Susceptibility of Opioid Peptides and Myofibrillar Proteins to Carp Cathepsin L

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The ability of carp cathepsin L to degrade opioid peptides and separate myofibrillar proteins was examined. The enzyme differentially released fragments from leucine-enkephalin, α-neoendorphin, and dynorphin, in which the common cleavage site of the enzyme was determined to be a Gly2−Gly3 bond. A Leu5−Arg6 bond in α-neoendorphin and dynorphin was also commonly cleaved by the enzyme. At a protein ratio of the enzyme to myofibrillar protein of 1:500, the preferences of the enzyme appeared to be myosin > tropomyosin > troponins T, I, and C > actin > α-actinin. Myosin heavy chain was completely degraded into some fragments by the enzyme within 30 min, and all of them underwent further degradation during subsequent incubation. Although troponins and tropomyosin were also completely degraded by the enzyme within 24 h, a small amount of actin and a large amount of α-actinin remained undegraded.

Keywords: *Carp; cathepsin L; Cyprinus carpio; myofibrillar proteins; opioid peptides*

INTRODUCTION

Post-mortem muscle tenderization of terrestrial and aquatic animals results from proteolytic degradation of muscular structures by endogenous proteinases (Penny, 1980; Etherington, 1984; Brown, 1986; Greaser, 1986; Asghar and Bhatti, 1987). Similar phenomena are observed in fish *surimi* paste during gel formation (Chang-Lee et al., 1989; Morrissey et al., 1993) and in white muscle of chum salmon *Oncorhynchus keta* during spawning migration (Mommsen et al., 1980; Konagaya, 1982; Nomata et al., 1985).

The proteinases responsible for these phenomena are considered to be lysosomal proteinases, in particular cathepsins B and L. A variety of hydrolytic actions have so far been studied about fish cathepsin B on synthetic substrates (Lee et al., 1996; Aranishi et al., 1997a), oligopeptides (Hara et al., 1988; Yamashita and Konagaya, 1990b), and myofibrils (Hara et al., 1988; Jiang et al., 1996, 1997) and about fish cathepsin L on synthetic substrates (Lee et al., 1996; Aranishi et al., 1997b), insulin B-chain (Yamashita and Konagaya, 1990a), and myofibrils (Yamashita and Konagaya, 1990c; An et al., 1994; Jiang et al., 1996, 1997). These studies indicate that cathepsin L, which expresses more potent endopeptidase activity than cathepsin B, is likely the best candidate for proteolytic breakdown of muscular structures during both ante-mortem and post-mortem states (Yamashita and Konagaya, 1991).

There has been, however, little elucidated about the specificity of fish cathepsin L for peptide bonds in oligopeptides and its preferences to individual myofibrillar proteins. The work presented in this paper is designed to examine the proteolytic actions of carp *Cyprinus carpio* cathepsin L on three opioid peptides having partially common structures and on separate myofibrillar proteins.

MATERIALS AND METHODS

Materials. Cultured carps (*C. carpio*, body weight of 800−900 g) were obtained from a commercial fish supplier (Nagasaki, Japan), and ordinary muscle was collected after decapitation. Carp cathepsin L was purified to homogeneity from the muscle according to the described method (Aranishi et al., 1997b), and carbobenzoxy-L-phenylalanyl-L-arginyl-4-methylcoumaryl-7-amide (Peptide Institute, Inc., Osaka, Japan) hydrolysis was monitored to identify its activity throughout purification. After the activity in the crude homogenate was concentrated by solid $(NH_4)_2SO_4$ at 20−80% saturation and then separated from the bulk of contaminating proteins by serial chromatographies, its final purification was accomplished by affinity chromatography on Blue-Sepharose CL-6B (Pharmacia Biotech., Uppsala, Sweden). Carp myosin (Tsuchiya and Matsumoto, 1975) and tropomyosin (Seki and Konno, 1975) and the mixture of actin and troponins (Konno and Seki, 1975) were also separated from the muscle according to each indicated method. Red sea bream α-actinin was a gift from Dr. Tachibana (Nagasaki University, Japan). Leucine-enkephalin, α-neoendorphin, and dynorphin were purchased from Wako Pure Chemicals Ind., Ltd. (Osaka, Japan), and L-3-carboxy-*trans*-2,3-epoxypropionyl-L-leucine-4-guanidinobutylamide (E-64) was the product of Peptide Institute, Inc. (Osaka, Japan). All other chemicals used were of analytical grade.

