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A CALCIUM-ACTIVATED PROTEASE POSSIBLY INVOLVED IN MYOFIBRILLAR PROTEIN TURNOVER

ISOLATION OF A LOW-CALCIUM-REQUIRING FORM OF THE PROTEASE

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Summary

Two forms of a calcium-activated neutral protease were isolated and purified from porcine skeletal muscle. The two forms of the protease differ markedly in their requirement for calcium with the low-calcium-requiring form showing one-half maximal activation at 45 μM calcium while the high-calcium-requiring form shows one-half maximal activation at 0.74 mM calcium. Additionally, they chromatograph differently on DEAE-cellulose, exhibit different mobilities in electrophoresis in a nondenaturing buffer, are affected differently by certain divalent cations, and have slightly different pH dependencies. Despite these differences, the purified forms of the calcium-activated protease co-chromatograph in gel permeation chromatography, have identical banding patterns on sodium dodecyl sulfate (SDS)-polyacrylamide gels, cross-react with an antibody directed against the 80 000-dalton subunit of the calcium-activated protease we originally purified from skeletal muscle (Dayton, W.R., Goll, D.E., Zeece, M.G., Robson, R.M. and Reville, W.J. (1976) Biochemistry 15, 2150—2158), and have identical effects on the ultrastructure of myofibrils. The high-calcium-requiring protease purified in this study is very likely identical to the calcium-activated protease we originally purified from skeletal muscle. The properties of the low-calcium-requiring form of the protease suggest that it is the form of the enzyme that is active in vivo.

Abbreviation IgG, immunoglobulin G

Introduction

Calcium-activated neutral proteolytic activity has been isolated from many tissues including brain [1], skeletal muscle [2], cardiac muscle [3,4], smooth muscle [5,6] and hen oviduct [7]. In addition, calcium-activated proteases have been purified from mammalian skeletal muscle [8,9] and chicken skeletal muscle [10]. Even so, the function of the calcium-activated protease(s) in the cell is not well understood. Additionally, there is disagreement concerning the subunit composition of the enzyme from skeletal muscle [9,11].

Purified calcium-activated protease from skeletal muscle causes partial degradation of myofibrils, attacking the Z-disk and hydrolyzing troponin-T, troponin-I, C-protein and tropomyosin [12]. It is therefore possible that the calcium-activated protease may be involved in degradation of myofibrillar proteins in muscle [12] and also in non-muscle tissue. This theory is supported by reports that the amount of calcium-activated protease isolated from rapidly atrophying muscle tissue (where myofibril degradation is proceeding rapidly) is several-fold greater than the amount isolated from normal muscle tissue [13]. Additionally, immunocytochemical localization of the calcium-activated protease has shown that the protease is located in the myofibril [15,16]. Our studies [16] using a monospecific antibody directed against the 30 000-dalton subunit of the calcium-activated protease to localize the enzyme have shown that the protease is located specifically in the Z-disk of the myofibril and also in or near the sarcolemma. Localization of the calcium-activated protease in the Z-disk, a myofibrillar structure shown to be extremely susceptible to calcium-activated protease-catalyzed hydrolysis, strongly supports the theory that the protease plays a role in myofibril degradation.

A major problem in understanding the physiological role of the calcium-activated protease(s) has been the fact that in in vitro assays the protease(s) requires the presence of 1—2 mM Ca^{2+} in order to express maximum activity [2,8,9]. This is higher than the levels of free calcium normally thought to be present in muscle cells [17]. Consequently, a recent report that cardiac muscle contains both the calcium-activated protease requiring millimolar levels of calcium and a calcium-dependent proteolytic activity detectable in the presence of physiologically obtainable calcium concentrations [3] was of extreme interest. Although the two calcium-activated proteolytic activities detected in cardiac muscle [3] are similar in some properties, it was not clear whether or not they were related since neither protease was purified. We report here the purification of both the low-calcium and high-calcium-requiring proteases. The purified proteases are immunologically cross-reactive and have identical banding patterns on SDS-polyacrylamide gels. Consequently, it appears that the two calcium-activated proteolytic activities present in porcine skeletal muscle are due to two forms of the same basic calcium-activated proteolytic enzyme. One form requires high calcium for activity while the other form is active at much lower calcium concentrations approaching the free Ca^{2+} concentrations attained in muscle cells during contraction. Additionally, it appears that the high-calcium-requiring form is identical to the calcium-activated protease we originally purified from porcine skeletal muscle [8].

