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MODE OF DEGRADATION OF MYOFIBRILLAR PROTEINS BY AN ENDOGENOUS PROTEASE, CATHEPSIN L

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The mode of degradation of myofibrils and their constituent proteins by cathepsin L (EC 3.4.22.15) of rabbit skeletal muscle was studied. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis showed that cathepsin L degraded myosin heavy chain, α-actinin, actin, troponin T and troponin I assembled in myofibrils and produced mainly fragments of 160 000 and 30 000 daltons in the acidic pH region. This degradation was most intense around pH 4. Degradation of myosin in the isolated state by cathepsin L resulted in the disappearance of the heavy chain and the decrease of light chains 1, 2 and 3, producing fragments of 160 000, 92 000, 83 000 and 60 000 daltons. The degradation of the heavy chain was most severe at pH 4.2. Cathepsin L degraded actin into fragments of 40 000, 37 000 and 30 000 daltons. This action was most intense at pH 4.7. Tropomyosin was not degraded. Troponin T and troponin I were degraded into fragments of 30 000 and 13 000 daltons at pH 3.7–6.7, which were degraded further into smaller fragments. Troponin C was not degraded. α-Actinin was degraded into several fragments, the major one of which showed an M_r of 80 000. This degradation was most intense at pH 3.0–3.5.

Introduction

Tissue proteinases have been considered to play an important role in the degradation of intracellular proteins, but the proteolytic system(s) in skeletal muscle has yet to be defined. A few proteinases capable of degrading myofibrillar proteins have been identified from skeletal muscle. Yasogawa et al. [1] isolated a serine protease from rat skeletal muscle which was active in a slightly basic pH region towards myosin heavy chain, troponin, tropomyosin and actin but not towards α-actinin or M-protein. A Ca^{2+}-activated neutral protease (CAF) purified from porcine skeletal muscle by Dayton et al. [2] removes the Z-line from myofibrils and degrades tropomyosin, troponin and C-protein, but shows no action on myosin, actin or α-actinin. Similar actions were reported for the Ca^{2+}-activated neutral proteases purified from chicken skeletal muscle by Ishiura et al. [3] and from rabbit skeletal muscle by Azanza et al. [4], respectively. As to acid proteinases, Schwartz and Bird [5] reported that lysosomal endopeptidases, cathepsin B (EC 3.4.22.1) and D (EC 3.4.23.5), purified from rat skeletal muscle degraded myosin heavy chain. Robbins et al. [6] reported that cathepsin D partially purified from bovine skeletal muscle degraded myosin.

In the previous work [7] we purified another myofibrillar protein-degrading protease from rabbit skeletal muscle and regarded it as muscle cathepsin L since it resembled cathepsin L found in liver by Kirschke et al. [8] in several properties. This paper describes in detail the modes of the degradation by cathepsin L of myofibrils and their constituent proteins revealed using electrophoresis.

Materials

Rabbit muscles (longissimus dorsi) were used to prepare cathepsin L, myofibrils and their constituent proteins.

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Pepstatin A was purchased from the Peptide Institute, Osaka, Japan. DEAE-Sephadex A-50, Sephadex G-75 and G-200 were products of Pharmacia Fine Chemicals, Sweden. Ultrogel AcA-54 was from LKB, Sweden.

Methods

Preparation of cathepsin L. Cathepsin L was prepared according to the method described previously [7]. The crude enzyme extracted from muscle homogenate at pH 3.7 for 2 h was fractionated with $(NH_4)_2SO_4$. The precipitate between 25 and 65% saturation was applied to a Sephadex G-75 column and then to a phosphocellulose column. The active fractions obtained were purified using columns of Sephadex G-75 and Ultrogel AcA-54. The purified enzyme was shown to be almost homogeneous with polyacrylamide gel electrophoresis. The unit of cathepsin L activity was determined by the assay of its Ca-sensitivity-removing activity towards myofibrils, essentially according to the method described in the previous paper [7]. The amount which reduced the Ca-sensitivity of 0.4 mg myofibrils by 1% on incubation at pH 5.0 and 25°C for 1 h was expressed as one unit of activity.

Preparation of myofibrils and their constituent proteins. Myofibrils were prepared as described by Yang et al. [9].

