Biochimica et Biophysica Acta, 662 (1981) 202–209
Elsevier/North-Holland Biomedical Press

BBA 69414

PURIFICATION OF CATHEPSIN D FROM RABBIT SKELETAL MUSCLE AND ITS ACTION TOWARDS MYOFIBRILS

AKIHIRO OKITANI [a], TERUYO MATSUMOTO [b], YOHKO KITAMURA [b] and HIROMICHI KATO [a]

[a] *Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo and* [b] *Tokyo Kasei University, Tokyo (Japan)*

(Received May 12th, 1981)

Key words: Cathepsin D; Myofibril; Protein degradation; (Rabbit skeletal muscle)

Cathepsin D (EC 3.4.23.5) was purified from rabbit skeletal muscle using acetone-dried muscle powder as starting material. After the acetone-dried powder was extracted with 0.2 mM ATP, the extract was fractionated with acetone and subjected to DEAE-Sephadex A-50 and Sephadex G-100 column chromatography. Rechromatography on a Sephadex G-100 column resulted in a purified preparation. SDS-polyacrylamide gel electrophoresis of the purified enzyme showed one major band of 42 000 daltons and some bands of contaminants. Since gel filtration also indicated a value of 42 000 daltons for the enzyme, it was concluded that muscle cathepsin D has no subunit structure. The enzyme acted optimally towards myofibrils around pH 3, resulting in the degradation of the myosin heavy chain and production of a 30 000-dalton component.

Introduction

It is well known that a lysosomal protease, cathepsin D, (EC 3.4.23.5) is distributed throughout animal tissues, although its role remains to be clarified in protein catabolism and postmortem proteolysis [1]. While cathepsin D has been purified to a reasonably homogeneous state from spleen [2–6] and liver [7–9], a purified preparation of muscle cathepsin D has not as yet been obtained [9–13], probably due to its lower content in muscle than in other tissues [9]. In the present work, we have examined the effectiveness of several purification methods and found that the acetone-dried muscle powder is the most suitable source for obtaining purified cathepsin D of the highest homogeneity reported so far. The purified enzyme allowed us to examine its possible subunit structure and the action towards myofibrillar proteins.

Materials and Methods

Materials. DEAE-Sephadex A-50 and Sephadex G-100 were obtained from Pharmacia Fine Chemicals, Sweden, Diafilter G-10T membrane for ultrafiltration was from Bioengineering Co., Japan. Pepstatin was from the Peptide Institute, Japan. Aldolase, catalase, bovine serum albumin, pepsin, ovalbumin, chymotrypsinogen and cytochrome *c* were purchased from Boehringer Mannheim GmbH, F.R.G. Bovine hemoglobin was from Difco Lab., U.S.A. Muscle (longissimus dorsi) was obtained from a rabbit carcass immediately after death and minced with a meat chopper.

Preparation of myofibrils. Myofibrils prepared as described by Yang et al. [14] were washed additionally twice with 0.16 M KCl/0.2% Triton X-100 and used as the substrate for the enzyme assay.

Preparation of cathepsin D. All preparations were carried out at 4°C. Acetone-dried muscle powder was prepared according to the method of Straub [15]

with the slight modification made by Suzuki and Fujimaki [11], yielding about 70 g from 1 kg minced muscle. The muscle powder was extracted with 2 l cold 0.2 mM ATP with stirring for 1 h, and then the mixture was centrifuged. The resultant supernatant was mixed with 3 M KCl to give a final concentration of 0.1 M KCl and the mixture left to stand for 30 min to allow the actin to polymerize. The polymerized actin solution was fractionated with acetone pre-cooled to −20°C. The fraction precipitating from 33–55% acetone was collected by centrifugation and dialyzed against 10 mM phosphate buffer (pH 7.0). The dialysate clarified by centrifugation was subjected to DEAE-Sephadex A-50 column chromatography.

Preparation of cathepsin D from KCl-extracts of muscle was carried out essentially according to the method of Fukushima et al. [13] who used chicken muscle.

