Identification of the Proteinases Responsible for the Postmortem Degradation of Myofibrillar Protiens

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It has been reported by many researchers that proteolysis undergoes in postmortem muscles resulting in both increases of free amino acids and oligopeptides and liberation of a 30,000-Mr fragment from myofibrillar proteins. Some researchers suggest that such degradation of the proteins may be associated with tenderization of meat[1]. In the previous papers[2,3] we reported that rabbit skeletal muscle showed three peaks of the auto-proteolytic activity each of which was around pH 3, 4.5 and 7. The pH 7 peak was ascribable to the action of a Ca^{2+}-activated neutral proteinase(CAF)[2]. The pH 3 peak and the pH 4.5 peak were ascribable to the action of cathepsin D and to the action of thiol proteinase(s), respectively[3]. Our recent work[4] revealed that muscle contained two thiol proteinases, i.e. cathepsins B and L, which were active in the acidic pH region.

In the present work we prepared cathepsins B, D and L of a highly-purified state from rabbit skeletal muscle and examined those enzymatic actions towards myofibrils and the various isolated myofibrillar proteins, in order to determine which one of those enzymes is responsible for the postmortem degradation of myofibrillar proteins in the muscles with normal ultimate pH values(around 5.5).

MATERIALS AND METHODS

Materials: The muscle(longissimus dorsi) of rabbit was removed from the carcass immediately after slaughter and used to prepare all the enzymes, myofibrils and various myofibrillar proteins. The psoas muscle was used to prepare glycerinated muscle fibers. Myofibrils were prepared as described by Yang et al.[5]. Myosin, actin, tropomyosin, troponin and α-actinin were prepared by the methods of Richards et al.[6], Rees et al.[7], Mueller [8], Ebashi et al.[9] and Masaki et al.[10], respectively.

Measurement of enzyme activities: Cathepsin L was prepared and its activity unit was determined by the method described previously[4]. Cathepsin B activity was determined incubating with 0.5 mM benzoyl-DL-Arg-β-naphthyl-amide(BANA) in the presence of 50 mM phosphate buffer containing 0.4 mM EDTA and 0.3 mM dithiothreitol, as previously reported[4]. One unit of the activity hydrolyzed 1 μmol of substrate/min at pH 6.2 and 37°C. Cathepsin D activity was determined incubating with urea-denatured hemoglobin(0.5 %) in the presence of 50 mM sodium acetate-HCl buffer, as previously reported[11]. The amount of the enzyme releasing one μg tyrosine equivalent for 1 hr at pH 4.0 was expressed as one unit.

Enzyme treatment on myofibrils and myofibrillar proteins: The buffers used were sodium acetate-HCl(below pH 4.8) and Tris-acetate(above pH 5.0). The concentration of each enzyme in the treating medium was comparable or lower than the value roughly estimated for the in situ muscle.

Electrophoresis: SDS-polyacrylamide gel electrophoresis(SDS-PAGE) was carried out by the method of Weber and Osborn[12] using 5, 7.5 or 10 % gels containing 0.1 % SDS.

Protein concentration: The protein concentration of the enzymes was determined by the method of Lowry et al. [13]. Bovine serum albumin was used as a standard.

RESULTS

Purification of cathepsin B and its properties

Minced muscles were homogenized with 2 vol. of 3 % NaCl solution containing 1 mM EDTA and 15 mM HCl. After the pH was adjusted to 3.7 with 1 N HCl, the homogenates were stirred for 2 hr at 4°C, and then centrifuged. The resultant supernatant(the crude extract) was fractionated with ammonium sulfate. The precipitates between 45 % and 65 % saturation were collected and dialyzed against 10 mM sodium acetate-HCl buffer(pH 5.0) containing 0.1 M NaCl and 1 mM EDTA(solution A). The dialysate was subjected to Sephadex G-75 column chromatography using solution A. The active fractions eluted from the column were pooled and subjected to P-cellulose column chromatography using a linear gradient of NaCl concentration in solution A for elution. The activity was eluted around 0.25 M NaCl. The active fractions were pooled and subjected to Sephadex G-100 column chromatography after concentration. The most active fractions eluted from the column were used as the purified cathepsin B. One hundred grams of muscles gave 68 μg of the purified enzyme with a specific activity of 45.3 units/ml. Approximately 120-fold purification was achieved from the crude extract with an overall yield of 3.6 %. Although SDS-PAGE revealed that the purified enzyme was contaminated with impurities, some properties of the enzyme were preliminarily examined. The optimum pH for BANA hydrolysis was around 6.2. On standing at 37°C for 3 hr the enzyme activity was stable at pH 6.0, but lost by 80 % at pH 7. The Mr was between 20,000 and 30,000. The activity was completely inhibited with 0.1 mM antipain. These properties are very similar to those of other tissues cathepsin B[14].