Myosin was prepared as described by Perry [10] and purified further by DEAE-Sephadex A-50 column chromatography as described by Richards et al. [11]. Actin was prepared according to the method of Mommaerts [12] and purified further by Sephadex G-200 column chromatography as described by Rees and Young [13]. Alpha-actinin was prepared by the method of Masaki and Takaiti [14]. Troponin was prepared as described by Ebashi et al. [15]. Tropomyosin was prepared from the residue obtained after the extraction of actin from the acetone-dried muscle powder according to the method of Mueller [16].

The enzyme treatment of myofibrils and their constituent proteins. The substrate protein (1.5 mg/ml myofibrils or 120–900 μg/ml of each of the constituent proteins) was incubated with cathepsin L (50–200 units/ml) at 37°C in 10 mM Tris-acetate buffer, pH 5.0/0.1 M NaCl/1 mM EDTA/0.3 mM dithiothreitol/5 mM NaN_3/0.1 mM pepstatin. Myosin was incubated in the solution described above containing 0.6 M NaCl in place of 0.1 M NaCl. For enzyme treatment at various pH values, 50 mM sodium acetate-HCl buffer (below pH 4.8) or 50 mM Tris-acetate buffer (above pH 5.0) was used. Pepstatin was added to block cathepsin D type activity, if any, bound to substrate proteins.

At a given time a 0.2 ml-portion was removed from the incubation mixture and mixed with 0.1 ml of 60 mM sodium phosphate buffer, pH 7.2/5.24% SDS/32.4% 2-mercaptoethanol/20.2% glycerol/0.029% bromophenol blue and then boiled for 3 min to stop the enzymatic reaction.

Protein determination. Protein concentrations of myofibrils and their constituent proteins were determined by the biuret method using bovine serum albumin as a standard.

Acrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out as described by Weber and Osborn [17] using 5, 7.5, 10 or 15% gels containing 0.1% SDS. Electrophoresis with polyacrylamide gels containing both 0.1% SDS and 8 M urea was carried out as described by Sender [18]. The gels were stained with Coomassie brilliant blue R-250.

Results

Degradation of myofibrils

Fig. 1a shows electrophoretic patterns of myofibrils incubated with cathepsin L at pH 5.0. As the time of incubation increased, the intensity of the band corresponding to myosin heavy chain decreased markedly. A thick band of a degradation product with an M_r of 160 000 and several thin ones having M_r values between 50 000 and 100 000 appeared concomitantly. The intensity of the bands corresponding to α-actinin and actin, respectively, decreased considerably on 22 h incubation (gel 6). A band of 30 000 daltons appeared after 2 h incubation (gel 3) and a band of 37 000 daltons appeared after 4 h incubation (gel 4). Both bands increased in density up to 22 h. The band corresponding to troponin I disappeared within 2 h incubation (gel 3). The band pattern around tropomyosin changed noticeably during the incubation, indicating the degradation of tropomyosin and/or troponin T, both of which comigrated under the conditions of electrophoresis as shown in Fig. 1a. Thus, SDS-polyacrylamide gel electrophoresis