Preparation of cathepsin D from Triton-extracts of muscle was performed as follows: 1 kg minced muscle was homogenized with 2 vol. 3% NaCl and the mixture stirred for 1 h, after adjusting the pH to 3.8. The mixture was centrifuged and the resultant residue was extracted with 1 l 0.2% Triton X-100/3% NaCl, with stirring, for 1 h. After centrifugation, the supernatant obtained was fractionated with $(NH_4)_2SO_4$. The precipitates between 25 and 65% saturation were collected and dialyzed against 10 mM phosphate buffer (pH 7.0). The dialysate was applied to a DEAE-Sephadex A-50 column.

Enzyme assay. Cathepsin D activity was assayed using myofibrils and urea-denatured hemoglobin as the substrates. Urea-denatured hemoglobin was prepared by incubating 2% native hemoglobin at 25°C for 1 h in the presence of 3 M urea. The enzyme solution (0.5 ml) was mixed with 0.25 ml 2% denatured hemoglobin and 0.25 ml 0.2 M sodium acetate/HCl buffer. After incubation at 37°C for 1 h, the reaction was stopped by adding 1 ml 6% trichloroacetic acid and the mixture was filtered. Non-protein nitrogenous compounds in the filtrate were determined by the procedure of Lowry et al. [16]. The amount of the enzyme releasing 1 μg tyrosine equivalent over 1 h at pH 4.0 was regarded as one unit. The myofibrils (2.5 mg/ml) were incubated with the enzyme in 20 mM sodium acetate/HCl buffer/0.18 M NaCl/10 mM NaN_3 at 37°C for 16–22 h. The reaction was stopped by adding SDS and proteolytic degradation of the myofibrils was examined by SDS-polyacrylamide gel electrophoresis.

Electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn [17] using 7.5% gels. The gels were stained with Coomassie brillant blue R-250.

Molecular weight determination. The molecular weight of the enzyme was determined by gel filtration and SDS-polyacrylamide gel electrophoresis, using Sephadex G-100 and 7.5% polyacrylamide gels, respectively. The protein standards for chromatography were bovine serum albumin (M_r = 67 000), pepsin (M_r = 34 000), chymotrypsinogen (M_r = 25 000), and cytochrome *c* (M_r = 12 500). For the electrophoresis, bovine serum albumin, ovalbumin (M_r = 45 000), chymotrypsinogen, cytochrome *c*, and the subunits of catalase (M_r = 60 000) and aldolase (M_r = 40 000) were used.

Protein determination. Absorbance at 280 nm was used to monitor the protein peaks on chromatography. The protein concentration of the enzyme was determined by the method of Lowry et al. [16] and that of myofibrils was determined by the biuret method [18], using bovine serum albumin as the standard.

Results

Purification of cathepsin D from acetone-dried muscle powder

When the acetone fraction prepared by our method was applied to a column of DEAE-Sephadex A-50, most of the cathepsin D activity was eluted at 0.12 M NaCl (Fig. 1). At this concentration of NaCl, the protein eluted early as a narrow peak, while the enzyme activity started to elute later, and formed a broad peak. The active fractions were pooled and concentrated by ultrafiltration. Sucrose (final concentration: 10%) was added to the enzyme solution, prior to concentration, to prevent inactivation of the enzyme during the concentration step.

The concentrated enzyme solution was dialyzed against 10 mM acetate buffer (pH 4.0)/0.1 M NaCl and then applied to a Sephadex G-100 column equilibrated with the same buffer. Most of the protein was eluted in tubes 30–40 and was most abundant in tube 35 (Fig. 2), whereas the enzyme activity was

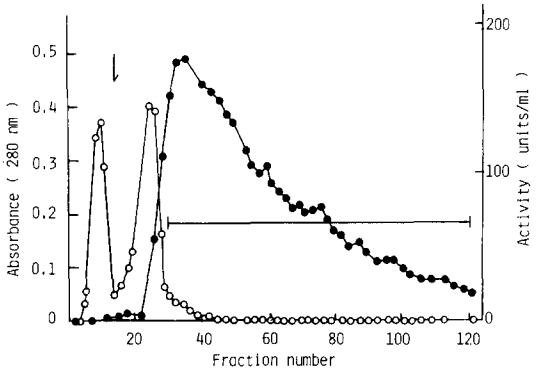

Fig. 1. DEAE-Sephadex A-50 column chromatography of cathepsin D. The acetone fraction of the enzyme (36.5 mg protein, 53.7 ml) was applied to a column (3 × 21 cm) equilibrated with 10 mM phosphate buffer (pH 7.0). Elution of the enzyme with 10 mM phosphate buffer (pH 7.0)/0.12 M NaCl was started at the point indicated by the arrow (20-ml fractions). ○———○, absorbance at 280 nm; ●———●, activity.

