 1 min of reaction at 35 °C.

Preparation of Actomyosin. Crude actomyosin (AM) was extracted from the dorsal muscle of mackerel according to the method of Noguchi and Matsumoto (1970). After dilution with 10 volumes of deionized water, the AM was centrifuged at 5000*g* for 20 min. The precipitates were dissolved in 50 mM phosphate buffer (pH 6.0) containing 0.6 M NaCl and stored at 4 °C until needed. For degradation by cathepsins, AM was suspended in 50 mM sodium acetate or 50 mM phosphate buffer at various pH levels. The protein concentration was finally adjusted to around 1 mg/mL using the same buffer solution.

Degradation of Actomyosin by Cathepsin B at 37 °C. AM in 50 mM phosphate buffer (pH 6.0) was incubated with various concentrations of cathepsin B at 37 °C for 3 or 6 h. The reaction mixtures were 0.5 mL and contained 2 mM DTT, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM pepstatin, 0.2 mg of AM, and various concentrations of cathepsin B (0, 0.56, 1.12, 1.68, 2.24, and 2.80 units, respectively) in 50 mM phosphate buffer.

AM was incubated with cathepsin B under various pH values at 37 °C for 12 h. The reaction mixtures were 0.5 mL and contained 2 mM DTT, 1 mM EDTA, 0.1 mM pepstatin, 0.2 mg of AM, and 7 units of cathepsin B in either 50 mM sodium acetate buffer (pH 4.0, 4.5, 5.0, and 5.5) or 50 mM phosphate buffer (pH 6.0, 6.5, 7.0, and 7.5). Degradation of AM was assayed by means of SDS−PAGE.

Degradation of Actomyosin by Cathepsin L at 37 °C. AM was incubated with cathepsins L and L-like at various pH values. The reaction mixture was 0.5 mL and contained 2 mM DTT, 1 mM EDTA, 0.2 mg of AM, and 0.72 unit of cathepsin L or L-like in 50 mM sodium acetate buffer (pH 4.0, 4.5, 5.0, 5.5, and 6.0). After 2 h of incubation at 37 °C, the resulting samples were subjected to SDS−PAGE analysis.

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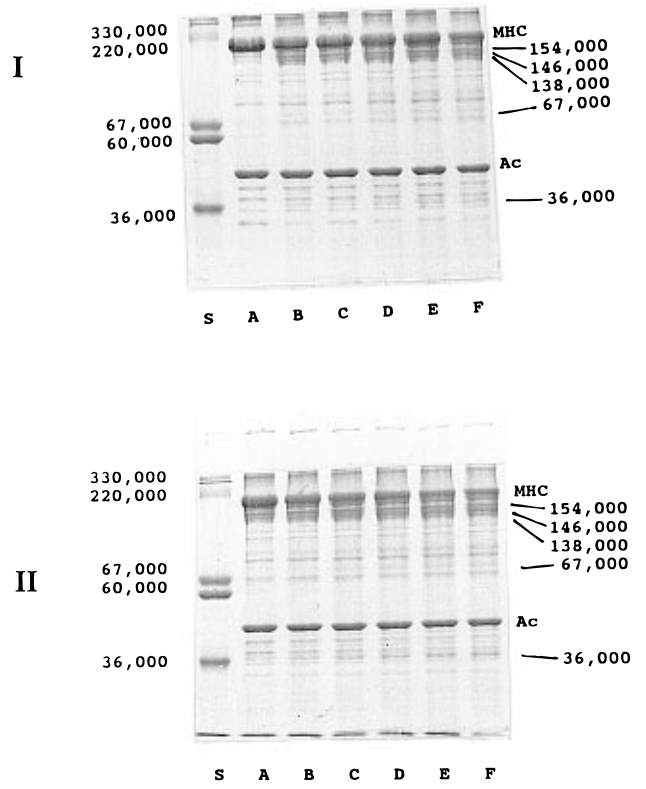

Figure 1. Effect of cathepsin B on the degradation of mackerel AM incubation at 37 °C, pH 6.0, for 3 (I) or 6 h (II). Lanes: S, standard proteins of HMW; A, without cathepsin B; B, with 0.56 unit; C, with 1.12 units; D, with 1.68 units; E, with 2.24 units; F, with 2.80 units of cathepsin B. MHC, myosin heavy chain; Ac, actin.