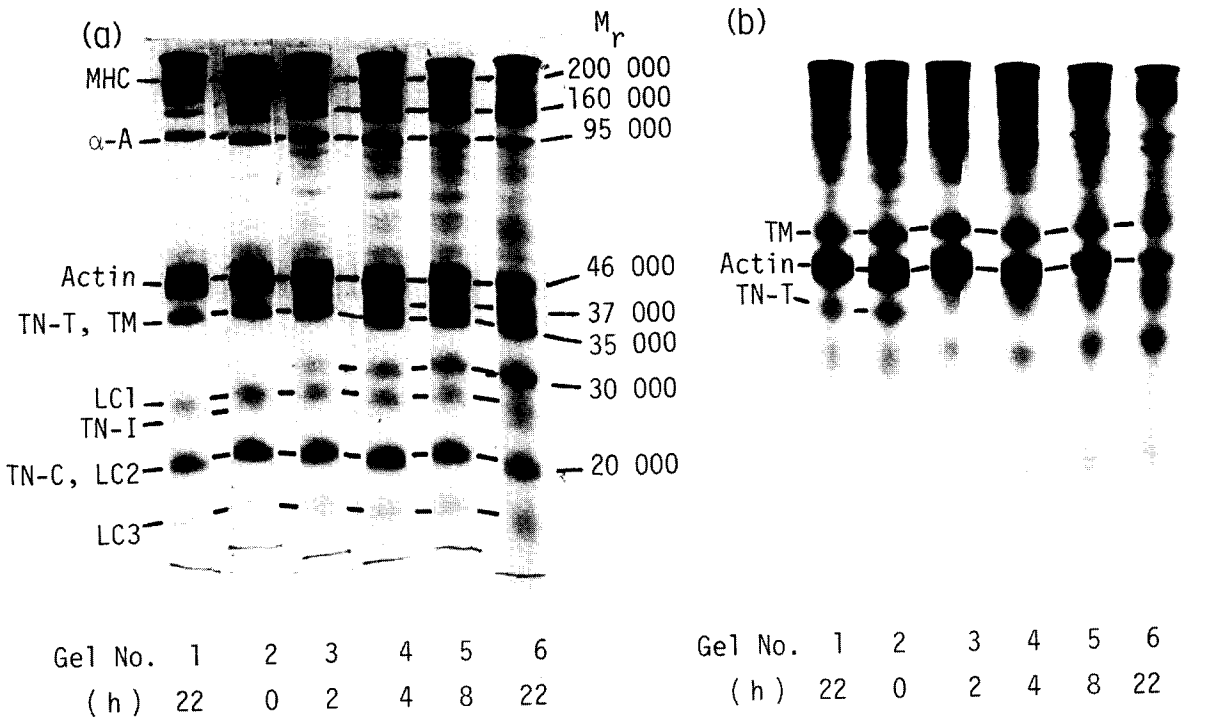

Fig. 1. Electrophoretograms of myofibrils incubated with cathepsin L at pH 5.0. Myofibrils (1.5 mg/ml) were incubated with cathepsin L (200 units/ml) in 10 mM Tris-acetate buffer (pH 5.0)/0.1 M NaCl/1 mM EDTA/0.3 mM dithiothreitol/5 mM NaN_3/0.1 mM pepstatin at 37°C for the period indicated below each gel. (a) The incubated myofibrils (50 μg protein) were applied to 7.5% polyacrylamide gels containing 0.1% SDS. Gel 1 is the myofibrils incubated without cathepsin L. (b) The incubated myofibrils (75 μg protein) were applied to 10% polyacrylamide gels containing 0.1% SDS and 8 M urea. Gel 1 is the myofibrils incubated without cathepsin L. MHC, myosin heavy chain; α-A, α-actinin; TN-T, troponin T; TM, tropomyosin; LC1, myosin light chain 1; TN-I, troponin I; TN-C, troponin C; LC2 and LC3, myosin light chains 2 and 3, respectively.

in the presence of 8 M urea was employed to clarify the changes in tropomyosin and troponin T. Since in the presence of urea tropomyosin migrates slower than actin, troponin T can be separated from tropomyosin [18] (gel 1 in Fig. 1b). Fig. 1b shows that the degradation of troponin T assembled in myofibrils occurred within 2 h incubation, whereas tropomyosin did not change after 22 h incubation.

The extent of cathepsin L-induced degradation of myofibrils (1.5 mg/ml) was examined as a function of pH on 24 h incubation with 70 units/ml enzyme. The degradation of myosin heavy chain and the generation of 160 000- and 30 000-dalton fragments were observed at pH 3.0–6.7. In a narrower range of pH than the above, i.e., pH 3.0–5.1, a degradation product of 80 000 daltons appeared. Actin was digested at pH 4.0–5.7. All these changes induced by cathepsin L were most intense at pH 4.0.

Degradation of isolated myofibrillar proteins

When the treatment of myosin with cathepsin L was carried out at pH 5.0, the band of myosin heavy chain decreased with the increase in the incubation time and almost disappeared on 22 h incubation (Fig. 2a). At an early stage of the degradation (gel 3) a fragment of approx. 160 000 daltons appeared, and at the next stage 92 000- and 83 000-dalton fragments emerged (gels 4 to 6). Finally, the 22 h incubation gave 83 000- and 60 000-dalton fragments as the major degradation products from myosin heavy chain.

The changes of myosin light chains treated with the enzyme at pH 5.0 were investigated using more concentrated gels (15%) as shown in Fig. 2b. The bands of myosin light chains 1, 2 and 3 decreased in density as the time of incubation increased.