Determination of Protein. Protein concentration was determined according to the method of either Lowry et al. (1951) or Smith et al. (1985) utilizing a BCA protein assay kit (Pierce Chemical Co., Rockford, IL) referring to the manufacturer's instructions, both with bovine serum albumin as a standard.

Digestion of Opioid Peptide. The reaction mixture consisted of 400 nmol of opioid peptide and the enzyme at a protein ratio of 250:1 in 750 μL of 50 mM sodium acetate buffer (pH 5.5) containing 1 mM dithiothreitol and 1 mM EDTA. The reaction was carried out at 37 °C and then terminated after

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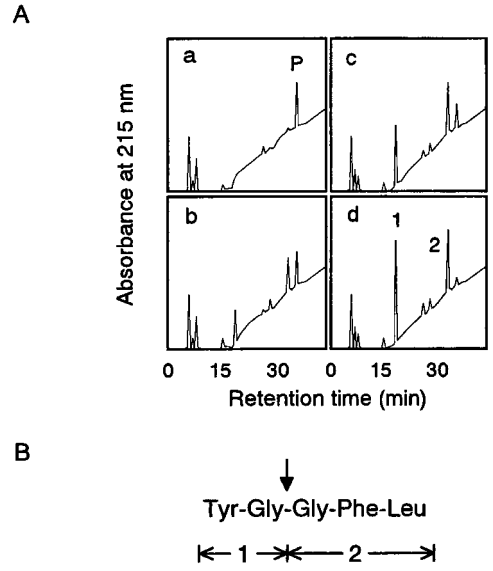

Figure 1. Action of carp cathepsin L on leucine-enkephalin. (A) RP-HPLC of leucine-enkephalin digested by cathepsin L. The mixture of peptide (P) and the enzyme was incubated at pH 5.5 and 37 °C for 0 (a), 30 (b), 60 (c), and 180 min (d), and resultant products were separated by RP-HPLC on a μBond-Pak C_{18} column and numbered according to the order of their elution. (B) Cleavage pattern of leucine-enkephalin by cathepsin L. The resultant products were analyzed for amino acid composition, and their amino acid sequences were deduced by reference to the structure of the substrate. The arrow indicates the cleavage site.

30, 60, and 180 min by addition of 5 μL of 2.7 mM E-64. The mixture was applied to reversed-phase high-performance liquid chromatography (RP-HPLC) on a Waters μBond-Pak C_{18} (Millipore Co., Bedford, MA) under a linear gradient of 0−50% acetonitrile in 0.1% trifluoroacetic acid in a Waters model 600 HPLC system. Elution was followed at 215 nm, and peptide eluate was separately collected and analyzed for amino acid composition after lyophilization and hydrolysis with 6.0 N HCl at 110 °C for 24 h. The control reaction was processed in the same manner with the exception that 2.7 mM E-64 was previously added to the reaction mixture.

Degradation of Myofibrillar Protein. The reaction mixture consisted of myofibrillar protein and the enzyme at a protein ratio of 500:1 in 1 mL of 25 mM sodium acetate buffer (pH 5.5) containing 2 mM cysteine and 1 mM EDTA. The reaction was carried out at 37 °C and then terminated after 10, 30, and 60 min and 24 h by boiling with an equal volume of a sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) application buffer containing 5.0% 2-mercaptoethanol. A portion of the mixture corresponding to 40 μg of the substrate protein content was applied to SDS−PAGE, which was performed according to by the method of Weber and Osborn (1969), using either a 5.0% separating gel for the assay of myosin degradation or a 12.5% separating gel for the assay of other proteins degradation. Proteins in the gel were stained with Coomassie Brilliant Blue R-250. The control reaction was processed in the same manner with the exception that the reaction mixture did not contain the enzyme.