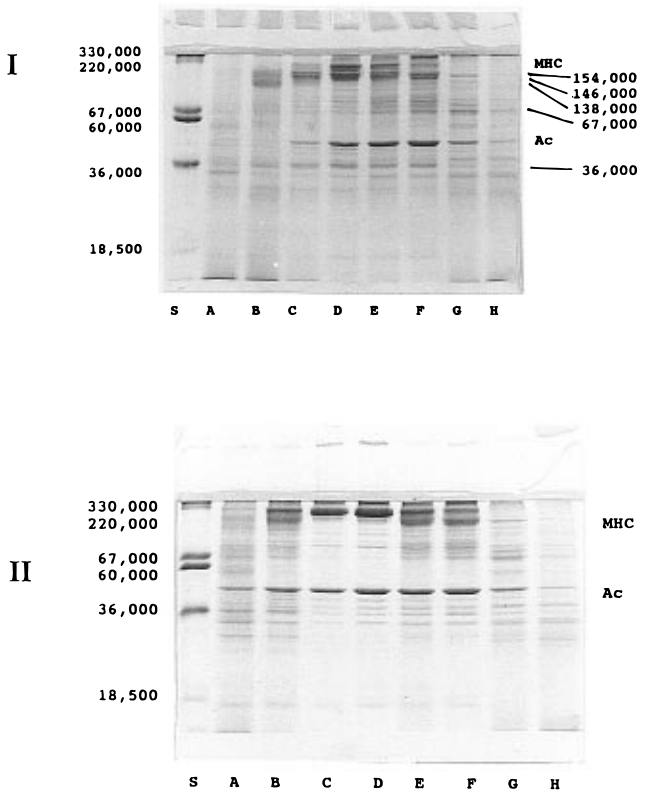

Figure 2. Effect of pH on the degradation of mackerel AM incubated with (I) or without cathepsin B (II) at 37 °C for 12 h. Lanes: S, standard proteins of HMW; A, pH 4.0; B, pH 4.5; C, pH 5.0; D, pH 5.5; E, pH 6.0; F, pH 6.5; G, pH 7.0; H, pH 7.5. MHC, myosin heavy chain; Ac, actin.

Degradation of AM by cathepsin L in the presence of 0.6 M NaCl under similar conditions was also investigated.

Comparison of the Degradation of Actomyosin by Mackerel Cathepsins B, L, L-like, and X at 25 °C. To compare the susceptibility of AM to mackerel cysteine proteinases under postmortem conditions of fish muscle, AM was incubated with 10 units of cathepsins B, L, L-like, and X. In the hydrolysis experiment by cathepsins B and X, the reaction mixture was 0.5 mL and contained 2 mM DTT, 1 mM EDTA, 0.2 mg of AM, and either 10 units of cathepsins B or X in 50 mM sodium acetate buffer (pH 5.0) or 50 mM phosphate buffer (pH 6.0). In the hydrolysis experiment with cathepsins L and L-like, the reaction mixture was also 0.5 mL and contained 2 mM DTT, 1 mM EDTA, 0.2 mg of AM, and either 2.5 units of cathepsin L or 10 units of cathepsin L-like in 50 mM sodium acetate buffer (pH 5.0) or 50 mM phosphate buffer (pH 6.0). After 3 h of incubation at 25 °C, all samples were subjected to SDS−PAGE analysis.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). After being incubated with or without mackerel cysteine proteinases, 3 mL of the protein samples was dissolved in 3 mL of dissociation buffer [final concentration: 62.5 mM Tris-HCl buffer, 2% SDS, and 5% β-mercaptoethanol (β-Me); pH 6.8], and then heated at 100 °C for 3 min. The resulting samples (30 μL) were subjected to 10% polyacrylamide gel electrophoresis in the presence of 1% SDS according to the method of Laemmli (1970). Gels were fixed, stained, and destained as described by Neuhoff et al. (1988). High (thyroglobulin, 330 kDa; ferritin, 220 kDa; albumin, 67 kDa; catalase, 60 kDa; and lactate dehydrogenase, 36 kDa) and low MW (phosphorylase *b*, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa) calibration kits were mixed and used as standard markers.