The effect of pH on the activity of cathepsin L (50

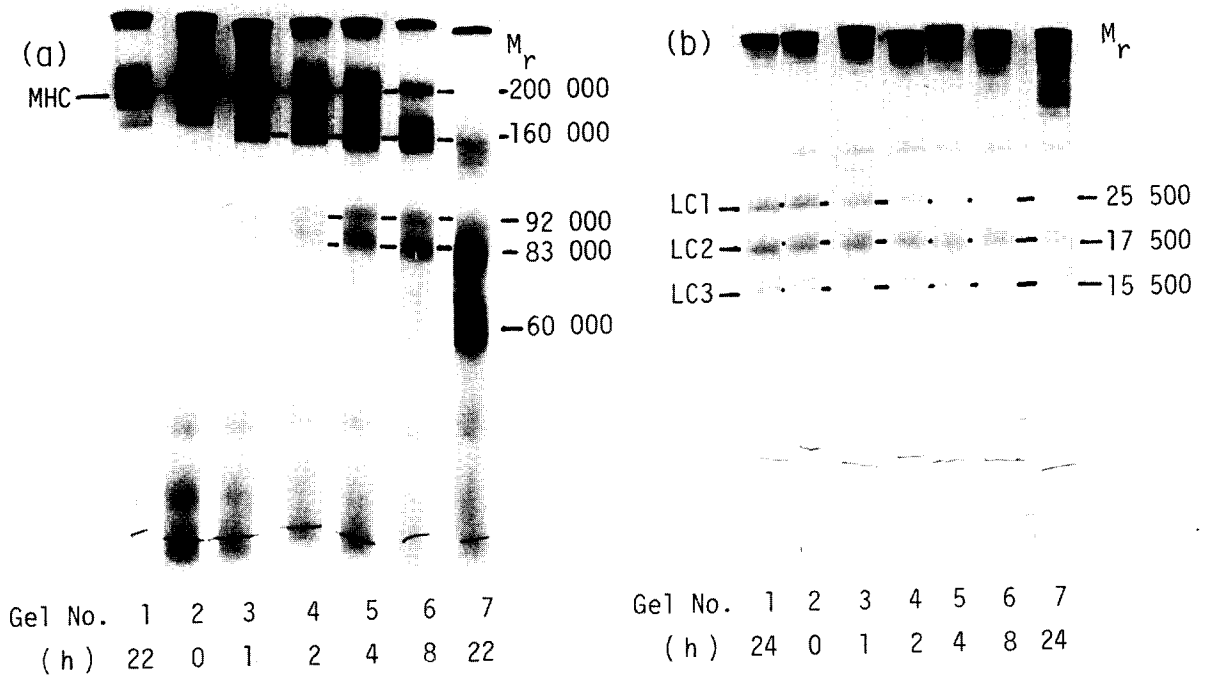

Fig. 2. SDS-polyacrylamide gel electrophoretic patterns of myosin incubated with cathepsin L at pH 5.0. (a) Myosin (450 μg/ml) was incubated with cathepsin L (140 units/ml) in 10 mM Tris-acetate buffer (pH 5.0)/0.6 M NaCl/1 mM EDTA/0.3 mM dithiothreitol/5 mM NaN_3/0.1 mM pepstatin at 37°C for the period indicated below each gel. The incubated myosin (20 μg) was applied to a 5% polyacrylamide gel containing 0.1% SDS. Gel 1 is the myosin incubated without cathepsin L. (b) Myosin (900 μg/ml) was incubated with cathepsin L (50 units/ml) in the same medium as in (a) at 37°C for the period indicated below each gel. The incubated myosin (30 μg) was applied to a 15% polyacrylamide gel containing 0.1% SDS. Gel 1 is the myosin incubated without cathepsin L. MHC, LC1, LC2 and LC3 are the same as in Fig. 1.

units/ml) towards isolated myosin (300 μg/ml) was examined with 4 h incubation. At pH 3.6–5.9 cathepsin L degraded myosin heavy chain into 160000-, 92000- and 83000-dalton fragments, and its action was most severe at pH 4.2. The degradation of the light chains occurred at pH 5.0–5.6 after 24 h incubation.

Electrophoretograms of actin treated with cathepsin L at pH 5.0 for various times are shown in Fig. 3. As the incubation time increased, the band of actin decreased in intensity. Its fragments of 40000 and 30000 daltons appeared at an early stage, and an additional band of 37000 daltons emerged at a later stage.

