Role of Pepstatin-Sensitive Proteases on the Postmortem Changes of Tilapia (*Tilapia nilotica* X *Tilapia aurea*) Muscle Myofibrils†

Shann-Tzong Jiang,*,‡ Yuh-Tai Wang,‡ Bih-Suey Gau,‡ and Ching-San Chen§

Department of Marine Food Science, National Taiwan Ocean University, Keelung, Taiwan 20224, ROC, and Institute of Botany, Academia Sinica, Nankung, Taipei, Taiwan 11529, ROC

To investigate the pepstatin-sensitive proteases in fish muscle tenderization, tilapia myofibrils (mf) (G-I), myofibrils with crude proteases added (G-II), and myofibrils with crude proteases and pepstatin added (G-III) at pH 5.5, 6.0, and 6.5 were incubated at 4 °C for 2.5–3.5 days. The increasing rate of the degree of myofibril fragmentation (DMF) during incubation was higher in G-I and G-II than in G-III, while the activity of pepstatin-sensitive proteases (cathepsin D was used as a marker enzyme) of G-II maintained an almost constant level. Evidence from phase mirographs also indicated that the proteolysis of myofibrils of G-I and G-II was much more severe than that of G-III. Disappearance of α-actinin on SDS–PAGE profiles of myofibrils (G-I) and myofibrils with crude enyzmes (G-II) during incubation at pH 5.5 and 6.0 demonstrated that the proteolysis of Z-disk by pepstatin-sensitive proteases occurred in postmortem myofibrils. However, the dispersed myosin heavy chain on SDS–PAGE profiles of all samples during incubation at pH 6.5 suggested that the activity of pepstatin-sensitive proteases decreased markedly at this pH condition and did not contribute to the myofibril degradation.

INTRODUCTION

Tenderness is a good parameter for the evaluation of fish quality, since the duration of rigor mortis of fish is much shorter than that of land animals (Suyama and Konosu, 1987). Some studies (Koohmaraie et al., 1986, 1988a,b) have been carried out to accelerate the tenderization of lamb and bovine muscle, with the subsequent intention to save the space and costs of aging. In the case of fish, however, because of the short period of tenderization, it is necessary to clarify the mechanisms further to retard the tenderization and consequently keep the fish quality.

Although tenderization is considered to be caused by the disappearance of Z-disks, dissociation of actomyosin complex, destruction of connectin, and denaturation of collagen (Robbins et al., 1979; Hatori, 1986; Suyama and Konosu, 1987; Koohmaraie et al., 1986, 1988a), the mechanisms of postmortem tenderization are still unclear (Koohmaraie et al., 1986, 1988a,b). Many changes during postmortem tenderness have generally been assumed to arise from the release of endogenous muscle proteases which are active at postmortem pH (Robbins et al., 1979). The proteases indigenous to skeletal muscle possibly include the Ca-dependent proteases located in sarcoplasm and cathepsins located in lysosomes (Asghar and Hendrickson, 1982; Declan et al., 1986; Ouali et al., 1987; Koohmaraie et al., 1988a,b). Many researchers recently reported that lysosomal enzymes were not involved in the myofibrillar protein breakdown in rat, bovine, and lamb muscle (Lowell et al., 1986; Furuno and Goldberg, 1986; Goodman, 1987; Koohmaraie et al., 1988a,b). However, the effects of pepstatin-sensitive proteases (mainly cathepsin D) were ignored, since the pepstatin-sensitive proteases are maximally active at pH around 3.0 (Okitani et al., 1981) and, therefore, the activity should be decreased at pH around 5.5–6.5, which is the postmortem pH in fish and shellfish. The question arising at this point is whether the pepstatin-sensitive proteases must be maximally active to cause sufficient damage to the myofibrils to produce the observed postmortem tenderness. According to Marsh (1981), the breaking of only one sarcomere in every 250 could result in a significant improvement in beef tenderness. In addition, Huang and Tappel (1971) reported that cathepsin D is the most important within the cathepsins in endogenous protein degradation since it initiates the protein hydrolysis and produces peptide fragments that can then be further broken down by the other cathepsins. On the basis of this point, it would seem likely that the pepstatin-sensitive lysosomal enzymes are involved in postmortem changes. This study aimed to investigate the effects of these proteases on fish myofibrils at pH around 5.5–6.5.

MATERIALS AND METHODS

Tilapia (*Tilapia nilotica* X *Tilapia aurea*) purchased from an aquatic farm in southern Taiwan were kept alive and transported to the laboratory. After being eviscerated, headed and washed, samples were used for crude enzyme extraction and myofibril preparation.