RESULTS

Action of Carp Cathepsin L on Opioid Peptides. The actions of carp cathepsin L on leucine-enkephalin, α-neoendorphin, and dynorphin were examined at pH 5.5 and 37 °C, and the cleavage sites of the enzyme in these peptides were determined. Figure 1A shows that the enzyme cleaved leucine-enkephalin to release two fragments within 30 min and finally converted most of the peptide to these two fragments after 180 min. Two resultant fragments, which formed after enzyme digestion, indicate a single cleavage site in leucine-enkephalin. According to amino acid composition analysis, these resultant fragments consisted of two residues including Tyr and Gly and of three residues including Gly, Phe, and Leu. By reference to the structure of leucine-enkephalin, the enzyme cleaved this peptide at a Gly2−Gly3 bond (Figure 1B). Figure 2A shows that the enzyme slightly cleaved α-neoendorphin to release several fragments for 60 min and finally to release seven major fragments after 180 min. By reference of the substrate structure and by amino acid analysis of these resultant fragments, the enzyme cleaved α-neoendorphin at six sites (Figure 2B). Large amounts of two fragments split at a Leu5−Arg6 bond (fragments 2 and 5) appeared, indicating that it is the main cleavage site of the enzyme. In addition, four peptide bonds from Tyr1 to Leu5 were all determined to be the cleavage sites in α-neoendorphin. Figure 3A shows that the enzyme nearly completely cleaved dynorphin to release two major fragments (fragments 3 and 5) and three minor fragments (fragments 2, 4, and 6) within 30 min and that some of these resultant fragments were further degraded to lower products during subsequent incubation. This peptide seems to undergo enzyme digestion at the highest rate among the tested peptides. From amino acid analysis and the substrate structure, the enzyme cleaved dynorphin at four sites, Gly2−Gly3, Leu5−Arg6, Arg6−Arg7, and Arg7−Ile8 (Figure 3B). The first and second cleavage sites are consistent with the sites in other tested peptides and with the site in

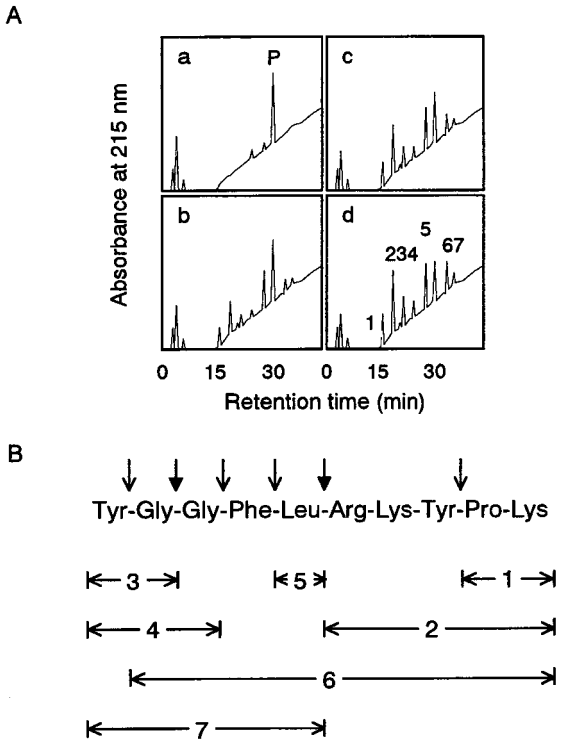

Figure 2. Action of carp cathepsin L on α-neoendorphin. (A) RP-HPLC of α-neoendorphin digested by cathepsin L. The mixture of peptide (P) and the enzyme was incubated at pH 5.5 and 37 °C for 0 (a), 30 (b), 60 (c), and 180 min (d), and resultant products were separated by RP-HPLC on a μBond-Pak C_{18} column and numbered according to the order of their elution. (B) Cleavage pattern of α-neoendorphin by cathepsin L. The resultant products were analyzed for amino acid composition, and their amino acid sequences were deduced by reference to the structure of the substrate. The bold arrows and arrows indicate the major cleavage sites and cleavage sites, respectively.