eluted in tubes 35–50 and was highest in tube 41. The elution of protein standards on the same column implied that tubes 35 and 41 corresponded to 67 000 and 42 000 daltons, respectively. In order to locate a myofibrillar protein-degrading activity during the chromatography step shown in Fig. 2, myofibrils

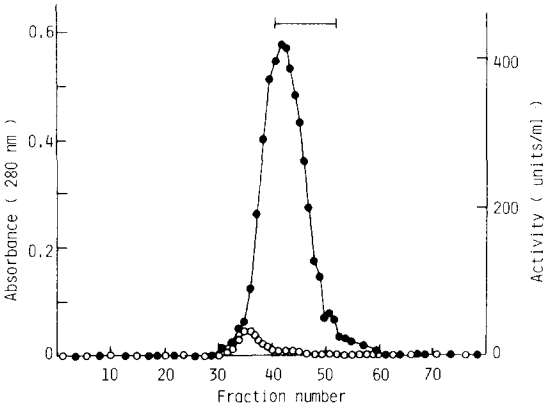

Fig. 2. First Sephadex G-100 column chromatography of cathepsin D. The active fractions from the DEAE-Sephadex A-50 column, indicated with the bar (———) in Fig. 1, were pooled and concentrated. The concentrated protein solution (17 mg protein, 15 ml) was applied to a column (3.6 × 74 cm) equilibrated with 10 mM sodium acetate/HCl buffer (pH 4.0)/0.1 M NaCl, and eluted with the same solution (10-ml fractions). ○———○, absorbance at 280 nm; ●———●, activity.

were incubated with each fraction at pH 3.8 and 37°C for 16 h and then subjected to SDS-polyacrylamide gel electrophoresis. Remarkable changes were observed in the myofibrils treated with the fractions having the hemoglobinolytic activity, where myosin heavy chain was degraded and a new band of 30 000 daltons appeared. Since the elution position of the myofibrillar protein-degrading activity coincided with that of the hemoglobinolytic activity, both activities could be attributed to cathepsin D.

The proteins eluted from the Sephadex G-100 column in Fig. 2 were examined by SDS-polyacrylamide gel electrophoresis (Fig. 3). Tube 35, with a low cathepsin D activity, showed a major 67 000-dalton band and tube 37 showed three protein bands of 67 000, 44 000 and 42 000 daltons. As the elution proceeded, the 67 000- and 44 000-dalton proteins

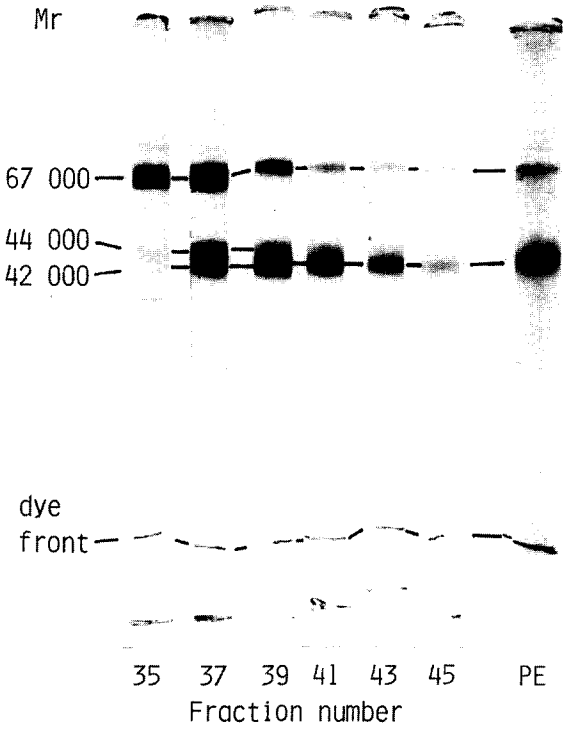

Fig. 3. SDS-polyacrylamide gel electrophoretic patterns of the fractions eluted from the first Sephadex G-100 column shown in Fig. 2. After concentration, fractions 35 (8 μg protein), 37 (13 μg), 39 (9 μg), 41 (6 μg), 43 (4 μg) and 45 (3 μg) were loaded on gels. Gel PE contains the purified enzyme (5 μg protein) eluted from the second Sephadex G-100 column.