Preparation of Crude Enzymes. This fish meat was homogenized with 9 volumes of precooled acetone (−20 °C). The homogenate was filtered through a Büchner funnel and washed with precooled acetone again and once with ether. The acetone powder was air-dried overnight at room temperature and stored at −20 °C until use. The crude enzymes were then extracted with 10 volumes of 2% KCl solution from 20 g of acetone powder. After homogenizing, centrifugation was performed under 13000*g* for 30 min at 0 °C. The residue was washed with 10 volumes of 2% KCl solution again. The collected supernatants were used as crude enzymes in this experiment. All the preparations were carried out at 0–4 °C.

† This research work was supported by the National Science Council of Republic of China under Grant NSC 78-0406-B019-10.

* To whom correspondence should be addressed.
‡ National Taiwan Ocean University.
§ Institute of Botany, Academia Sinica.

0021-8561/90/1438-1464$02.50/0

Table I. Effect of the Pepstatin-Sensitive Enzymes on the Degree of Myofibril Fragmentation[a] during Incubation at 4 °C

pH	h at 4 °C	treatments[b] G-I	G-II	G-III
6.5	0	28.30cA[c]	28.30cA	28.30cA
	12	33.72bB	43.87bA	36.02bB
	84	66.02aB	91.54aA	41.44aC
6.0	0	22.72cA	22.72cA	22.72cA
	36	61.62dB	76.54aA	41.20bC
	60	75.25aA	78.13aA	50.97aB
5.5	0	22.72cA	22.72cA	22.72cA
	36	61.63bB	76.57bA	40.53aC
	60	87.97aA	81.27aA	44.66aB

[a] The degree of myofibril fragmentation (DMF) is expressed as the percentage ratio of the sum of myofibrils with four and less than four sarcomeres to the total myofibrils. [b] G-I: myofibrils suspended in a buffer containing 20 mM sodium acetate–hydrochloride, 0.09 M NaCl, and 10 mM NaN_3 (pH 5.5 6.0, and 6.5). G-II: myofibrils suspended in a buffer containing 20 mM sodium acetate–hydrochloride, 0.09 M NaCl, and 10 mM NaN_3 (pH 5.5, 6.0, and 6.5), with crude enzyme (7.8 units of cathepsin D/mg). G-III: myofibrils suspended in a buffer containing 20 mM sodium acetate–hydrochloride, 0.09 M NaCl, and 10 mM NaN_3 (pH 5.5, 6.0, and 6.5), with crude enzyme (7.8 units of cathepsin D/mg) and pepstatin (1 μg/mL). [c] Values in the same row bearing unlike uppercase letters differ significantly ($P < 0.05$). At each pH condition, values in the same column bearing unlike lowercase letters differ significantly ($P < 0.05$).

Preparation of Myofibrils. Minced fish meat was homogenized with 10 volumes of buffer I (0.03 M KCl, 0.04 M Tris-HCl, and 4.5 mM EDTA, pH 7.2) for 2 min. After centrifugation at 600*g* for 15 min, the residue was homogenized with 10 volumes of buffer II (0.08 M KCl, 0.04 M Tris-HCl, and 4.5 mM EDTA, pH 7.0) for 60 s. After centrifugation under the same conditions, the upper half layer of sediment was removed and resuspended in 10 volumes of buffer II by using a stir rod and passed through a nylon strainer (6 mesh) with four layers of cheesecloth to remove the connective tissue and debris. The filtrate was sedimented at 600*g* for 15 min and washed with buffer II again. The precipitate was suspended in 100 mL of buffers III, IV, and V (20 mM sodium acetate–hydrochloride, pH 5.5, 6.0 and 6.5, respectively, containing 0.09 M NaCl and 10 mM NaN_3) and passed through a nylon strainer (6 mesh) with four layers of cheesecloth. The filtrate was used as myofibrils.

Effect of Pepstatin-Sensitive Enzymes on the Fragmentation of Myofibrils. The myofibrils were suspended in buffers III, IV, and V (blank, G-I), suspended in buffers III, IV, and V with crude enzymes (7.8 units of cathepsin D/mg) (test, G-II), and suspended in buffers III, IV, and V with crude enzyme and pepstatin (1 μg/mL) (control, G-III). The resultant myofibrils were incubated at 4 °C for 2.5–3.5 days. The changes in the degree of myofibril fragmentation (DMF), analysis of sodium dodecyl sulfate discontinuous polyacrylamide gel electrophoresis (SDS–PAGE), and phase microscopy of the isolated myofibrils were investigated. In addition, the changes in the activity of cathepsin D (as a marker enzyme) and quantity of myofibrillar protein were also measured to evaluate the effects on the degradation of myofibrils.