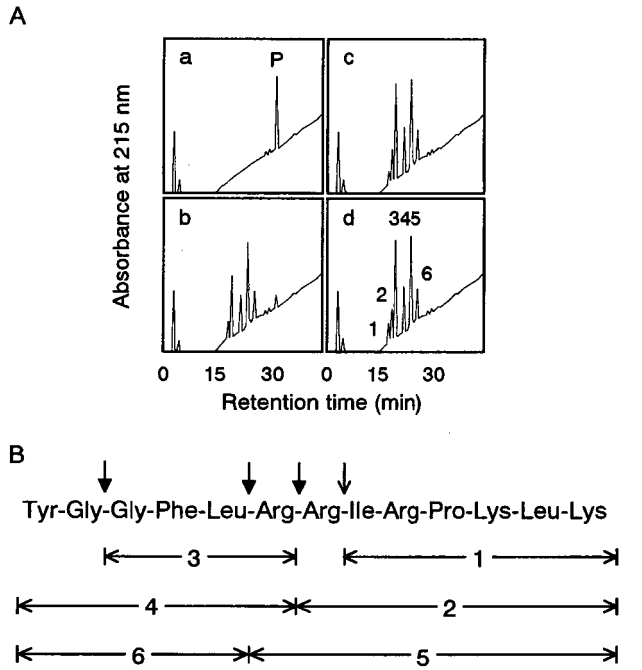

Figure 3. Action of carp cathepsin L on dynorphin. (A) RP-HPLC of dynorphin digested by cathepsin L. The mixture of peptide (P) and the enzyme was incubated at pH 5.5 and 37 °C for 0 (a), 30 (b), 60 (c), and 180 min (d), and resultant products were separated by RP-HPLC on a μBond-Pak C_{18} column and numbered according to the order of their elution. (B) Cleavage pattern of dynorphin by cathepsin L. The resultant products were analyzed for amino acid composition, and their amino acid sequences were deduced by reference to the structure of the substrate. The bold arrows and arrows indicate the major cleavage sites and cleavage sites, respectively.

α-neoendorphin, respectively. The RP-HPLC profiles and the structure of the resultant fragments indicate that fragment 1 is likely released by the enzyme from either fragment 2 or fragment 5 as time proceeded after 30 min. The sites that the enzyme cleaved preferentially were determined to be Gly2−Gly3, Leu5−Arg6, and Arg6−Arg7 bonds in dynorphin.

Action of Carp Cathepsin L on Myofibrillar Proteins. The actions of carp cathepsin L on carp myosin, actin, troponins, tropomyosin, and red sea bream α-actinin were examined at pH 5.5 and 37 °C and analyzed by SDS−PAGE. Figure 4 shows the change of myosin due to enzymatic activity during 24 h. The enzyme completely degraded myosin heavy chain (MHC) into some fragments with molecular weight (M_r) below 140 000 within 30 min. The largest fragment (M_r 140 000) was mostly degraded into lower products for 60 min, and the majority of other fragments (M_r from 110 000 to 140 000) also disappeared within 24 h. This was accompanied by the appearance of large quantities of two resultant proteins with M_r of 47 000 and 39 000. These two resultants are most likely secondary products derived from the initial products, which are directly released from MHC by the enzyme. The degradation of MHC was also found in the control fractions with no addition of cathepsin L, but it seemed to be much slower than that in the test fractions. Figure 5 shows the change of actin and troponins T, I, and C due to enzymatic activity. Although these proteins in the control fractions commonly underwent slow degradation during 24 h, all troponins and the majority of actin in the test fractions disappeared at 60 min and