Materials and Methods

Isolation of calcium-activated proteolytic activity from muscle tissue Extraction of the calcium-activated proteolytic activity from porcine skeletal muscle was done essentially as described previously [8], except that the extraction buffer was 50 mM Tris-acetate, pH 8.0/4 mM EDTA. Both the low- and high-calcium-requiring proteolytic activities and an inhibitor of the calcium-activated protease(s) precipitated between 30 and 40% $(NH_4)_2SO_4$ saturation from the 15 000 × *g* supernatant of the whole muscle homogenate The precipitate was dissolved in 20 mM Tris-acetate, pH 7.5/1 mM EDTA/10 mM 2-mercaptoethanol and dialyzed against this buffer for 12 h This solution was then clarified at 105 000 × *g* for 1 h and added to DEAE-cellulose that had been equilibrated against dialysis buffer. After allowing time for the protein to bind to the DEAE-cellulose, the cellulose was allowed to precipitate and the supernatant was decanted. The cellulose was subsequently washed twice with 3 vol (v/v) dialysis buffer and was then packed into a 4 × 85 cm column Protein concentrations were determined using the biuret method [19] or the method of Lowry et al. [20].

Chromatographic procedures. Sepharose 6B (Pharmacia Fine Chemicals, Inc.) and Ultrogel (LKB Instruments, Inc.) were used for gel-permeation chromatography. Ion-exchange chromatography was done using DEAE-cellulose (Whatman DE-52). Column dimensions, protein loads, flow rates and buffer systems for individual columns are given in the appropriate figure legend.

Electrophoretic procedures Analytical slab-gel electrophoresis in the presence of SDS was done according to the procedure of Laemmli [21]. Gels were stained in 0.1% Coomassie blue/50% methanol/7% glacial acetic acid solution and were destained electrophoretically.

Preparative polyacrylamide slab-gel electrophoresis in non-denaturing buffer was done according to the procedure of Maurer [22] as described previously [8], except that a dual vertical slab-gel electrophoresis cell (Bio-Rad Laboratories) and an LKB 2103 power supply were used. 1 mg protein (either high-calcium-requiring or low-calcium-requiring protease that in each case had been partially purified chromatographically) was loaded onto a slab gel and electrophoresed at 14 mA/gel while the gel was being cooled to 4° C by cold water passing through the electrophoresis cell At the completion of the run, the polyacrylamide containing each individual protein band was removed and disrupted mechanically to form a slurry which was then placed in a separate cylindrical gel tube containing a 3.5% polyacrylamide plug Electrophoresis for 8 h at 4° C and 2 mA/gel was sufficient to move the protein out of the slurry through the plug and into a dialysis tubing which was fitted over the end of the gel tube The protein obtained in this way was then dialyzed overnight against 1 mM $KHCO_3$/5 mM 2-mercaptoethanol/5 mM EDTA. Each sample was then assayed for the calcium-activated proteolytic activity for homogeneity using SDS-polyacrylamide slab-gel electrophoresis.

Purification of the 80 000-dalton subunit of the calcium-activated protease was done as described above using preparative SDS-polyacrylamide slab-gel electrophoresis [21].

Protease assays. Calcium-activated proteolytic activity was assayed using either casein or myofibrils as the substrate. The details of these assays have been described previously [8]. Calcium dependence and pH dependence of the calcium-activated proteolytic enzyme were determined using casein or myofibrils as a substrate. The methodology for these assays has been reported in detail previously [9].

Immunological procedures The high-calcium-requiring protease which we originally isolated from skeletal muscle was purified to near homogeneity using chromatographic procedures described previously [8]. To prepare highly purified 80 000-dalton subunit for use in antibody production, chromatographically purified calcium-activated protease was further purified using SDS-polyacrylamide slab-gel electrophoresis as described previously in this section. The homogeneity of the electrophoretically-purified 80 000-dalton subunit preparation was confirmed using analytical SDS-polyacrylamide gel electrophoresis (Fig. 6). Approx. 75 μg of the electrophoretically-purified 80 000-dalton subunit was emulsified in an equal volume of Freund's complete adjuvant and injected directly into the popliteal lymph node of a rabbit. This procedure was repeated 21 days after the first injection except that the antigen was emulsified in incomplete adjuvant. Rabbits injected in this way produced antisera with a high titer directed against the 80 000-dalton subunit. This antisera was frozen in liquid nitrogen and stored at —60°C until needed. The IgG fraction was precipitated from the antisera at 50% $(NH_4)_2SO_4$ saturation and further purified using DEAE-cellulose column chromatography [23,24].