Determination of Protein Concentration. Protein concentration was determined using a protein−dye binding method (Bradford, 1976) with γ-globulin as standard.

RESULTS AND DISCUSSION

Degradation of Actomyosin by Cathepsin B at 37 °C. Comparing the electrophoregrams of mackerel actomyosin (AM) incubated with and without cathepsin B, the intensity of myosin heavy chains (MHC; MW 200 kDa) on AM with cathepsin B decreased. Several new bands with MW of 154, 146, 138, 67, and 36 kDa were observed after 3 h of incubation at 37 °C (Figure 1-I, lanes B−F). The degradation progressed during 6 h of incubation (Figure 1-II, lanes B−F). The AM *per se* was not degraded after 3 h of incubation at 37 °C (Figure 1-I, lane A) but was slightly degraded after 6 h of incubation (Figure 1-II, lane A). This could have been due to the contamination of endogenous proteinases. With actomyosin, however, actin seemed to be resistant to the proteolysis by cathepsin B. These data suggest that cathepsin B could strongly hydrolyze the MHC of AM but did not hydrolyze actin.

To further investigate whether the degradation of AM was caused by purified cathepsin B or contaminated endogenous proteinases, AM at various pH values was incubated with or without cathepsin B at 37 °C for 12 h. From the electrophoregrams of AM incubated with cathepsin B (70 units/mg of AM), the breakdown of MHC occurred at all samples (Figure 2-I, lanes A−H). However, degradation of MHC of AM incubated without cathepsin B was also observed at pH 4.0−7.5, except at pH 5.0 and 5.5 (Figure 2-II, lanes C and D). This phenomenon could have been because of the contamination of some of the endogenous proteinases in mackerel AM. From the pH specificity of the proteinases, degradation of MHC at pH 4.0−4.5 might be due to the proteolysis by acid proteinases (such as cathepsins), while that which occurred at pH 6.0−7.5 might be due

to the action of neutral or alkaline proteinases (such as calpains). Although the pH optima for cathepsins B and L are not at 4.0−4.5 or 6.0−7.5, cathepsin B still has 50% activity at pH 4.0 and more than 40% activity at pH 7.5 (Jiang et al., 1994a). Cathepsin L still has more than 60% activity at pH 4.0 (Lee et al., 1993). Since 0.1 mM pepstatin and 1 mM EDTA had been added to inhibit the hydrolysis by endogenous cathepsin D and calpain, respectively (Jiang et al., 1991, 1993;Wang et al., 1993) during the incubation of AM without cathepsin B (Figure 2-II), the degradation of AM without cathepsin B (Figure 2-II) might be caused by the endogenous cathepsins B and/or L but not by cathepsin D or calpain. This postulation could also be confirmed by the progressive degradation of AM with purified cathepsin B after 12 h of incubation at various pH values (Figure 2-I).

In this study, the appearance of the fragments with MW of 138 and 67 kDa (Figure 1-I) seemed to correspond to HMM (140 kDa) and LMM (77 kDa), respectively. The molecular masses of subfragment 1 of heavy chain (S_1), heavy meromyosin (HMM), light meromyosin (LMM), and rod of myosin subfragments were 90, 140, 77, and 120 kDa, respectively, estimated by SDS−PAGE (Margossian and Lowey, 1982). From this result, the cross-linking of HMM and LMM on AM was likely susceptible to mackerel cathepsin B. This phenomenon is similar to the observation by Dufour et al. (1989), who demonstrated that rat liver cathepsin B hydrolyzed rabbit myosin into HMM and LMM. However, Noda et al. (1981) found the MHC of rabbit myosin was easily degraded by rat liver cathepsin B into several fragments with MW of 175, 170, 160, and 145 kDa. The differences in the degradation profiles of rabbit myosin by cathepsin B might be due to the difference in proteinase sources.