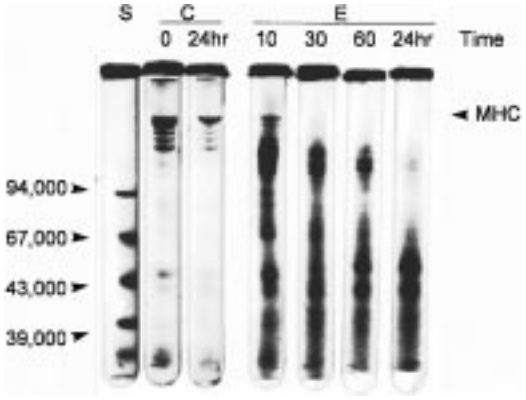

Figure 4. Action of carp cathepsin L on carp myosin. The mixture of myofibrillar protein and the enzyme (E) was incubated at pH 5.5 and 37 °C for 10 (10), 30 (30), and 60 min (60) and 24 h (24 h), and the resultant products and standard proteins (S) were applied to SDS−PAGE using a 5.0% separating gel under reducing condition. The control mixture without additional enzyme (C) was processed in the same manner.

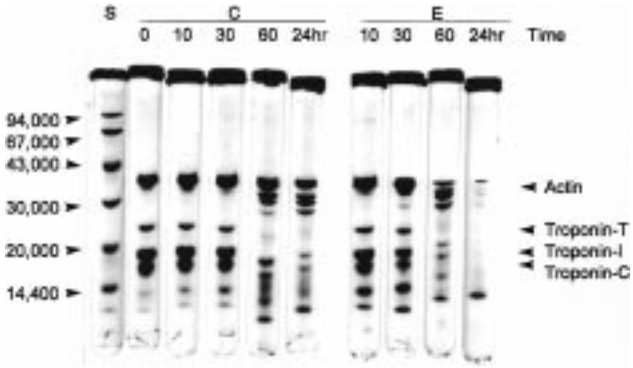

Figure 5. Action of carp cathepsin L on carp actin and troponins. The mixture of myofibrillar proteins and the enzyme (E) was incubated at pH 5.5 and 37 °C for 10 (10), 30 (30), and 60 min (60) and 24 h (24 h), and the resultant products and standard proteins (S) were applied to SDS−PAGE using a 12.5% separating gel under reducing condition. The control mixture without additional enzyme (C) was processed in the same manner.

24 h, respectively. Figure 6 shows the change of tropomyosin due to enzymatic activity, and similar slow degradation was observed in the control fractions. This protein in the test fractions was degraded by the enzyme to mainly release a small fragment with M_r of 17 000 within 60 min, and thereafter, the degradation proceeded to yield a remarkable increase in this or slightly lower M_r proteins for 24 h. Figure 7 shows the change of α-actinin due to enzymatic activity, and it underwent negligible degradation in the control fraction. This protein in the test fractions was marginally degraded by the enzyme during 24 h, but traces of an intermediate product with M_r of 44 000 appeared within 60 min. This product was further degraded into a lower product with M_r of 42 000 by subsequent incubation.

DISCUSSION

The digestive action of carp cathepsin L was tested on three opioid peptides having the same amino acid sequence of five residues from the N terminus. In this common structure, the enzyme cleaved a Gly2−Gly3 bond in three peptides (Figures 1−3) and additionally Tyr1−Gly2, Gly3−Phe4, and Phe4−Leu5 bonds only in α-neoendorphin (Figure 2). Moreover, a Leu5−Arg6