Sodium Dodecyl Sulfate–Polyacrylaminde Gel Electrophoretic Analyses (SDS–PAGE). At a definite time interval, 5 mL of myofibril solutions of each group was removed and incubated at 100 °C for 3 min in a buffer consisting of 2% SDS, 5% 2-mercaptoethanol, and 62.5 mM Tris-HCl (pH 6.8). The solubilized proteins were analyzed with discontinuous SDS–PAGE according to the method of Laemmli and Favre (1973). After running in 25 mM Tris–glycine buffer (pH 8.3) for 1 h, the gel was silver stained (Robilloud et al., 1988).

Determination of the Activity of Cathepsin D. The acid-denatured hemoglobin which was prepared by dissolving 5% native hemoglobin (Difco Laboratory, Detroit, MI) in 0.06 N HCl and dialyzed against deionized water overnight was employed as substrate. The enyzme solution (0.5 mL) was mixed with 0.5 mL of 5% acid-denatured hemoglobin and 1.5 mL of McIlvaine buffer (pH 3.0). After incubation at 37 °C for 1 h, the reaction was stopped by adding 2.5 mL of 5% trichloroacetic acid (TCA). The reaction mixtures were stood at room temperature for 45 min and filtered through Whatman No. 42 filter paper. Nonprotein nitrogenous compounds in the filtrate were determined according to the method of Lowry et al. (1951). One nanomole of tyrosine equivalent releasing within 1 h at pH 3.0 was defined as 1 unit.

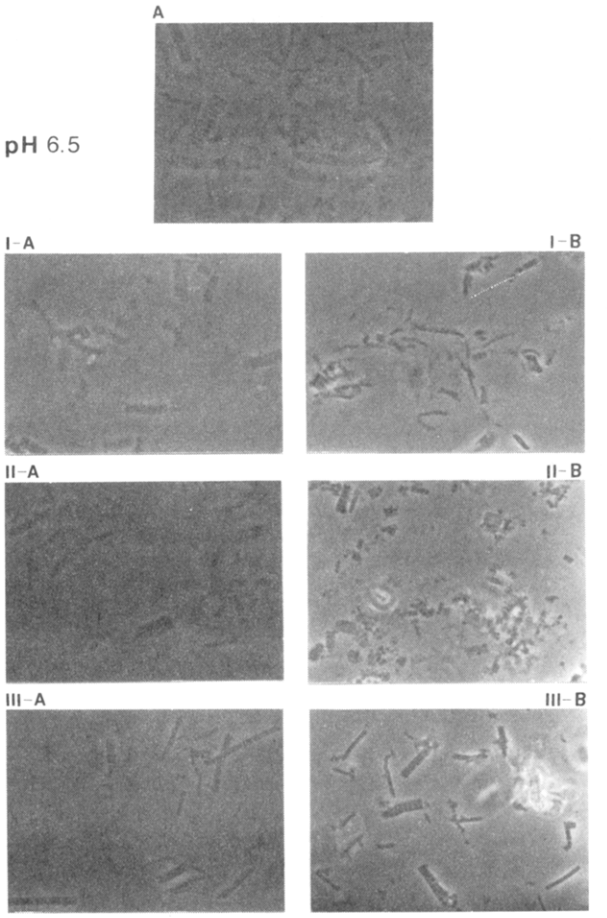

Figure 1. Effect of the pepstatin-sensitive enzymes on the phase micrograph of myofibrils during incubation at 4 °C at pH 6.5. (A) Before incubation. I-A, II-A, and III-A were the phase micrographs of G-I, G-II, and G-III after 1.5 days of incubation. I-B, II-B, and III-B were the phase micrographs of G-I, G-II, and G-III after 2.5 days of incubation.

Phase Microscopy. The phase microscopy was performed to determine the degree of myofibril fragmentation (DMF) and the change in number of myofibril sarcomeres. A drop of myofibril suspension was placed onto a glass slide and then covered by a coverslip. Excess fluid was blotted with bibulous paper. Finally the prepared slide was examined by phase contrast optics in a Nikon microscope. The DMF was expressed as the percentage ratio of the sum of myofibrils with four and less than four sarcomeres to the total myofibrils.