Degradation by Cathepsin L at 37 °C. At pH 4.0−5.5, cathepsin L degraded the MHC into several fragments with MW of 164 and 155 kDa, in the absence of NaCl (Figure 3-I, lanes A−D), and 164, 155, 41, and 37 kDa in the presence of 0.6 M NaCl, respectively (Figure 3-II, lanes A−D). No significant changes in electrophoretical profiles of AM incubated without cathepsin L at pH 5.0 and 5.5 were obtained after 2 h of incubation at 37 °C (Figure 3-I and 3-II, a−d). The degradation profile of MHC by cathepsin L was similar to that by cathepsin B, but the actin was found to be susceptible to cathepsin L at pH 4.0 in the absence of NaCl (Figure 3-I, lane A). According to Matsukura et al. (1981), rat liver cathepsin L, except for the MHC, could degrade myosin light chains (LC). However, cathepsin L from mackerel muscle could degrade both actin and MHC (Figure 3-I, lane A).

According to SDS−PAGE on the degradation profiles of AM, the MHC was severely degraded at pH 4.0−5.5 both with and without NaCl (Figure 3-I and 3-II, lanes A−D) and completely degraded after 2 h of incubation at pH 4.0 in the absence of 0.6 M NaCl (Figure 3-I, lane A). These phenomena suggest that NaCl could affect the hydrolytic activity of cathepsin L on AM and the hydrolysate patterns. On the other hand, NaCl might induce conformational changes and increase the hydrophobicity of AM, which consequently cause the AM to be more susceptible to cathepsin L at pH 5.5. Therefore, some minor proteolytic fragments, such as 37 and 41 kDa, appeared on the SDS−PAGE electrophoregram after 2 h of incubation with cathepsin L at 37 °C and pH 5.5 (Figure 3-II, lane D).

Cathepsin L is capable of hydrolyzing the major muscle structural proteins, such as connectin, nebulin,

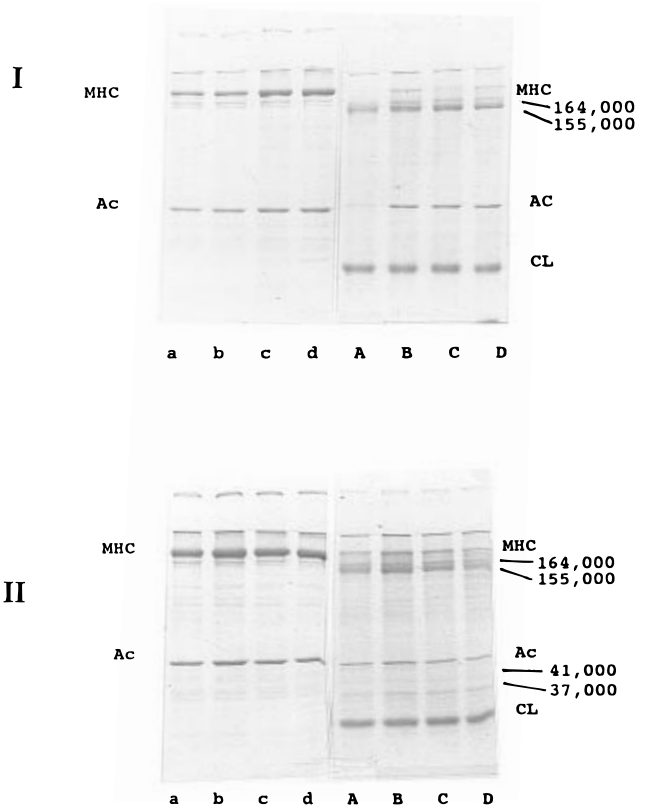

Figure 3. Effect of 0.6 M NaCl (I, without; II, with) on the degradation of mackerel AM incubated with cathepsin L at various pH values, 37 °C, for 2 h. Lanes: A and a, pH 4.0; B and b, pH 4.5; C and c, pH 5.0; D and d, pH 5.5; A−D, with cathepsin L (CL); a−d, without cathepsin L. MHC, myosin heavy chain.

myosin, collagen, α-actinin, and troponins T and I (Yamashita and Konagaya, 1991). The MHC of rabbit AM could be degraded into several fragments of 160, 92, 83, and 60 kDa by rat liver cathepsin L (Matsukura et al., 1981) or 130, 120, 90, 85, 31, and 30 kDa by rabbit liver cathepsin L (Mikami et al., 1987). According to Dufour et al. (1989), rat liver cathepsin L cleaved rabbit myosin at the head−rod junction, while the cathepsin L from mackerel muscle in our study seemed to attack on the HMM−LMM junction.