TABLE I

PURIFICATION OF CATHEPSIN D FROM ACETONE-DRIED MUSCLE POWDER

The yields of protein and enzyme activities are those obtained from 1 kg tissue.

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield of activity (%)	Purification (-fold)
Crude extract	4960	277000	55.9	100	1
Acetone fraction	38.0	141000	3720	50.9	66.2
DEAE-Sephadex A-50	21.4	112000	5230	40.1	93.6
Sephadex G-100	0.67	29000	43600	10.5	781
2nd Sephadex G-100	0.147	11000	74900	4.0	1340

decreased and the 42000-dalton protein increased. Since the 42000-dalton band was the major band in the most enzymatically active tube (tube 41), it can be regarded as cathepsin D.

The fractions (tubes 40–52) with high specific activities were pooled, and then subjected to rechromatography on a Sephadex G-100 column after concentration to 1/50 vol. The position of the protein

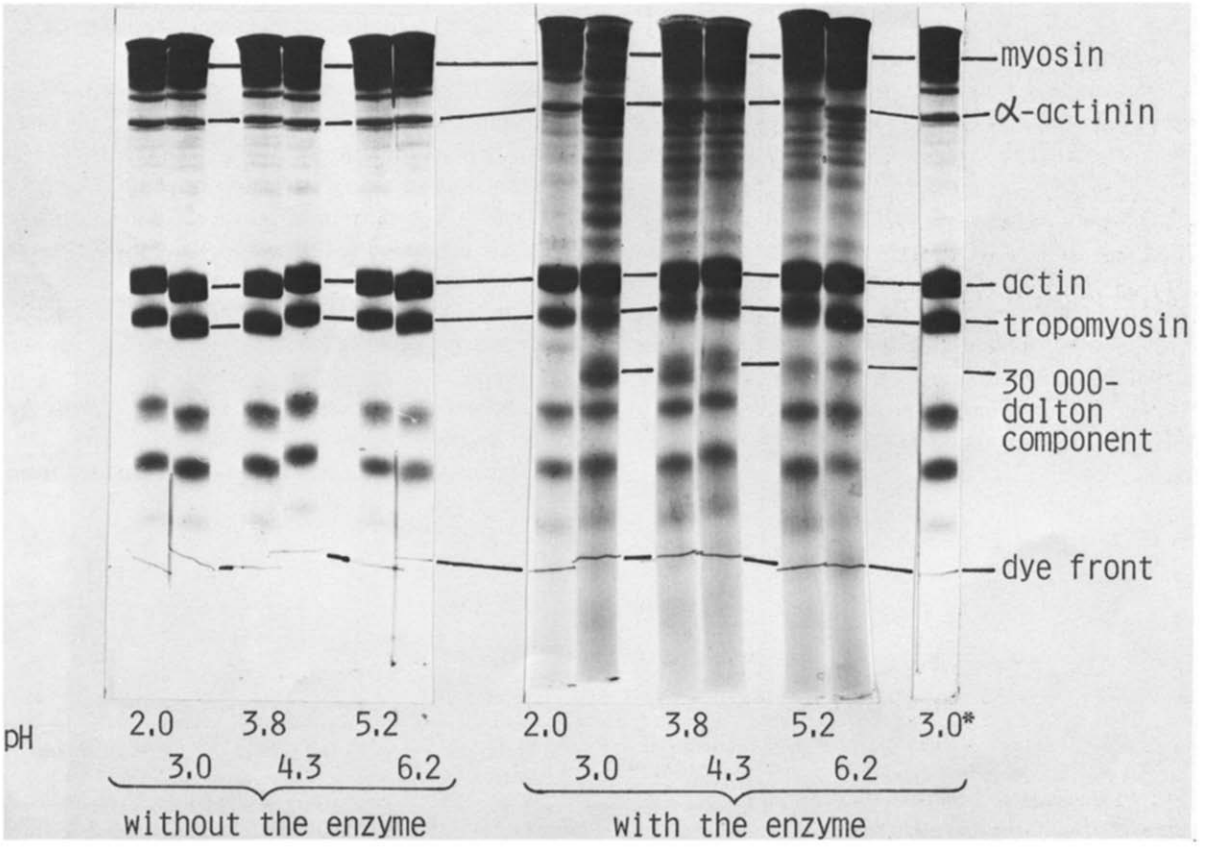

Fig. 4. Effect of pH on the activity of cathepsin D towards myofibrils. 1 mg myofibrils was incubated with or without 63 units of the enzyme in 0.4 ml 20 mM sodium acetate/HCl buffer/0.18 M NaCl/10 mM NaN_3 at 37°C and the pH indicated below each gel for 22 h. The treated myofibrils (93 μg) were subjected to SDS-polyacrylamide gel electrophoresis. * shows the myofibrils treated with the enzyme at pH 3.0 in the presence of 0.1 mM pepstatin.

peak coincided with that of the activity peak. When the most active fraction was examined by SDS-polyacrylamide gel electrophoresis, it still contained some impurities with molecular weights higher than 42000 (Fig. 3). Further purification tried, using a third Sephadex G-100 chromatography, to completely eliminate these bands resulted in a drastic reduction of the enzyme yield. Since these impurities did not interfere to examine the action of cathepsin D towards myofibrils we used the enzyme obtained by the second Sephadex G-100 chromatography for further studies (Table I). The fractionation with acetone resulted in a marked increase of the specific activity, because the bulk of foreign protein, i.e., actin, was removed by 33% acetone. Only 0.147 mg of the purified enzyme was obtained from 1 kg muscle, and 1340-fold purification was achieved from the crude extract to the purified enzyme.