Statistic Analysis. Duncan's multiple range test was used for the statistical analysis.

RESULTS AND DISCUSSION

Effect of Pepstatin-Sensitive Proteases on the Myofibril Fragmentation. Myofibrils (G-I), myofibrils with crude proteases (G-II), and myofibrils with crude proteases and pepstatin (G-III) were incubated at 4 °C for 2.5–3.5 days at pH 6.5, 6.0, and 5.5. The degree of myofibril fragmentation (DMF) of all samples increased with the duration of incubation (Table I). The increasing rate of DMF was highest in G-II and then G-I and G-III in this order.

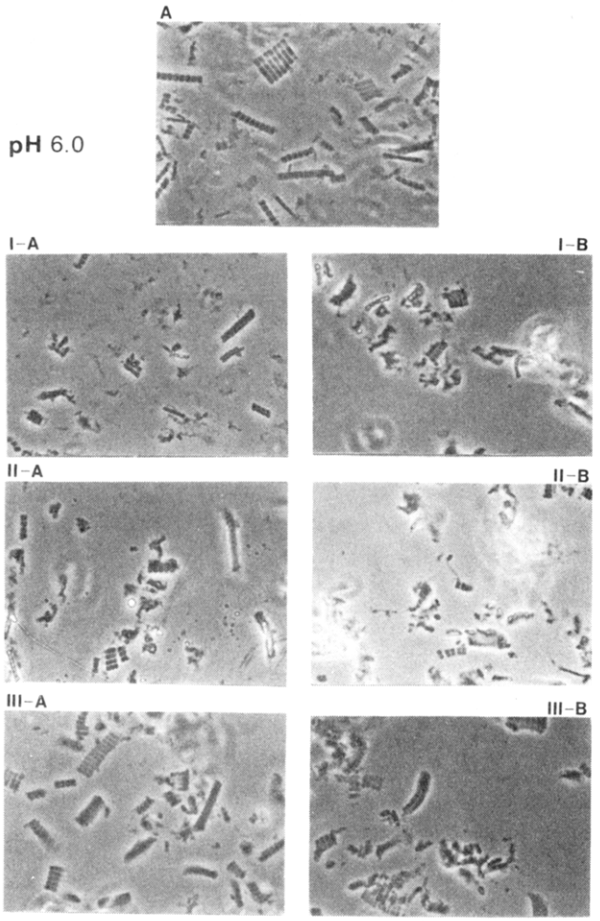

Figure 2. Effect of the pepstatin-sensitive enzymes on the phase micrograph of myofibrils during incubation at 4 °C at pH 6.0. (A) Before incubation. I-A, II-A, and III-A were the phase micrographs of G-I, G-II, and G-III after 1.5 days of incubation. I-B, II-B, and III-B were the phase micrographs of G-I, G-II, and G-III after 2.5 days of incubation.

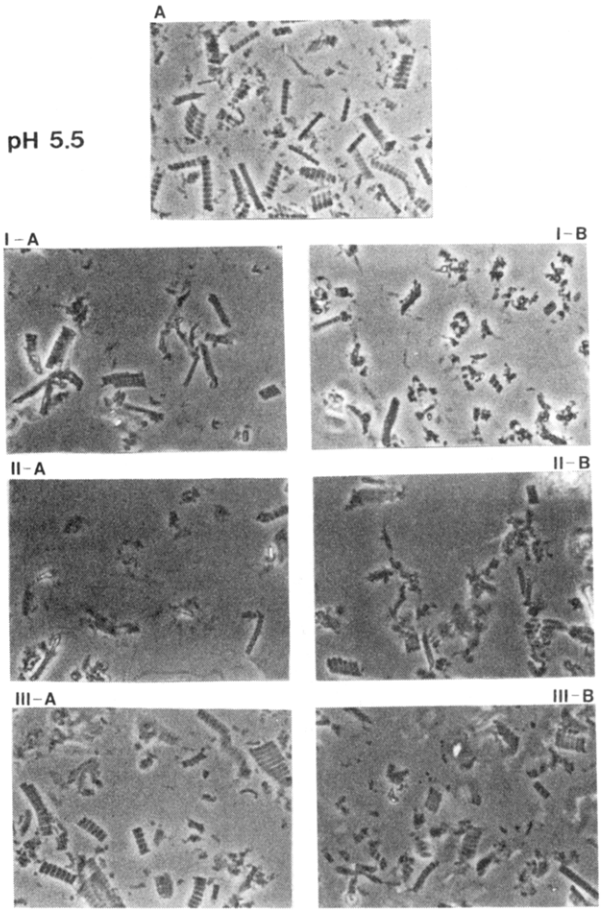

Figure 3. Effect of the pepstatin-sensitive enzymes on the phase micrograph of myofibrils during incubation at 4 °C at pH 5.5. (A) Before incubation. I-A, II-A, and III-A were the phase micrographs of G-I, G-II, and G-III after 0.5 day of incubation. I-B, II-B, and III-B were the phase micrographs of G-I, G-II, and G-III after 3.5 days of incubation.