Double diffusion was carried out in 1% agarose (Induboise A45, L'Industrie Biologique Francaise 5. A. Gennevilliers, France) containing 0.01% Merthiolate (Eli Lilly) and borate-buffered saline, pH 8.5 [25]. The immunodiffusion plates were allowed to incubate in a moist chamber for 24 h at room temperature. The plates were then rinsed in three changes of 0.15 M NaCl each day for three days, rinsed in distilled water for 2 h with one change and dried by placing a piece of wet filter paper over the plate for at least 15 h [26]. Dried plates were stained for 30 min with 0.25% Coomassie brilliant blue in a 5 . 1 . 4 mixture of methanol/acetic acid/water [27]. The plates were destained in the same solution without the stain.

Calcium-activated protease treatment of myofibrils Myofibrils prepared from porcine skeletal muscle according to the procedure of Goll et al. [18] were treated with purified calcium-activated protease as described previously [12]. Control and treated myofibrils were then prepared for electron microscopy using a procedure described in detail elsewhere [13]. Specific details of individual calcium-activated protease treatments are given in the appropriate figure legend.

Results

Chromatographic purification of calcium-activated proteolytic activity

The 4 × 85 cm DEAE-cellulose column to which the crude calcium-activated protease had been bound (Materials and Methods) was eluted with a linear KCl gradient (Fig. 1). Two peaks of calcium-activated proteolytic activity elute from the column as the gradient is developed. One peak elutes a 70 mM KCl

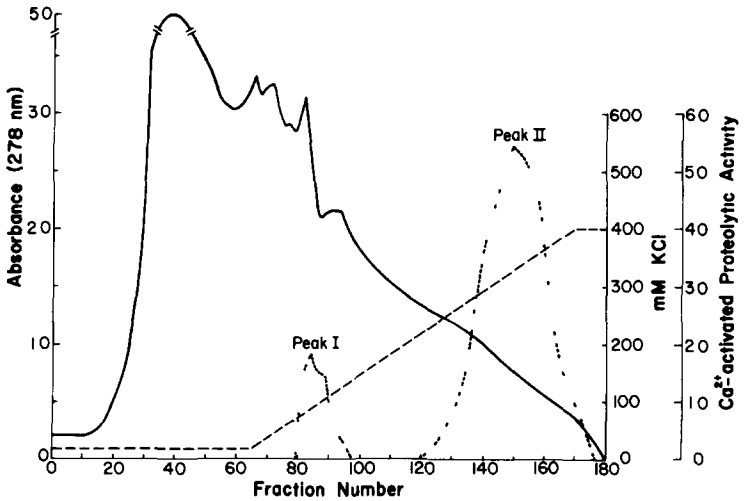

Fig 1 Elution profile of calcium-activated protease activity from a 4 0 X 85 cm DEAE-cellulose column Prior to pouring the column, the DEAE-cellulose was mixed with 31 059 mg of the protein fraction salting out between 30 and 40% $(NH_4)_2SO_4$ saturation from the 15 000 X *g* supernatant of the whole muscle homogenate The bound protein was eluted at 60 ml/h with a 2000 ml continuous gradient from 10 to 400 mM KCl in 20 mM Tris-acetate, pH 7 5/1 mM EDTA/10 mM 2-mercaptoethanol 20-ml fractions were collected ------, KCl concentration in the eluant, , Ca^{2+}-activated proteolytic activity (adjusted absorbance units) determined using casein as a substrate, ——— absorbance at 280 nm Individual tubes in peak I and peak II were pooled as indicated and salted out between 0 and 45% $(NH_4)_2SO_4$ saturation to give the peak I and the peak II calcium-activated protease fractions

(peak I protease) and another elutes at 220 mM KCl (peak II protease). Although both the peak I and peak II proteases are activated by calcium, the peak I protease requires substantially lower levels of calcium than does the