Properties of the purified cathepsin D

The molecular weight determined by gel filtration was 42000. Since this value was the same as estimated by SDS-polyacrylamide gel electrophoresis (Fig. 3), it has been concluded that the cathepsin D molecule is a single polypeptide chain of 42000 daltons.

The pH dependence of the activity was examined using urea-denatured hemoglobin and myofibrils as the substrates. Cathepsin D hydrolyzed hemoglobin optimally at pH 3.2, and myofibrils at pH 3.0 (Fig. 4). The myofibrillar protein-degrading activity of the enzyme was completely inhibited by pepstatin (Fig. 4).

Purification of cathepsin D from the salt-soluble fraction of muscle

The salt-soluble fraction of muscle was discarded in the above method, although several researchers [9,10,12,13] employed 1–2% KCl for the extraction of muscle cathepsin D. Thus, it is important to determine if such different starting materials give the same enzyme as obtained from the acetone-dried muscle powder. KCl-extracts of muscle were prepared and fractionated with $(NH_4)_2SO_4$. The resultant active fraction was loaded on a DEAE-Sephadex A-50 column and eluted with a linear gradient of NaCl. The activity was eluted around 0.12 M NaCl. When the active fractions were pooled and subjected to Sephadex G-100 chromatography after concentration, the activity was eluted in a single peak at the position corresponding to a molecular weight of 42000. The active fractions were pooled again and subjected to rechromatography on a Sephadex G-100 column.

Table II presents summarized data of the purification using the 2% KCl-extracts. The enzyme obtained from the rechromatography on a Sephadex G-100 column showed remarkably lower specific activity and yield as compared with the enzyme obtained from the acetone-dried muscle powder. The enzyme with such low specific activity was shown to be contaminated by abundant impurities by SDS-polyacrylamide gel electrophoresis.

Extraction of cathepsin D bound to myofibrils by Triton X-100

The fact that cathepsin D could be extracted from

TABLE II

PURIFICATION OF CATHEPSIN D FROM 2% KCl-EXTRACTS OF MUSCLE

The yields of protein and enzyme activities are those obtained from 1 kg tissue.