According to the phase micrograph, the myofibrils after 12 h of incubation at 4 °C at pH 6.5 were still intact. Although the proteolysis occurred in G-I and G-III, the myofibrils were still longer than that of G-II even after 3.5 days of storage. The rupture of myofibrils of samples with crude proteases (G-II) severely occurred after 3.5 days of storage (Figure 1). However, the proteolysis of myofibrils occurred in the blank (G-I) and test (G-II) samples with pH of 6.0 and 5.5 after 1.5 days of incubation at 4 °C (Figures 2 and 3), while the myofibrils with more than four sarcomeres were clearly observed in control samples (G-III) at both pH 6.0 and 5.5. After 2.5 days of storage, severe proteolysis was found in G-I and G-II; less was observed in G-III (Figures 2 and 3). These data suggested that the pepstatin inhibited the proteolysis of myofibrils at pH 6.0 and 5.5 during incubation.

Robbins et al. (1979) and Okitani et al. (1981) reported that the disappearance of Z-disk of myofibrils and proteolysis of myofibrillar proteins occurred during 22–24 h of incubation of myofibrils with cathepsin D at 25 and 37 °C, pH 2.0–6.2. However, according to the studies on pork, chicken, lamb, and beef (Etherington et al., 1987; Koohmaraie et al., 1988a,b), the lysosomal proteases did not contribute to the postmortem tenderization. This seems to be contrary to the present results. But, on studying the lysosomal proteases, Ueno et al. (1981, 1986, 1988a,b), Hara et al. (1987), and Sakata et al. (1985) found two different types of proteases that hydrolyze hemoglobin at pH 4.0; one was pepstatin insensitive and had a broad activity on proteins (possibly cathepsins B, H, S, and L), and the other one was strongly inhibited by pepstatin and corresponded to cathepsin D. In the studies by Etherington et al. (1987) and Koohmaraie et al. (1988a,b), attention was paid to the action of cysteine proteases (cathepsins B, H, and L) and the action of pepstatin-sensitive protease might be ignored. Therefore, the present data suggested that the pepstatin-sensitive proteases were involved in the myofibrils fragmentation.

Changes of Pepstatin-Sensitive Proteases Activity during Incubation at 4 °C. After 2.5–3.5 days of incubation at 4 °C at various pH values (5.5, 6.0, and 6.5), the cathepsin D activity (as a marker enzyme) of myofibrils (G-I), myofibrils with crude proteases (G-II), and myofibrils with crude proteases and pepstatin (G-III) decreased significantly (Table II). However, the cathepsin D activity differences between G-II and G-III at various pH values almost maintained a constant level during incubation (Table II). These data suggested that, at pH 5.5, 6.0, and 6.5 conditions, the pepstatin-sensitive proteases were stable at 4 °C.

According to the study by Toldra and Etherington (1988), no significant decease in cathepsin D activity of pork was observed during 20 days of storage at 4 °C. The result of their study seems to be in accordance with the present results. This might be because the pepstatin-insensitive proteases of tilapia, which could hydrolyze the hemoglobin at 37 °C, pH 3.0 (Ueno et al., 1988b), existed in myofibril solutions and inactivated during incubation, which consequently decreased the overall cathepsin D activity.