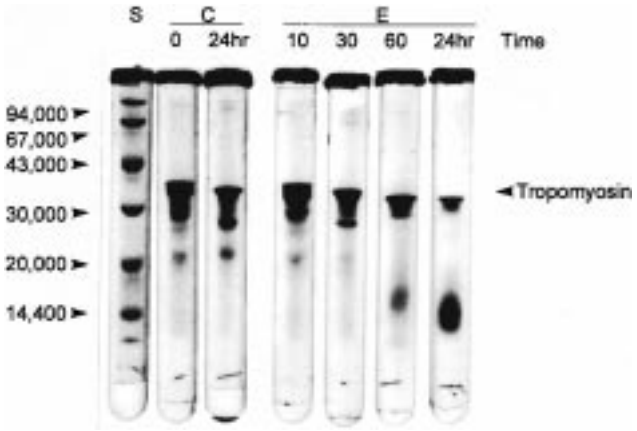

Figure 6. Action of carp cathepsin L on carp tropomyosin. The mixture of myofibrillar protein and the enzyme (E) was incubated at pH 5.5 and 37 °C for 10 (10), 30 (30), and 60 min (60) and 24 h (24 h), and the resultant products and standard proteins (S) were applied to SDS−PAGE using a 12.5% separating gel under reducing condition. The control mixture without additional enzyme (C) was processed in the same manner.

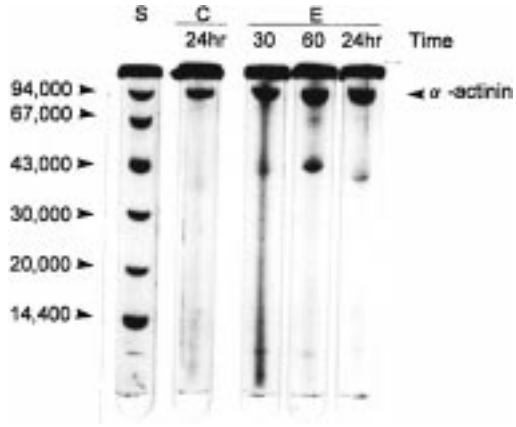

Figure 7. Action of carp cathepsin L on red sea bream α-actinin. The mixture of myofibrillar protein and the enzyme (E) was incubated at pH 5.5 and 37 °C for 10 (10), 30 (30), and 60 min (60) and 24 h (24 h), and the resultant products and standard proteins (S) were applied to SDS−PAGE using a 12.5% separating gel under reducing condition. The control mixture without additional enzyme (C) was processed in the same manner.

bond was determined to be the common and major cleavage site in α-neoendorphin and dynorphin, because of the appearance of large amounts of fragments split at this site (Figures 2 and 3). Marks and Berg (1987) report the digestion of opioid peptides, such as leucine-enkephalin, methionine-enkephalin, and their analogues, by rat brain cathepsin L. This enzyme cleaves a Gly2−Gly3 bond in all tested peptides having the same sequence of four residues from the N terminus. In PH-P8 corresponding to octapeptide of dynorphin from the N terminus, it additionally cleaves Arg6−Arg7 and a Arg7−Ile8 bonds. These results are very consistent with carp cathepsin L, indicating that similar hydrolytic actions of the carp and rat enzymes occur on peptide sequences having a hydrophobic group at the P_2 position. The major cleavage of a Leu5−Arg6 bond by the carp enzyme in α-neoendorphin and dynorphin is a typical example (Figures 2 and 3), but it is not confirmed in the digestion of PH-P8 and other peptides by the rat enzyme. Otherwise, carp cathepsin B cleaves leucine-enkephalin at a Gly3−Phe4 bond and α-neoendorphin at Leu5−Arg6 and Lys7−Tyr8 bonds, whereas fair amounts of the substrate peptides remain undigested after 24 h (Hara et al., 1988). The cleavage of a Leu5−Arg6 bond in α-neoendorphin is common between carp cathepsins L and B, but the tested peptides are cleaved at more sites and larger amounts by cathepsin L than cathepsin B (Figures 1 and 2). This is certainly caused by a potent endopeptidase activity and a broad range action of cathepsin L on peptide bonds.