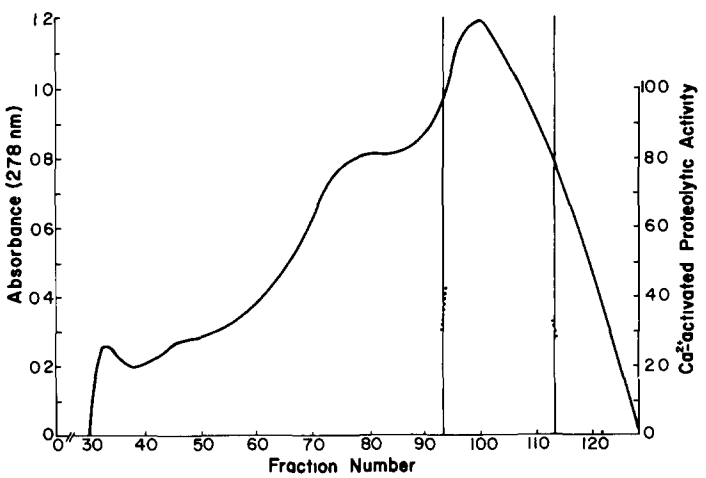

Fig 2 Elution profile of the DEAE-cellulose-purified, peak I protease from a 4 0 X 85 cm 6% agarose column The column was load with 1872 mg of the DEAE-cellulose-purified, peak I protease in 50 ml and was eluted with 20 mM Tris-acetate, pH 7 5/1 mM EDTA/1 mM NaN_3 at 30 ml/h 10-ml fractions were collected , Ca^{2+}-activated proteolytic activity (adjusted absorbance units) determined using casein as a substrate, ———, absorbance at 280 nm The indicated fractions were pooled and used in the next purification step

TABLE I

SUMMARY OF TOTAL PROTEIN YIELD, SPECIFIC CALCIUM-ACTIVATED PROTEASE ACTIVITY AND TOTAL CALCIUM-ACTIVATED PROTEASE ACTIVITY AT EACH STEP IN PURIFICATION OF THE PEAK I PROTEASE FROM PORCINE SKELETAL MUSCLE

Figures are for a typical calcium-activated protease preparation beginning with 12 000 g porcine skeletal muscle Specific Ca^{2+}-activated protease activity is expressed as absorbance units at 278 nm/mg protein per h as measured in the caseinolytic assay Total Ca^{2+}-activated protease activity is total absorbance units at 278 nm in the casein assay

Fraction	Total protein (mg)	Specific Ca^{2+}-activated protease activity	Total Ca^{2+}-activated protease activity	Purification (-fold)
Crude extract	1 727 645	— *	—	—
P_{30-40}	31 059	— *	—	55 6 **
1st DEAE-cellulose	1872 5	3 12	5842 2	922 7 **
6% agarose	772 4	7 32	5654 2	2168 3
2nd DEAE-cellulose	248	15 72	3899	4667 1
Ultrogel AcA-34	156 4	20 0	3128	5900 8
Prep electrophoresis	—	50	—	14 802

* Presence of an inhibitor of the calcium-activated protease in these fractions prevents accurate measurement of protease activity

** Calculated from reduction in total protein of these steps (assuming total recovery of the protease) since the presence of an inhibitor in these fractions prevents measurement of calcium-activated protease activity

peak II protease. This result is consistent with reports [3] that an extract obtained from canine heart contained two calcium-activated proteolytic activities. Neither of these activities were purified, however.

The peak II calcium-activated proteolytic activity appeared very similar to the calcium-activated proteolytic activity isolated from skeletal muscle tissue by Busch et al. [2] and later purified from porcine skeletal muscle by Dayton et al. [8] and was consequently purified using the procedure developed for this protease [8]. The peak II protease co-purified with the calcium-activated protease we originally purified from porcine skeleton muscle [8] throughout this procedure. Additionally, the banding pattern of chromatographically-purified peak II protease on polyacrylamide gels in either SDS or in nondenaturing buffer was identical to that already reported [8,9] for the calcium-activated protease we originally purified from porcine skeletal muscle.

Peak I proteolytic activity was further purified by chromatography on a 4 × 85 cm 6% agarose column. The calcium-activated proteolytic activity eluted from the column as shown in Fig. 2. This step resulted in a 2.3-fold increase in the calcium-activated proteolytic activity/mg enzyme protein in the caseinolytic assay (Table I).