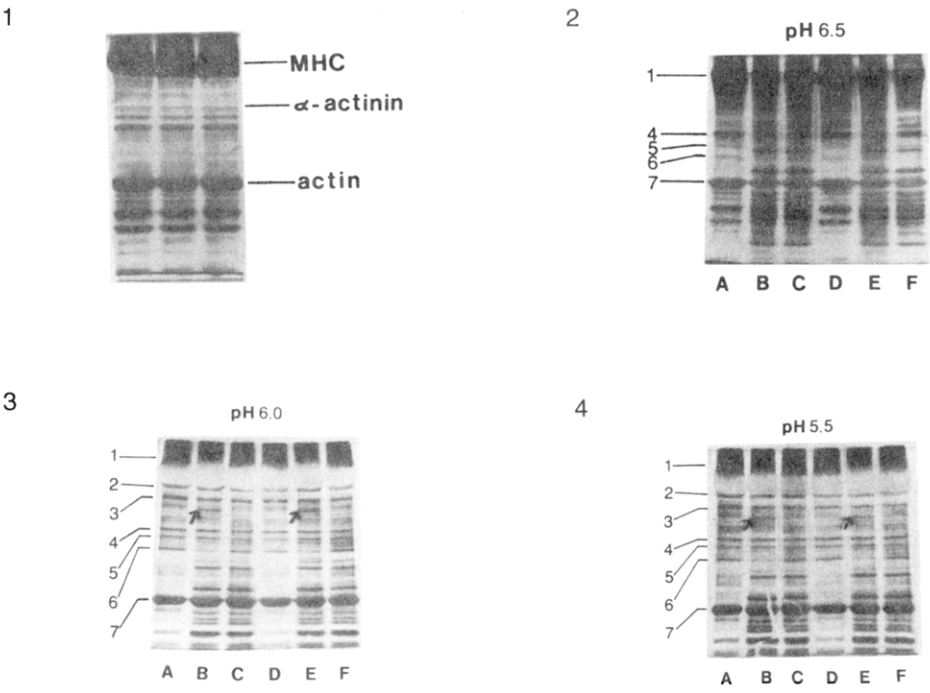

Figure 4. Effect of the pepstatin-sensitive enzymes on the electrophoretic profiles of myofibrils during incubation at 4 °C at pH 6.5 (2), 6.0 (3), and 5.5 (4) (1) Myofibril before incubation. (Lanes A) Blank, after 1.5 days of incubation. (Lanes B) Control, after 1.5 days of incubation with crude enzyme and pepstatin. (Lanes C) Test, after 1.5 days of incubation with crude enzyme. (Lanes D) Blank, after 2.5 days of incubation. (Lanes E) Control, after 2.5 days of incubation with crude enzyme and pepstatin. (Lanes F) Test, after 2.5 days of incubation with crude enzyme.

Table II. Activity[a] of Cathepsin D (as a Marker Protease) in Myofibril Solutions during Incubation at 4 °C

		treatments[b]			
pH	h at 4 °C	G-I	G-II	G-III	D[c]
6.5	12	11.53aB[d]	33.78aA	12.84aB	20.94
	84	6.10bC	29.74bA	8.94dB	20.80
6.0	36	8.20aC	39.16aA	25.80aB	13.36
	60	5.16bB	19.13bA	5.47dB	13.66
5.5	36	9.72aC	36.58aA	21.55aB	15.03
	60	7.29dB	22.77bA	7.89dB	14.88

[a] The enzyme activity is expressed as units/milligram. [b] Refer to the footnote of Table I. [c] The activity difference between G-II and G-III. [d] Values in the same row bearing unlike uppercase letters differ significantly ($P < 0.01$). At each pH condition, values in the same column bearing unlike lowercase letters differ significantly ($P < 0.01$).

Effect of Pepstatin-Sensitive Protease on the Electrophoretic Pattern of Myofibrils. According to the SDS–PAGE analysis, no distinct changes in myosin heavy chain (band 1), M-protein (band 2), C-protein (band 3), and actin (band 7) of myofibrils was observed during incubation at 4 °C at pH 6.0 and 5.5 (Figure 4, parts IV-3 and IV-4). No significant change in the subunit marked by arrow (identified as α-actinin) in samples B and E, which were incubated with pepstatin added, was found; however, α-actinin in samples A (blank) and C (with crude enzyme added) was proteolyzed after 1.5 days of incubation and further proteolyzed after 2.5 days of incubation (Figure 4, parts IV-3 and IV-4). More severe proteolysis on α-actinin occurred at pH 5.5 than at pH 6.0. No significant change in α-actinin on SDS–PAGE profiles of all myofibrils at pH 6.5 was observed; however, the marked proteolysis on MHC occurred (Figure 4, part IV-2). Although the optimal pH of pepstatin-sensitive proteases (mainly cathepsin D) varied from 2.0 to 5.0 (Asghar and Hendrickson, 1982; Asghar and Bhatti, 1987; Bond and Butler, 1987), these proteases still had activities at pH 5.5 and 6.0 in this study.