When the enzyme obtained from the P-cellulose column was incubated with myofibrils at pH 5.0 and 37°C for 24 hr, no change was observed with the SDS-PAGE of the treated myofibrils, indicating that muscle cathepsin B hardly degraded myofibrillar proteins.

Purification of cathepsin D and its action towards myofibrillar proteins

We tried to obtain muscle cathepsin D of a highly-purified state using three kinds of starting materials, i.e. acetone-dried muscle powder prepared by the method of Straub[15], KCl(2 %)-extracts of whole muscle homogenates and Triton X-100(0.2 %)-extracts from the KCl-extracted residue. The acetone-dried muscle powder was shown to be most recommendable. The enzyme was extracted from this material with 0.2 mM ATP. The crude extract obtained was kept at 0.1 M KCl for 30 min to allow actin to polymerize. The polymerized actin solution was fractionated with acetone. The precipitates between 33 % and 55 % acetone were collected and dialyzed against 10 mM phosphate buffer(pH 7.0). The dialysate was subjected to DEAE-Sephadex A-50 column chromatography using the above buffer. The active fractions eluted with the buffer containing 0.12 M NaCl were pooled and then subjected to Sephadex G-100 column chromatography using 10 mM sodium acetate-HCl buffer(pH 4.0) containing 0.1 M NaCl. The active fractions eluted from the column were pooled and subjected again to Sephadex G-100 column chromatography. The active fractions obtained by this step were used as the purified enzyme. SDS-PAGE revealed that the enzyme was a single polypeptide chain of 42,000 daltons. One hundred grams of muscles gave 15 μg of the purified enzyme with a specific activity of 74,900 units/ml. A 1340-fold purification was achieved from