When actin (120 μg/ml) was incubated with cathepsin L (50 units/ml) for 2 h, it was degraded in a broad pH range from 3.4 to 7.6, and the degradation was most intense at pH 4.7.

On treatment with cathepsin L at pH 5.0 tropomyosin did not undergo any appreciable change even on 22 h incubation (Fig. 4). Incubation at other pH values for 20 h did not cause any change, either.

On treatment with cathepsin L at pH 5.0 troponin was degraded in the mode shown in Fig. 5. The troponin preparation used was contaminated with tropomyosin. However, this contaminant did not interfere with the change in troponin, because no change occurred in the band of tropomyosin with the enzyme treatment, as described above. With the progress of incubation time troponins T and I decreased, while troponin C remained unchanged. At an early stage fragments of 30000 and approx. 13000 daltons appeared (gels 3 to 6). After 4 h incubation the 30000-dalton fragment began to decrease while the 13000-dalton one still continued to increase (gel 7). After 8 h incubation the 13000-dalton fragment also began to be hydrolyzed into further smaller fragments. Incubation for 24 h com-

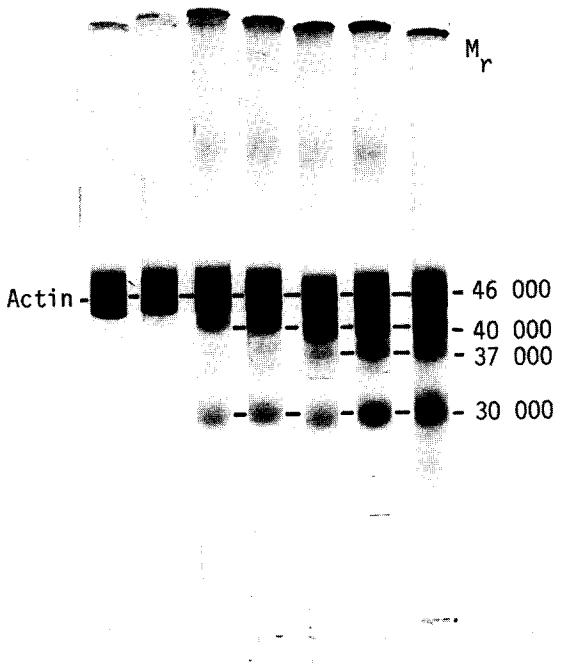

Fig. 3. Electrophoretograms of actin incubated with cathepsin L at pH 5.0. Actin (240 μg/ml) was incubated with cathepsin L (50 units/ml) in the same medium as in Fig. 1 at 37°C. The incubated actin (20 μg) was applied to a 7.5% polyacrylamide gel containing 0.1% SDS. Gel 1 is the actin incubated without cathepsin L.

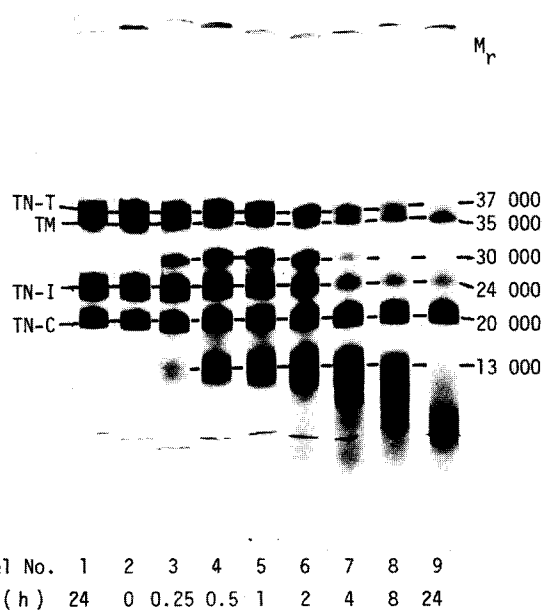

Fig. 5. Degradation of troponin by cathepsin L at pH 5.0. Troponin (600 μg/ml) was incubated with cathepsin L (80 units/ml) in the same medium as in Fig. 1 at 37°C. The incubated troponin (20 μg) was applied to a 10% polyacrylamide gel containing 0.1% SDS. Gel 1 is the troponin incubated without cathepsin L. TM, TN-T, TN-I and TN-C are the same as in Fig. 1.