From the phase micrograph and DMF measurements, it was conjectured that the myofibrils were proteolyzed by pepstatin-sensitive proteases at pH 6.5, 6.0, and 5.5. This phenomenon was further confirmed by the analysis of SDS–PAGE, which found that the α-actinin, involved in Z-disk, was hydrolyzed in the myofibrils incubated alone and with crude proteases added at pH 6.0 and 5.5 (Figure 4, parts IV-3 and IV-4). In spite of the myofibrils with or without pepstatin added, the MHC of all myofibrils incubated at pH 6.5 dispersed, and no significant difference on α-actinin occurred during incubation. This phenomenon suggested the degradation of postmortem myofibrils at pH 6.5 might be caused by pepstatin-insensitive proteases.

Previous studies (Robbins and Cohen, 1976; Robbins et al., 1979) reported that the Z-disk on the myofibrils of bovine semimembranous muscle was proteolyzed by cathepsin D. Some evidence (Robbins et al., 1979; Okitani et al., 1981; Ouali et al., 1987) demonstrated that myofibrils from different sources also proteolyzed at myosin heavy chain (MHC) by cathepsin D at various pH (2.0–6.2). The present study showed no distinct difference in MHC change between myofibrils with and without pepstatin added during incubation at 4 °C at pH 5.5 and 6.0. However, proteolysis on MHC occurred at pH 6.5. The postmortem changes in myofibrils before rigor mortis (pH 7.2–6.3) and during a later stage of autolysis (pH >6.3) are considered to be caused by pepstatin-insensitive proteases, since the activity of pepstatin-sensitive proteases markedly decreased at this pH. The proteolytic sites on α-actinin at pH 6.0 and 5.5 suggested that the pepstatin-sensitive proteases were involved in myofibril degradation during postrigor of fish muscle, since the incidence pH of postrigor was recognized to be around 5.8 (Nonaka et al., 1976).

LITERATURE CITED

Asghar, A.; Bhatti, A. R. Endogenous proteolytic enzymes in skeletal muscle: Their significance in muscle physiology and

during postmortem aging events in carcasses. *Adv. Food Res.* **1987**, *31*, 343–451.

Asghar, A.; Hendrickson, R. L. Postmortem stimulation of carcasses: Effects on biochemistry, biophysics, microbiology and quality of meat. *CRC Crit. Rev. Food Sci. Nutri.* **1982**, *18*, 1–58.

Bond, J. S.; Butler, P. E. Intracellular proteases. *Annu. Rev. Biochem.* **1987**, *56*, 333–364.

Declan, J. T.; Vivion, P. T.; Michael, G. H. Electrophoretic analysis of changes in beef myofibrillar proteins during the early postmortem period. *Biochem. Soc. Trans.* **1986**, *14*, 436–438.

Etherington, D. J.; Taylor, M. A. J.; Dransfield, E. Conditioning of meat from different species. Relationship between tenderizing and the levels of cathepsin B, cathepsin L, calpain I, calpain II and β-glucuronidase. *Meat Sci.* **1987**, *20*, 1–18.

Furuno, K.; Goldberg, A. L. The activation of protein degradation in muscle by Ca of muscle injury does not involve a lysosomal mechanism. *Biochem. J.* **1986**, *237*, 859–864.

Goodman, M. N. Differential effects of acute changes in cell Ca concentration on myofibrillar and non-myofibrillar proteins breakdown in the rat extensor digitorum longus muscle in vitro. *Biochem. J.* **1987**, *241*, 121–127.

Hara, K.; Takeda, T.; Sakai, K.; Ishihara, T. Existence of cysteine protease active in an acidic pH range in fish. *Nippon Suisan Gakkaishi* **1987**, *53*, 1295–1300.

Hatori, A. Aging of meat. *Kagaku To Seibutsu (Chemistry and Biology)* **1986**, *24*, 789–791.

Huang, F. L.; Tappel, A. L. Action of cathepsins C and D in protein hydrolysis. *Biochim. Biophys. Acta* **1971**, *236*, 739–748.

Koohmaraie, M.; Schollmeyer, J. E.; Dutson, T. R. Effect of low-calcium-requiring calcium activated factor on myofibrils under varying pH and temperature conditions. *J. Food Sci.* **1986**, *51*, 28–32.

Koohmaraie, M.; Babiker, A. S.; Merkel, R. A.; Dutson, T. R. Role of Ca-dependent proteases and lysosomal enzymes in postmortem changes in bovine skeletal muscle. *J. Food Sci.* **1988a**, *53*, 1253–1257.