Although the pH optimum of mackerel cathepsin L for the hydrolysis of Z-Phe-Arg-MCA was pH 5.0 (Lee et al., 1993), a more severe degradation of MHC was observed at pH 4.0 (Figure 3-I, lane A) than at pH 5.0 (Figure 3-I, lane C). Degradation of collagen by rat liver cathepsin L was over 5 times faster at pH 3.5 than at pH 6.0 (Kirschke et al., 1982). The pH optimum for the degradation of glomerular basement membrane by human kidney cathepsin L was 3.5 (Baricos et al., 1988), while that for myosin degraded by rabbit cathepsin L was 4.1 (Okitani et al., 1980). The difference in optimal pH might be due to the conformational changes of each substrates at different pH buffer systems. Generally, muscle proteins are stable at neutral pH. When the pH is away from the stable pH range, the sensitive regions buried in the interior of substrate molecules are exposed and subjected to proteolytic hydrolysis. This low pH for the hydrolysis of protein substrates was similar to that for hydrolysis of collagen by rat liver cathepsin L (Mason et al., 1984). Since the optimal pH for the solubilization of collagen by cathepsin L was 3.3, the collagen substrate was highly swollen and the cleavage sites were exposed and attacked by cathepsin L at pH below 4.0 (Mason et al., 1984). On the other hand, due to steric

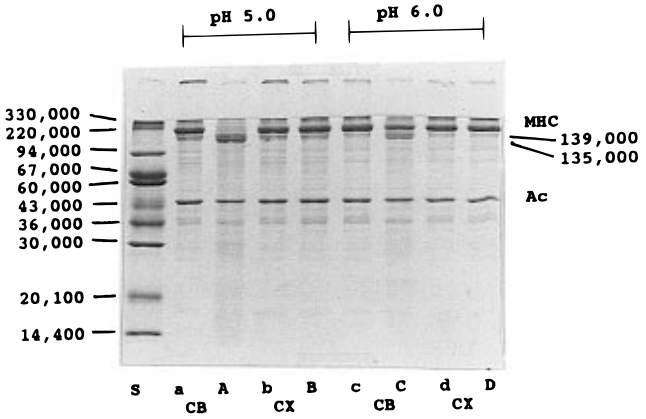

Figure 4. Effect of cathepsins B and X on the degradation of mackerel AM incubated at 25 °C, pH 5.0 or 6.0, for 3 h. Lanes: S, standard proteins of HMW and LMW; A and C, with cathepsin B (CB); B and D, with cathepsin X (CX); a−d, without proteinase. MHC, myosin heavy chain; Ac, actin.

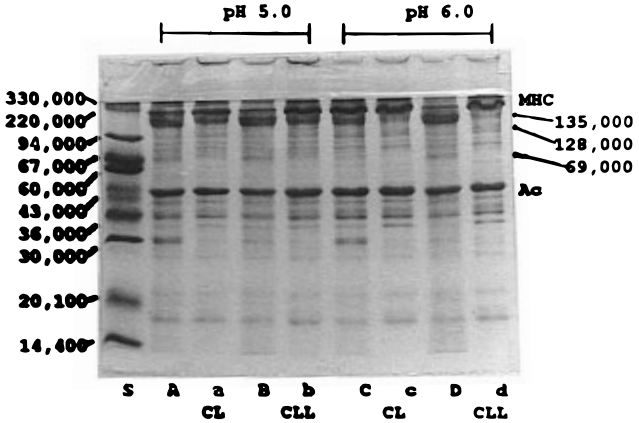

Figure 5. Effect of cathepsins L and L-like proteinase on the degradation of mackerel actomyosin incubated at 25 °C, pH 5.0 or 6.0, for 3 h. Lanes: S, standard proteins of HMW and LMW; A and C, with cathepsin L (CL); B and D, with cathepsin L-like proteinase (CLL); a−d, without proteinase. MHC, myosin heavy chain; Ac, actin.

hindrance the peptide substrates are much more easily accessed by the active sites of cathepsin L than are protein substrates. These make the pH optimum for Z-Phe-Arg-MCA higher than that for muscle protein substrates.