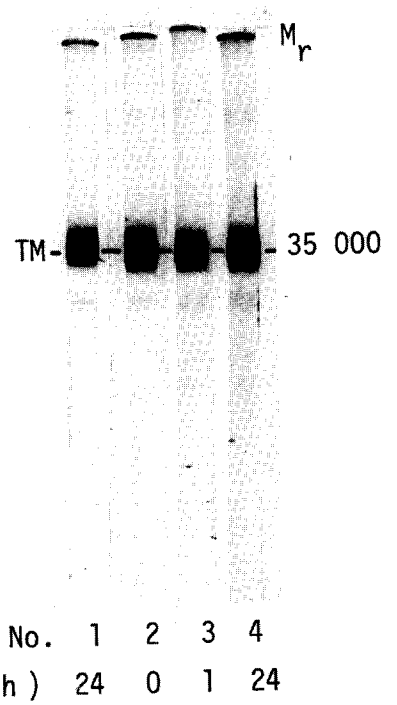

Fig. 4. Electrophoretograms of tropomyosin incubated with cathepsin L at pH 5.0. Tropomyosin (900 μg/ml) was incubated with cathepsin L (50 units/ml) in the same medium as in Fig. 1 at 37°C. The incubated tropomyosin (10 μg) was applied to a 7.5% polyacrylamide gel containing 0.1% SDS. Gel 1 is the tropomyosin incubated without cathepsin L. TM, tropomyosin.

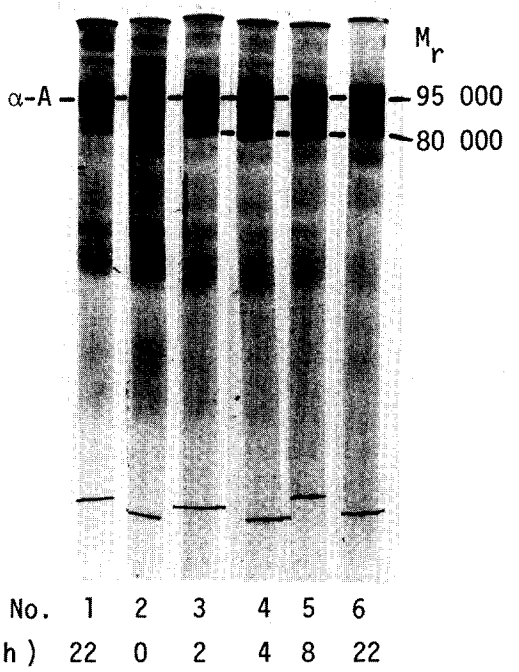

Fig. 6. Electrophoretograms of α-actinin incubated with cathepsin L at pH 5.0. α-Actinin (250 μg/ml) was incubated with cathepsin L (100 units/ml) in the same medium as in Fig. 1 at 37°C. The incubated α-actinin (16 μg) was applied to a 7.5% polyacrylamide gel containing 0.1% SDS. Gel 1 is the α-actinin incubated without cathepsin L. α-A, α-actinin.

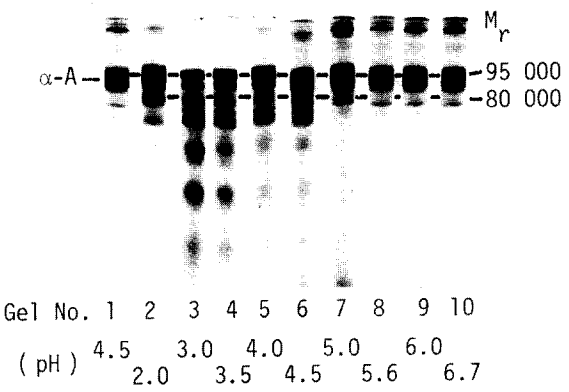

Fig. 7. Effect of pH on the degradation of α-actinin by cathepsin L. α-Actinin (150 μg/ml) was incubated with cathepsin L (80 units/ml) at 37°C and various pH values as indicated below each gel for 20 h in the presence of 50 mM sodium acetate-HCl buffer (below pH 4.5) or 50 mM Tris-acetate buffer (above pH 5.0)/0.1 M NaCl/0.3 mM dithiothreitol/1 mM EDTA/5 mM NaN_3/0.1 mM pepstatin. The incubated α-actinin (7.5 μg) was loaded on a 7.5% polyacrylamide gel containing 0.1% SDS. Gel 1 is the α-actinin incubated for 20 h without cathepsin L. α-A, α-actinin.

pletely eliminated troponin T and the 30 000- and 13 000-dalton fragments.