Carp cathepsin L acted differently on each of the separate myofibrillar proteins (Figures 4−7). MHC, troponins, and tropomyosin were completely degraded into small fragments by the enzyme at 30, 60, and 30−60 min, respectively, but a small amount of actin and a large amount of α-actinin remained undegraded after 24 h. The preferences of the enzyme were, therefore, determined to decrease from MHC, tropomyosin, troponins, actin, and α-actinin. The degradation of these proteins produced some regular fragments, such as two fragments (M_r 47 000 and 39 000) from MHC, a single fragment (M_r 15 000) from actin and troponins, a few fragments (M_r 13 000−17 000) from tropomyosin, and a single fragment (M_r 42 000) from α-actinin, via intermediate products. This indicates the presence of limited cleavage sites of the enzyme in each individual myofibrillar protein. On the other hand, there was slow degradation of MHC (Figure 4), actin and troponins (Figure 5), and tropomyosin (Figure 6) in the control fractions during 24 h, probably due to endogenous cathepsins (Yamashita and Konagaya, 1990c; An et al., 1994; Jiang et al., 1997). The electrophoregrams of the control actin and troponins for 24 h and the control tropomyosin for 24 h are similar to those of the test actin and troponins for 60 min and the test tropomyosin for 30 min, respectively. It seems likely that cathepsin L is the major cathepsin acting on control myofibrillar proteins.

Fish cathepsin L causes marked proteolysis of MHC in a mixture of myofibrillar proteins (Yamashita and Konagaya, 1990c; An et al., 1994; Jiang et al., 1996, 1997). The enzymes from Pacific whiting *Merluccius productus* (An et al., 1994) and mackerel *Scomber australasicus* (Jiang et al., 1996) degrade MHC initially into intermediate products with M_r around ∼140 000 and further into a few fragments with M_r between 30 000 and 45 000 at pH 5.5 and 55 °C and at pH 5.0−6.0 and 25 °C, respectively. This is very consistent with the degradation profile of isolated MHC by the carp enzyme at pH 5.5 and 37 °C (Figure 4). The chum salmon enzyme, however, releases only three larger fragments with M_r of 90 000, 150 000, and 160 000 from MHC at pH 6.5 and 15 °C (Yamashita and Konagaya, 1990c). Since these four fish enzymes share biochemical characteristics including hydrolytic activity (Yamashita and Konagaya, 1990a; Lee et al., 1993; Seymour et al., 1994; Aranishi et al., 1997b), the discrepancy of MHC degradation profiles between the chum salmon enzyme and is counterparts is likely caused by different reaction conditions, such as pH and temperature. As well as MHC, the enzymes from carp (Figures 5−7), whiting (An et al., 1994), and salmon (Yamashita and Konagaya, 1990c) commonly degrade troponins, tropomyosin, and α-actinin to different extents. This shows that various myofibrillar proteins under isolated and mixed conditions are susceptible to fish cathepsin L. Otherwise, the preferences of carp cathepsin B for myofibrillar proteins are determined to be MHC > troponin T > actin >

troponin I, and it is unable to degrade tropomyosin and troponin C (Hara et al., 1988). During MHC degradation, a small amount of MHC is degraded by the enzyme to release only large fragments with M_r of 150 000−170 000 for 60 min. These indicate that carp cathepsin L has a broader range of action on myofibrillar proteins and a stronger action on more sites of MHC than carp cathepsin B, as is stated in peptide digestion.

Carp cathepsin L has different actions on isolated myofibrillar proteins, similar to its fish counterparts on mixed myofibrils, such as *surimi* (An et al., 1994; Jiang et al., 1997), actomyosin (Jiang et al., 1996), and muscle homogenate (Yamashita and Konagaya, 1990c). By utilizing separate myofibrils in this work, the degradation process of each individual myofibrillar protein by cathepsin L eventually to resultant fragments via intermediate products can be monitored. In the previous studies, enzyme incubations with myofibrils are performed in <1 h (Yamashita and Konagaya, 1990c), 2.5 h (An et al., 1994), 3 h (Jiang et al., 1996), and 5 h (Jiang et al., 1997). Longer incubation for 24 h performed in this work could stimulate complete degradation of myofibrillar proteins by cathepsin L.

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