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield of activity (%)	Purification (-fold)
Crude extract	29418	253000	8.6	100	1
$(NH_4)_2SO_4$ fraction	1018	22400	22.0	8.80	2.5
DEAE-Sephadex A-50	110	19500	172	7.70	20.5
Sephadex G-100	7.0	6980	997	2.76	117
2nd Sephadex G-100	0.54	1020	1890	0.40	220

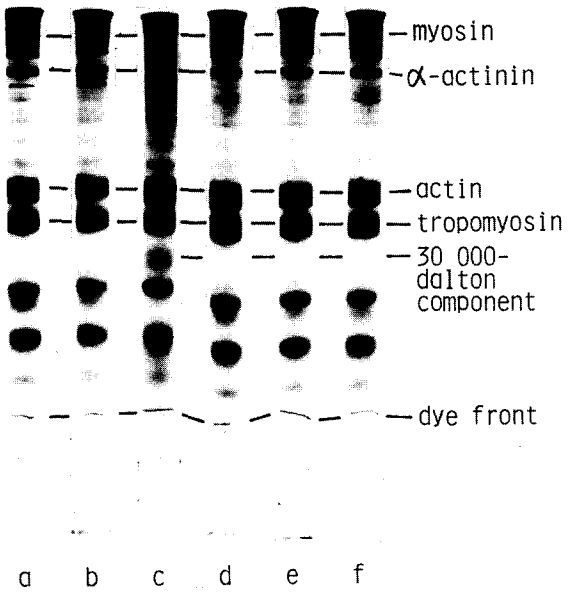

Fig. 5. Effect of Triton-washing on autolysis of myofibrils. Myofibrils (2.5 mg/ml) washed without (gels a, b and c) or with (gels d, e and f) 0.2% Triton X-100 were incubated in 20 mM sodium acetate/HCl buffer/0.18 M NaCl/10 mM NaN_3 at pH 3.8 and 37°C for 22 h. Myofibrils (62 μg) were subjected to SDS-polyacrylamide gel electrophoresis. a and d, before incubation; b and e, 22 h-incubation in the presence of 0.1 mM pepstatin; c and f, 22 h-incubation in the absence of pepstatin.

the acetone-dried muscle powder prepared from the residue obtained after the extraction of thick filament proteins with Straub solution (0.3 M KCl/0.1 M KH_2PO_4/0.05 M K_2HPO_4, pH 6.4) suggests that the enzyme was bound to thin filaments and/or the Z-line of myofibrils through hydrophobic bonding. Thus, the myofibrils prepared omitting Triton X-100-washing were incubated without the enzyme and examined by electrophoresis. As shown in Fig. 5, after incubation for 22 h at pH 3.8 and 37°C, degradation of myosin heavy chain and production of a 30 000-dalton component were observed. These changes were exactly the same as observed in the Triton-washed myofibrils treated with the purified cathepsin D as shown in Fig. 4. It was also demonstrated that such changes were blocked by the addition of pepstatin. On the other hand, no change was observed on incubation of the myofibrils washed with Triton. Furthermore, these results suggested that the crude enzyme extracted with Triton from salt-extracted muscle residues might be a good source for the purification of cathepsin D. Thus, the Triton-extracted crude enzyme was fractionated with $(NH_4)_2SO_4$, and then the active fractions was subjected to DEAE-Sephadex A-50 column chromatography under the same conditions as shown in Fig. 1. The active fractions eluted at 0.12 M NaCl were pooled and loaded on a Sephadex G-100 column. The cathepsin D activity was sometimes eluted in a single peak at the position corresponding to 42 000 daltons, but sometimes it was scattered between the position of the void volume and the position corresponding to about 10 000 daltons. Several separate experiments showed no reproducibility of the elution position of the cathepsin D activity extracted with Triton.