On treatment of troponin (600 μg/ml) with cathepsin L (60 units/ml) at various pH values for 2 h, the band intensities of troponins T and I decreased accompanying the appearance of the 30 000-dalton band between pH 3.7 and 6.7. The 13 000-dalton band was accumulated mostly with the treatments between pH 4.6 and 6.7.

When α-actinin was incubated with cathepsin L at pH 5.0, it was degraded into a 80 000-dalton fragment (Fig. 6). Roughly speaking, the degradation rate of α-actinin was comparable to that of actin but was slower than those of myosin heavy chain and troponin.

When the treatment of α-actinin with cathepsin L was carried out at various pH values for 20 h, degradation of α-actinin occurred at pH 2.0–5.0 (gels 2 to 7 in Fig. 7) and was most severe at pH 3.0–3.5 (gels 3 and 4). In the latter pH region several fragments appeared with M_r values lower than 80 000.

Discussion

The present work has shown that cathepsin L degrades myosin, actin, α-actinin, troponin T and troponin I assembled in myofibrils. Since these proteins isolated from myofibrils have also been demonstrated to be hydrolyzed by this enzyme, assembly into a myofibrillar structure does not seem to affect the susceptibility of the constituent proteins to cathepsin L. Moreover, the degradation mode of each protein assembled in myofibrils is assumed to be similar to that of the isolated one, because almost all the degradation products of the myofibrils treated with cathepsin L can be assigned by the degradation mode of the isolated proteins.

On the basis of the action towards myosin and actin, cathepsin L can be regarded as an acid proteinase, because these proteins are degraded most intensely around pH 4. However, it should be noted that its action towards troponin is profound at a pH value higher than 4.

There are some papers describing the mode of myosin degradation induced by tissue proteases. Myosin heavy chain is degraded by a serine protease into 100 000- and 88 000-dalton fragments [1], by cathepsin D into 150 000- and 110 000-dalton fragments [5], and by cathepsin B into a 150 000-dalton fragment [5]. The present work has shown that cathepsin L degrades myosin heavy chain initially into a 160 000-dalton fragment, which is hydrolyzed further into 92 000-, 83 000- and 60 000-dalton fragments. Since some of these degradation products show similarity in their molecular weights to those produced by other tissue proteases, some definite sites in the myosin heavy chain molecule seem commonly susceptible to various tissue proteases. Cathepsin L has been shown to degrade myosin light chains 1, 2 and 3, while the serine protease is known to hydrolyze light chain 2 [1].

There are few reports on muscle proteases capable of degrading actin. The serine protease was reported to degrade actin slowly in an isolated state producing no detectable fragments [1]. Neither Ca^{2+}-activated neutral proteases from three animal species [2–4] nor cathepsin D [6] is capable of degrading actin. Cathepsin L is thus the first intracellular protease reported so far in skeletal muscle to degrade actin, because the serine protease is not endogenous for muscle cells but for mast cells in muscle tissue [19].

Since α-actinin is known to be hardly degraded by the known muscle proteases, cathepsin L can be regarded as one of the few proteases capable of hydrolyzing α-actinin.

Tropomyosin was one of the proteins found not to be hydrolyzed by cathepsin L. This substrate specificity also distinguishes cathepsin L from the serine protease [1] and Ca^{2+}-activated neutral proteases [2,3], all of which are active towards tropomyosin.

Of all the myofibrillar proteins examined troponin appeared to be most susceptible to cathepsin L, which degraded it in a similar manner to that of CAF [2]. The serine protease is also known to degrade troponin T [1]. Troponin I was hydrolyzed by cathepsin L as well as by the serine protease [1] and Ca^{2+}-activated neutral proteases [2,3], while it was less susceptible than troponin T. There is no report on a muscle protease that degrades troponin C.

On the basis of all the results cathepsin L seems to be one of the important proteinases for the degradation of the myofibrillar proteins in living muscle. Especially, this enzyme appears to be essential for actin catabolism.