Koohmaraie, M.; Babiker, A. S.; Schroeder, A. L.; Merkel, R. A.; Dutson, T. R. Acceleration of postmortem tenderization in ovine carcasses through activation of Ca-dependent proteases. *J. Food Sci.* **1988b**, *53*, 1638–1641.

Laemmli, U. K.; Favre, M. Maturation of the head of bacteriophage T. *J. Mol. Biol.* **1973**, *80*, 575.

Lowell, B. B.; Ruderman, N. B.; Goodman, M. N. Evidence that lysosomes are not involved in the degradation of myofibrillar proteins in rat skeletal muscle. *Biochem. J.* **1986**, *234*, 237–240.

Lowry, H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

Marsh, B. B. Properties and behavior of prerigor meat. *Proc. Recent Meat Conf.* **1981**, *34*, 75–79.

Nonaka, J.; Hashimoto, Y.; Takashi, T.; Suyama, M. Postmortem changes of fish and shellfish. In *Marine Food Science (Suisan Shokuhin-Gaku)*; Koseisha, Koseikaku: Tokyo, 1976; pp 52–60.

Okitani, A.; Matsumoto, T.; Kitamura, Y.; Kato, H. Purification of cathepsin D from rabbit skeletal muscle and its action towards myofibrils. *Biochim. Biophys. Acta* **1981**, *662*, 202–209.

Ouali, A.; Garrel, N.; Obled, A.; Deval, C.; Valin, C. Comparative action of cathepsin D, B, H, L and of a new lysosomal cysteine proteinase on rabbit myofibrils. *Meat Sci.* **1987**, *19*, 83–100.

Rabilloud, T.; Carpentier, G.; Tarroux, P. Improvement and simplification of low-background silver staining of proteins by using sodium dithionite. *Electrophoresis* **1988**, *9*, 288–291.

Robbins, F. M.; Cohen, S. H. Effects of catheptic enzymes from spleen on the microstructure of bovine semimembranosus muscle. *J. Texture Stud.* **1976**, *7*, 137.

Robbins, F. M.; Walker, J. E.; Cohen, S. H.; Charterjee, S. Action of proteolytic enzymes on bovine myofibrils. *J. Food Sci.* **1979**, *44*, 1672–1677.

Sakata, K.; Matsumiya, M.; Mochizuki, A.; Otake, S. Thiol proteases in the ordinary muscle of pacific mackerel. *Bull. Jpn. Soc. Sci. Fish.* **1985**, *51*, 1865–1870.

Suyama M.; Konosu, A Postmortem changes of fish and shellfish. In *Marine Food Science (Suisan Shokuhin-Gaku)*; Koseisha, Koseikaku: Tokyo, 1987; pp 95–107.

Tokiwa, S. The preparation and observation of fish myofibrils. In *Suisan Seibutsukagaku, Shokuhingaku Jikensho*; Saito et al., Eds.; Koseisha, Koseikaku: Yokyo, 1974; pp 170–178.

Tokiwa, T.; Matsumiya, J. Fragmentation of Fish myofibril. Effect of storage condition and muscle cathepsins. *Bull. Jpn. Soc. Sci. Fish.* **1969**, *35*, 1099–1104.

Toldra, F.; Etherington, D. J. Examination of cathepsins B, D, H and L activities in dry cured hams. *Meat Sci.* **1988**, *23*, 1–7.

Ueno, R.; Morishita, T.; Takahashi, T. Intracellular distribution of enzymes and particle properties of lysosomes in carp muscle tissue. *Bull. Jpn. Soc. Sci. Fish.* **1981**, *47*, 207–214.

Ueno, R.; Liston, J.; Horiguchi, Y. Intracellular distribution of enzymes and particle properties of lysosomes in mackerel muscle tissue. *Bull. Jpn. Soc. Sci. Fish.* **1986**, *52*, 895–900.

Ueno, R.; Ikeda, S.; Horiguchi, Y. Characterization of pepstatin insensitive protease in mackerel white muscle. *Nippon Suisan Gakkaishi* **1988a**, *54*, 699–707.

Ueno, R.; Sakanaka, K.; Ikeda, S.; Horiguchi, Y. Purification of a pepstatin insensitive protease from mackerel white muscle. *Nippon Suisan Gakkaishi* **1988b**, 54, 691–697.

Received for review October 30, 1989. Revised manuscript received February 15, 1990. Accepted March 1, 1990.

Registry No. Proteinase, 9001-92-7; cathepsin D, 9025-26-7.