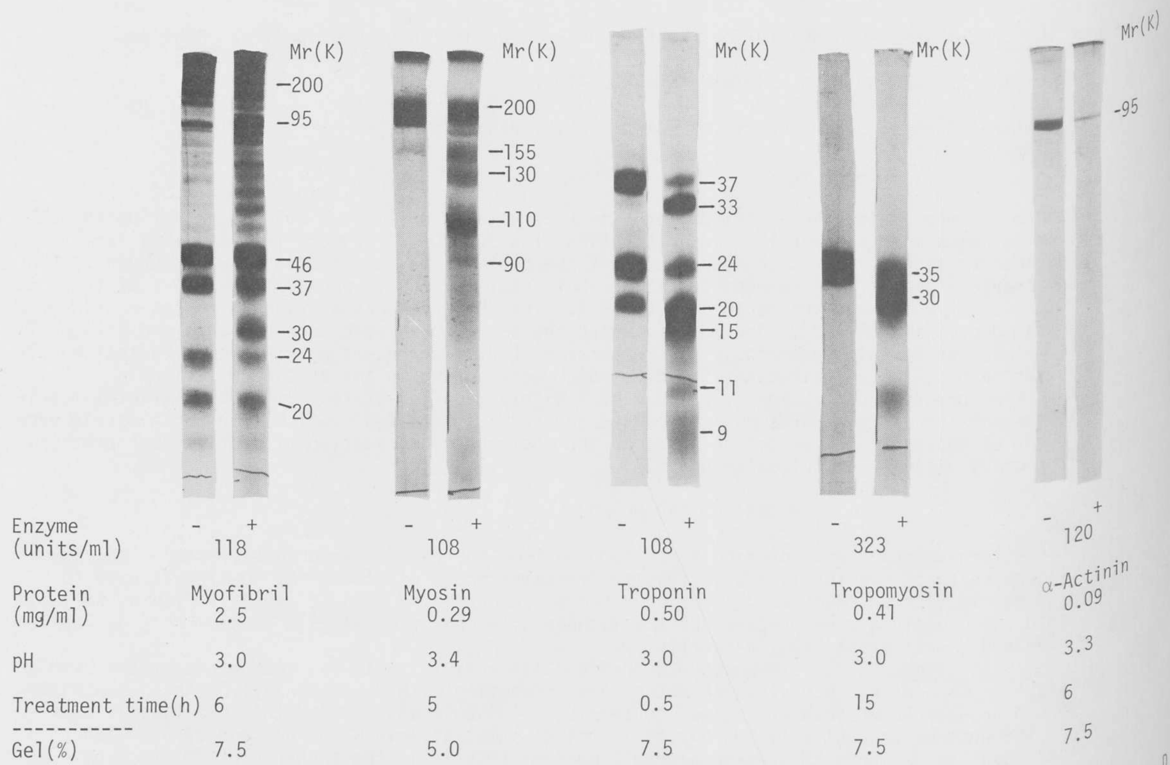


Fig.1. SDS-PAGE patterns of the myofibrils and the isolated myofibrillar proteins treated with cathepsin D at 37°C and around pH 3 in the presence of 20 mM sodium acetate-HCl buffer containing 0.1 M NaCl (for myofibril,0.18 M NaCl; for myosin, 0.6 M NaCl) and 10 mM NaN_3. Other conditions for treatment and gel concentrations were indicated below each gel.

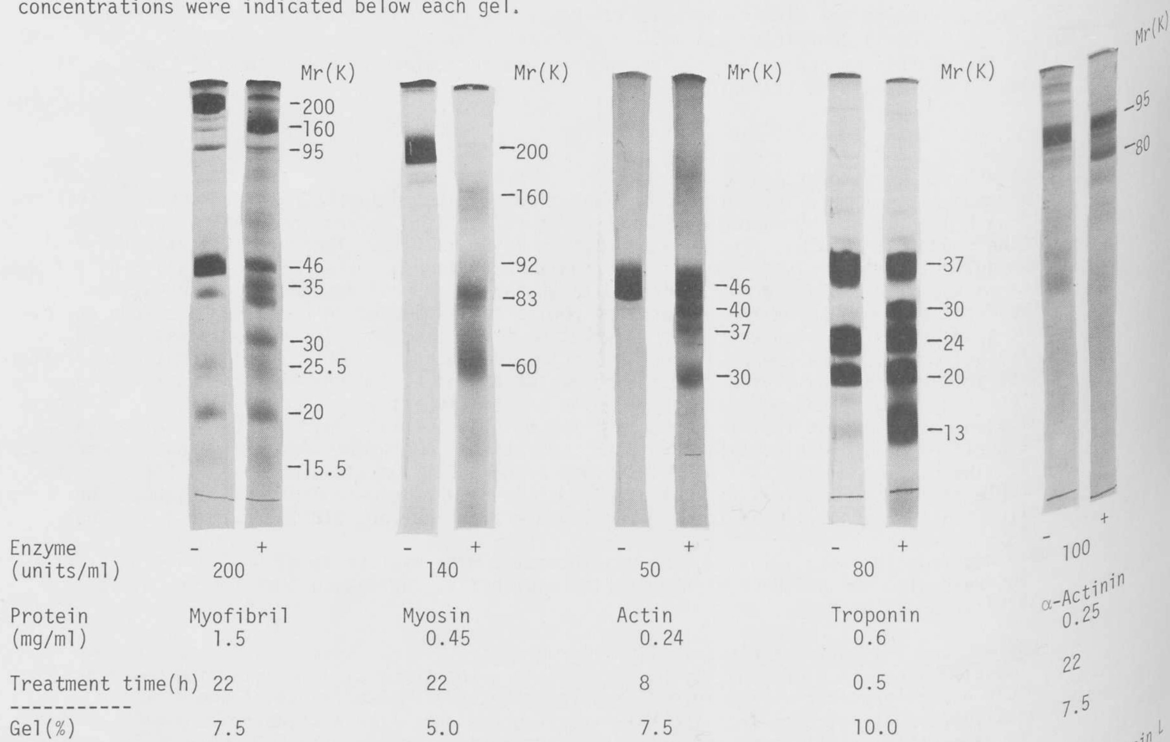


Fig.2. SDS-PAGE patterns of the myofibrils and the isolated myofibrillar proteins treated with cathepsin L at pH 5.0 and 37°C in the presence of 10 mM Tris-acetate buffer containing 0.1 M NaCl (for myosin, 0.6 M NaCl), 0.3 mM dithiothreitol, 1 mM EDTA, 5 mM NaN_3 and 0.1 mM pepstatin. Other conditions for treatment and gel concentrations were indicated below each gel.

the crude extract with an overall yield of 4.0 %.