Furthermore, cathepsin L is also assumed to play an important role in the autolysis of postmortem muscle. The presence of a 30 000-dalton component in SDS-polyacrylamide gel electrophoretic patterns from myofibrils during postmortem ageing of muscle was observed by Hay et al. [20], by Penny [21] and by Olson et al. [22]. Olson and Parrish [23], and MacBride and Parrish [24] reported that in tender meats the 30 000-dalton component was present, but it was absent in tough meats. Olson et al. [22] observed that CAF-degraded troponin T into a 30 000-dalton component in neutral pH regions and they thus considered that the development of the 30 000-dalton component in the stored muscle was due to the action of CAF. On the other hand, our present work has shown that cathepsin L acts on myofibrils and produces the 30 000-dalton component from troponin T and actin in an acidic pH region covering the normal ultimate (final) pH of postmortem muscles, i.e., pH 5.5–5.8. Penny and Ferguson-Pryce [25] recently measured the degradation rate of troponin T on the incubation of bovine muscle homogenates and showed that the rate was accelerated not by Ca^{2+} but by EDTA in a pH range of between 5 and 6. We have shown in the previous work [7] that EDTA is a potent enhancer for cathepsin L. These facts strongly suggest that the 30 000-dalton component developed on ageing of muscle with a normal ultimate pH is a product caused mainly by the action of cathepsin L. Furthermore it is probable that cathepsin L contributes to postmortem tenderization of meat, since Penny and Dransfield [26] have shown that the decrease in toughness is parallel with the loss of troponin T during conditioning of beef.

References

1 Yasogawa, N., Sanada, Y. and Katunuma, N. (1978) J. Biochem. 83, 1355–1360
2 Dayton, W.R., Goll, D.E., Stromer, M.H., Reville, W.J., Zeece, M.G. and Robson, R.M. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D.B. and Shaw, E., eds.), pp. 551–577, Cold Spring Harbor Laboratory, New York
3 Ishiura, S., Sugita, H., Suzuki, K. and Imahori, K. (1979) J. Biochem. 86, 579–581
4 Azanza, J.-L., Raymond, J., Robin, J.-M., Cottin, P. and Ducastaing, A. (1979) Biochem. J. 183, 339–347
5 Schwartz, W.N. and Bird, J.W.C. (1977) Biochem. J. 167, 811–820
6 Robbins, F.M., Walker, J.E., Cohen, S.H. and Chatterjee, S. (1979) J. Food Sci. 44, 1672–1677
7 Okitani, A., Matsukura, U., Kato, H. and Fujimaki, M. (1980) J. Biochem. 87, 1133–1143
8 Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S. and Bohley, P. (1977) Eur. J. Biochem. 74, 293–301
9 Yang, R., Okitani, A. and Fujimaki, M. (1972) Agric. Biol. Chem. 36, 2087–2095
10 Perry, S.V. (1955) Methods Enzymol. 2, 582–588
11 Richards, E.G., Chung, C.-S., Menzel, D.B. and Olcott, H.S. (1967) Biochemistry 6, 528–540
12 Mommaerts, W.F.H.M. (1951) J. Biol. Chem. 188, 559–565
13 Rees, M.K. and Young, M. (1967) J. Biol. Chem. 242, 4449–4458
14 Masaki, T. and Takaiti, O. (1969) J. Biochem. 66, 637–643
15 Ebashi, S., Wakabayashi, T. and Ebashi, F. (1971) J. Biochem. 69, 441–445
16 Mueller, H. (1966) Biochem. Z. 345, 300–321
17 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
18 Sender, P.M. (1971) FEBS Lett. 17, 106–110
19 Woodbury, R.G., Everitt, M., Sanada, Y., Katunuma, N., Lagunoff, D. and Neurath, H. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5311–5313
20 Hay, J.D., Currie, R.W. and Wolfe, F.H. (1973) J. Food Sci. 38, 987–990
21 Penny, I.F. (1976) J. Sci. Food Agric. 27, 1147–1155
22 Olson, D.G., Parrish, F.C., Jr., Dayton, W.R. and Goll, D.E. (1977) J. Food Sci. 42, 117–124
23 Olson, D.G. and Parrish, F.C., Jr. (1977) J. Food Sci. 42, 506–509
24 MacBride, M.A. and Parrish, F.C., Jr. (1977) J. Food Sci. 42, 1627–1629
25 Penny, I.F. and Ferguson-Pryce, R. (1979) Meat Sci. 3, 121–134
26 Penny, I.F. and Dransfield, E. (1979) Meat Sci. 3, 135–141