Discussion

The specific activity of the purified enzyme prepared from acetone-dried muscle was in the range of values reported for homogeneous cathepsin D from other tissues [6,9]. This means that the purification method employed has given muscle cathepsin D of the highest homogeneity reported so far, although the preparation still contains some impurities with molecular weights higher than 42 000. It cannot be excluded that these impurities are other proteases. However, the present work revealed that these impurities possessed no activity towards hemoglobin or myofibrillar proteins under the conditions where cathepsin D was operative, because none of these activities were observed at the elution position corresponding to their molecular weights on Sephadex G-100 column chromatography of the enzyme. These results also indicate that the enzyme preparation is free from a cathepsin D-like enzyme, i.e., cathepsin E, the molecular weight of which is 100 000 for the enzyme from rabbit bone marrow [19] and 305 000 from bovine spleen [20].

The similarity of the molecular weights suggests that the cathepsin D extractable with 2% KCl from the muscle homogenates is identical to the enzyme extractable from the acetone-dried muscle powder. Therefore, it appears that cathepsin D was partially released into the sarcoplasm and partially bound to myofibrils after rupture of lysosomes due to homogenization of the muscle. The effectiveness of Triton or acetone treatment in removing the enzyme

from myofibrils indicates that hydrophobic bonding occurs between these two substances. Noguchi and Kandatsu [21] reported that almost all the myofibril-bound hemoglobinolytic activity that could be measured at pH 3.8 could be extracted with Triton X-100. The present work suggests that such activity can be completely or partially ascribed to cathepsin D. Arakawa et al. [22] employed Triton X-100 to extract a myofibrillar protein-degrading protease from myofibrils, but it remains to be investigated whether it was cathepsin D or not. The acetone-dried muscle powder employed by Suzuki and Fujimaki [11] has been demonstrated to be a material superior to Triton- or KCl-extracts for the purification of muscle cathepsin D in both terms of specific activity and yield.

Affinity chromatography using pepstatin- or hemoglobin-conjugated Sepharose was used successfully for cathepsin D from other tissues [2,6]. However, these materials were not effective for purifying muscle cathepsin D because nonspecific binding of many impurities and a marked reduction of yield occurred.

One of the authors [23] estimated the molecular weight of rabbit skeletal muscle cathepsin D to be 29 000–30 000 by an equilibrium ultracentrifugation method. On the other hand, Schwartz and Bird [9] reported a value of 42 000 for rat skeletal muscle cathepsin D. In the present work, the value of 42 000 was obtained for the almost homogeneous enzyme by both gel filtration and SDS-polyacrylamide gel electrophoresis. Thus, the previous value of 29 000–30 000 is assumed to be incorrect, due to the large amount of impurities in the enzyme preparation used. Furthermore, the present results have presented the first evidence that muscle cathepsin D has no subunit structure, unlike the porcine spleen one reported by Huang et al. [5].

Muscle cathepsin D has been shown to act towards myofibrils optimally around pH 3, as it does similarly towards hemoglobin. The marked changes observed in the cathepsin D-treated myofibrils are degradation of myosin heavy chain and the appearance of a 30 000-dalton component. The degradation of myosin heavy chain is in accordance with the results of Schwarz and Bird [9]. They also reported that isolated actin was able to be hydrolyzed by rat skeletal cathepsin D. However, in the present work we could not observe any apparent degradation of actin in myofibrils. Further work is needed to clarify the cause of this discrepancy.

Several researchers [24–26] demonstrated that the appearance of a 30 000-dalton component was one piece of evidence of the postmortem proteolysis of skeletal muscles. A Ca^{2+}-activated neutral protease [27] and cathepsin L [28] were shown to produce such a component from myofibrillar proteins. Cathepsin D has also been demonstrated to be able to produce such a component. The postmortem pH of muscle decreased from around 7 at death to 5–6 at rigor. Therefore, if the above three proteinases are active in the postmortem muscle in situ, it is presumed that the Ca^{2+}-activated neutral protease is responsible for the production of the 30 000-dalton component at early postmortem stage, and then cathepsins L and D are responsible at the rigor stage. This presumption well explains our previous result [29] that the postmortem proteolysis of muscle having a pH lower or higher than 6.4 occurred more rapidly than in muscle at pH 6.4. Since cathepsin L has a higher optimum pH [28] than cathepsin D towards myofibrillar proteins, the former enzyme may be functioning more predominantly than the latter enzyme in the postmortem muscles of a normal ultimate pH (pH 5–6). This conception is supported by our previous observation [30] that there are two peaks of autoproteolytic activity at pH 3 and 4.5 on incubation of muscle homogenates in the acid pH region. Our conception is also in good accord with that presented by Penny and Ferguson-Pryce [31], who measured the pH dependence of the troponin T-degradation rate during the incubation of bovine muscle homogenates.