The optimum pH of the purified cathepsin D was around 3 for hemoglobin hydrolysis. The enzyme hydrolyzed myofibrils at pH 3-6, optimally around pH 3, resulting in the degradation of myosin heavy chain and the production of ca. 30,000-Mr fragments(Fig.1). On incubation with various isolated myofibrillar proteins at pH 2-6, the enzyme degraded optimally around pH 3 myosin heavy chain, troponin, α-actinin and tropomyosin but not actin. Fig.1 shows the degradation mode of each protein around pH 3 revealed with SDS-PAGE. The myosin heavy chain was degraded to 155,000-, 130,000-, 110,000- and 90,000-Mr fragments. Troponin T and troponin I were more susceptible to the enzyme than troponin C. The proteolytic products from these proteins showed the Mr of 33,000, 15,000, 11,000 and 9,000. Tropomyosin was degraded to ca. 30,000-Mr fragments. α-Actinin disappeared from the gel, although no clear degradation product was observed on the gel.

<u>Proteolytic action of cathepsin L towards myofibrillar proteins</u>

Cathepsin L hydrolyzed myofibrils at pH 3-7, optimally around pH 4, resulting in the degradation of myosin heavy chain and the production of a 30,000-Mr fragment(Fig.2). On incubation with various isolated myofibrillar proteins at pH 3-7, the enzyme degraded optimally at pH 4-6 myosin heavy chain, actin, troponin and α-actinin but not tropomyosin. Fig.2 shows the degradation mode of each protein at pH 5 revealed with SDS-PAGE. The myosin heavy chain was degraded to 160,000-, 92,000-, 83,000- and 60,000-Mr fragments. Actin was hydrolyzed to 40,000-, 37,000- and 30,000-Mr fragments. Troponin T and troponin I were more susceptible to the enzyme than troponin C. The Mr of the major proteolytic products from them were 13,000 and 30,000. α-Actinin was degraded to a 80,000-Mr fragment.

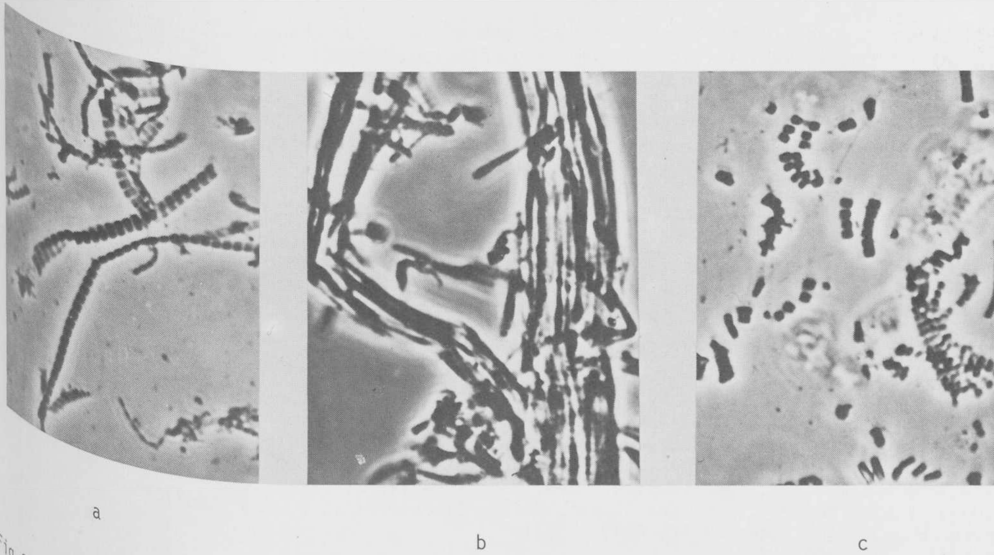
<u>Morphological changes in myofibrils on treatment with cathepsins D and L</u>

When myofibrils were incubated with cathepsin L at pH 4-7 and 4°C, the fragmentation of myofibrils was observed at pH 5-6 with a phase-contrast microscope(Fig.3). On the other hand, the treatment with cathepsin D at pH 4-6 did not bring about the fragmentation of myofibrils(Fig.3).

The glycerinated muscle fibers were treated with cathepsin L at pH 5.5 and 37°C for 16 hr and then subjected to electron microscopic observation. The result demonstrated that cathepsin L degraded Z-line and M-line of myofibrils and disturbed the lateral arrangement between myofibrils(Fig.4).