Comparison of the Degradation of Actomyosin by Cathepsins B, L, L-like, and X at 25 °C. Cathepsin B degraded MHC into two fragments with MW of 139 and 135 kDa after 3 h of incubation at 25 °C under pH 5.0 and 6.0 (Figure 4, lanes A and C, respectively). The degradation of AM by cathepsin B at pH 5.0 was faster than that at pH 6.0. However, the proteolytic fragments of AM by cathepsin B at 25 °C (Figure 4) were less than that at 37 °C (Figures 1 and 2). Although the optimal pH for cathepsin X was 6.0 for the hydrolysis of Z-Arg-Arg-MCA (Jiang et al., 1994b), no significant changes in SDS−PAGE profiles were observed on mackerel AM with cathepsin X at either pH 5.0 or 6.0 after 3 h of incubation at 25 °C (Figure 4, lanes B and D, respectively). When compared with cathepsins B and X, cathepsin X seemed to have no proteolytic activity on AM. However, according to Ouali et al. (1987), a new high MW cysteine proteinase from rat liver lysosomes revealed high proteolytic activity on MHC of rabbit myofibrils. The purified cathepsin X from mackerel muscle (Jiang et al., 1994b) was, therefore, considered to be different from the new cysteine proteinase obtained from rat liver (Ouali et al., 1987).

Cathepsin L degraded MHC into two fragments with MW of 135 and 128 kDa after 3 h of incubation at 25 °C under pH 5.0 and 6.0 (Figure 5, lanes A and C, respectively; component with MW of 30 kDa was cathepsin L). The proteolytic patterns of AM by cathepsin L at 25 and 37 °C were almost the same. The MHC of AM was degraded into three fragments with MW of 135, 128, and 69 kDa after 3 h of incubation with cathepsin L-like at both pH 5.0 and 6.0 (Figure 5, lanes B and D, respectively). The 135 000 and 69 kDa fragments seem likely to correspond to HMM and LMM, respectively. The result suggests that cathepsin L-like might have also attacked on the HMM−LMM junction of mackerel AM.

The degradation of native actomyosin by mackerel muscle cathepsins B and L in this study was similar to that of rabbit muscle cathepsins B (Okitani et al., 1980) and L (Schwartz and Bird, 1977), while Yamashita and Konagaya (1991) demonstrated that the susceptibility of denatured myofibrillar fragments and connective tissue against salmon cathepsins B and L was higher than that of native proteins. On the other hand, cathepsin L has shown the ability to degrade collagen and elastin, and its action on these substrates is severalfold greater than that of cathepsin B (Kirschke et al., 1982). Cathepsin L has also been found to be much more extensive than cathepsin B in degrading proteoglycan aggregates (Nguyen et al., 1990). Cathepsin B from rat liver could degrade rabbit F-actin at pH 5.0 (Schwartz and Bird, 1977). Cathepsin B from mackerel muscle degraded actin at pH 4.0−5.0 but did not degrade at pH 5.5−6.5 (around the pH of postmortem fish muscle) (Figure 2). Nevertheless, Okitani et al. (1980) demonstrated that myosin was degraded preferentially by rabbit muscle cathepsin L, and was not affected by cathepsin B. This discrepancy might be due to the different sources of enzyme, protein, and reaction conditions.

According to the proteolytic profiles of these cysteine proteinases on AM, there was a high MW component (MW ≥ 330 kDa) appearing on all of the figures. This might result from the cross-links of non-disulfide covalent bonds caused by transglutaminase during incubation (Jiang and Lee, 1992; Kamath et al., 1992) and/or from the contamination of titin or nebulin. Cathepsins B and L and cathepsin L-like proteinase degraded myosin heavy chain of AM. The hydrolytic rate on AM of these three cathepsins was faster at pH 5.0 than at 6.0. From the proteolytic patterns, actin seemed to be resistant to the hydrolysis by these three cathepsins in the absence of 0.6 M NaCl; however, it could be degraded by cathepsin L in the presence of 0.6 M NaCl. These data suggested that these three cathepsins might play important roles in the postmortem tenderization of mackerel. Although cathepsin X did not degrade native AM in this study, its effect on the denatured AM is interesting since the degradation of denatured proteins is highly influenced by the specific structural changes of the substrate rather than proteinase specificity (Muramoto and Seki, 1989).

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