References

1 Barret, A.J. (1977) in Proteinases in Mammalian Cells and Tissues (Barret, A.J., ed.), pp. 209–298, North-Holland Publishing Co., Amsterdam

2 Smith, R. and Turk, V. (1974) Eur. J. Biochem. 48, 245–254

3 Ducastaing, A., Azanza, J., Robin, J.M., Raymond, J. and Créach, P. (1976) Biochimie 58, 771–782

4 Cunningham, M. and Tang, J. (1976) J. Biol. Chem. 251, 4528–4536

5 Huang, J.S., Huang, S.S. and Tang, J. (1979) J. Biol. Chem. 254, 11405–11417

6 Yamamoto, K., Katsuda, N., Himeno, M. and Kato, K.

(1979) Eur. J. Biochem. 95, 459–467
7 Barret, A.J. (1970) Biochem. J. 117, 601–607
8 Wiederanders, B., Ansorge, S., Bohley, P., Broghammer, U., Kirschke, H. and Langner, J. (1976) Acta Biol. Med. Germ. 35, 269–283
9 Schwartz, W.N. and Bird, J.W.C. (1977) Biochem. J. 167, 811–820
10 Iodice, A.A., Leong, A.A. and Weinstock, I.M. (1966) Arch. Biochem. Biophys. 117, 477–486
11 Suzuki, A. and Fujimaki, M. (1968) Agric. Biol. Chem. 32, 975–982
12 Martin, C.B. and Whitaker, J.R. (1968) J. Food. Sci. 33, 59–64
13 Fukushima, K., Gnoh, G.H. and Shinano, S. (1971) Agric. Biol. Chem. 35, 1495–1502
14 Yang, R., Okitani, A. and Fujimaki, M. (1970) Agric. Biol. Chem. 34, 1765–1772
15 Straub, F.B. (1942) Studies Inst. Med. Chem. Univ. Szeged. 2, 3–15
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
17 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
18 Gornall, A.G., Bardawill, C.S. and David, M.M. (1949) J. Biol. Chem. 177, 751–766
19 Lapresle, C. (1971) in Tissue Proteinases (Barret, A.J. and Dingle, J.T., eds.), pp. 135–155, North-Holland Publishing Co., Amsterdam
20 Turk, V., Kregar, I. and Lebez, D. (1968) Enzymologia, 34, 89–100
21 Noguchi, T. and Kandatsu, M. (1970) Agric. Biol. Chem. 34, 390–394
22 Arakawa, N., Fujiki, S., Inagaki, C. and Fujimaki, M. (1976) Agric. Biol. Chem. 40, 1265–1267
23 Suzuki, A., Okitani, A. and Fujimaki, M. (1969) Agric. Biol. Chem. 33, 579–585
24 Hay, J.D., Currie, R.W. and Wolfe, F.H. (1973) J. Food Sci. 38, 987–990
25 Penny, I.F. (1976) J. Sci. Food Agric. 27, 1147–1155
26 Olson, D.G. and Parrish, F.C., Jr. (1977) J. Food Sci. 42, 506–509
27 Dayton, W.R., Reville, W.J. Goll, D.E. and Stromer, M.H. (1976) Biochemistry 15, 2159–2167
28 Okitani, A., Matsukura, U., Kato, H. and Fujimaki, M. (1980) J. Biochem. 87, 1133–1143
29 Okitani, A., Shinohara, K., Sugitani, M. and Fujimaki, M. (1973) Agric. Biol. Chem. 37, 321–325
30 Okitani, A., Matsukura, U., Otsuka, Y., Watanabe, M. and Fujimaki, M. (1977) Agric. Biol. Chem. 41, 1821–1823
31 Penny, I.F. and Ferguson-Pryce, R. (1979) Meat Sci. 3, 121–134