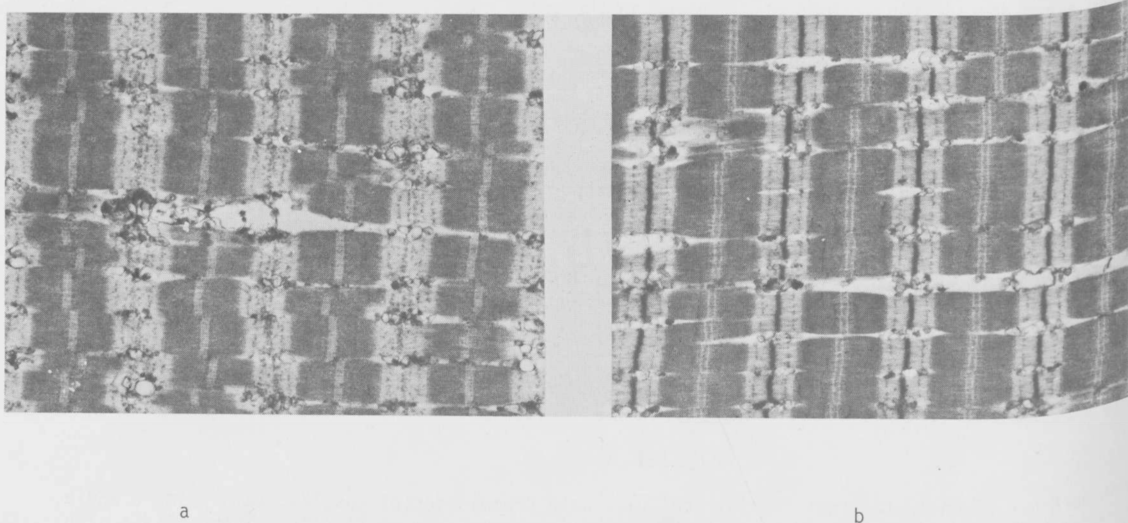
DISCUSSION

The present work has demonstrated that two intracellular acid proteinases of muscles, cathepsins D and L, can degrade some myofibrillar proteins and liberate 30,000-Mr fragments from myofibrils. Since CAF is also known to hydrolyze some myofibrillar proteins and liberate a 30,000-Mr fragment from myofibrils[16], we must now estimate the contribution rate of those three enzymes to determine the enzyme responsible for the postmortem degradation of myofibrillar proteins. We previously reported that the rate of proteolysis was lowest around pH 6.3 and it raised as increasing or decreasing of the pH from 6.3 on storage of either *in situ* muscles[17] or muscle homogenates[2]. Since normal ultimate pH of meats is around 5.5, it is thus most reasonable to conceive that cathepsin L is more operative in those meats than the other two enzymes. Cathepsin D and CAF probably contribute much to the proteolysis in the meats with an extremely-low ultimate pH(some of PSE meats) and in the



a　　　　b　　　　c

Fig.3. Phase-contrast micrographs of the myofibrils incubated without (a) and with cathepsin D (108 units/ml)(b) or cathepsin L(300 units/ml)(c) at pH 5.5 and 4°C for 6 days. Other conditions were the same as in Figs.1 and 2.



a b

Fig.4. Electron micrographs of the glycerinated muscle fibers incubated with (a) or without (b) cathepsin L(250 units/ml) at pH 5.5 and 37°C for 16 hr. Other conditions were the same as in Fig.2.

meats with a high ultimate pH(DFD meats), respectively. This conception is supported by the results of Penny and Ferguson-Pryce[18] who measured the pH-dependence of the troponin T-degradation rate on incubation of beef muscle homogenates.

The weakening of Z-line during conditioning is assumed to be one of the major changes associating with tenderization of meat. This change is substantiated by the fragmentation of myofibrils. CAF is shown to degrade the Z-line resulting in the fragmentation of myofibrils[16]. Now cathepsin L is also demonstrated to cause such a change. On the other hand, cathepsin D seems to be less responsible for such a change, because it does not cause the fragmentation of myofibrils. Therefore, if the weakening of the Z-line in the postmortem muscle is due to the action of a proteinase, cathepsin L should be more responsible for it than CAF, since the former has an optimum pH nearer to the muscle ultimate pH than the latter.

CONCLUSION

On the basis of all the results obtained so far by us and other researchers it can be concluded that cathepsin L is more responsible than the other known muscle proteinases, i.e. CAF, cathepsin B and cathepsin D, for the postmortem degradation of myofibrillar proteins in the muscles with normal ultimate pH values.

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