The pooled 6% agarose column fractions containing calcium-activated proteolytic activity were next loaded onto a 2.5 × 40 cm DEAE-cellulose column and eluted with a linear gradient (Fig. 3). Because it was necessary to drastically overload the initial DEAE-cellulose column this second DEAE-cellulose column yielded a significant purification of the calcium-activated proteolytic activity (Table I).

The calcium-activated proteolytic activity was then loaded onto a 2.5 × 85

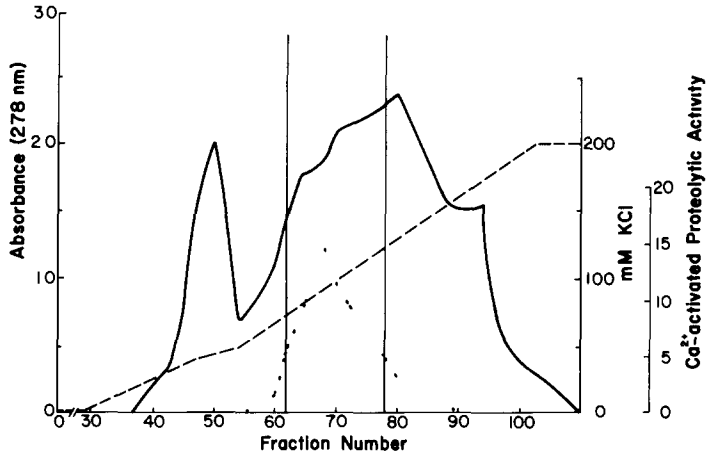

Fig 3 Elution profile of the 6% agarose-purified, peak I protease from a 2 5 X 40 cm DEAE-cellulose column The column was loaded with 772 4 mg 6% agarose purified, peak I protease in 400 ml and eluted at 30 ml/h with a 1000 ml continuous gradient from 0 to 200 mM KCl in 20 mM Tris-acetate, pH 7 5/1 mM EDTA/10 mM 2-mercaptoethanol 10-ml fractions were collected ------, KCl concentration in eluant, , Ca^{2+}-activated protease activity (adjusted absorbance units) determined using casein as a substrate, ———, absorbance at 280 nm The indicated fractions, containing protease activity, were pooled, salted out between 0 and 45% $(NH_4)_2SO_4$ saturation and used in the next purification step

cm AcA-34 column (Fig. 4). The peak I proteolytic activity, the peak II proteolytic activity and the calcium-activated protease we originally purified [8,9] elute from an AcA-34 column at the same volume indicating that their molecular size is similar.

Although the chromatographic procedures described resulted in a significant (106-fold) purification of the peak I calcium-activated proteolytic activity, the

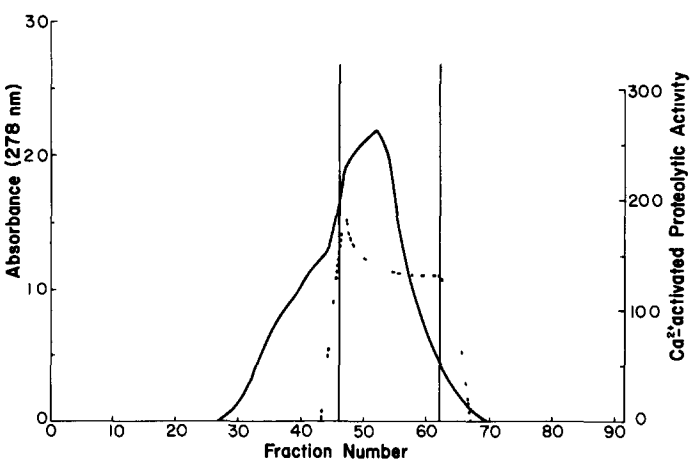

Fig 4 Elution profile of DEAE-cellulose-purified, peak II protease from a 2 5 X 85 cm Ultrogel AcA-34 column The column was loaded with 248 mg protein in 10 ml and was eluted with 20 mM Tris-acetate, pH 7 5/1 mM EDTA/1 mM NaN_3 at 20 ml/h Fractions of 7 5 ml were collected , Ca^{2+}-activated proteolytic activity (adjusted absorbance units) determined using casein as a substrate, ———, absorbance at 280 nm The indicated tubes, containing protease activity, were pooled

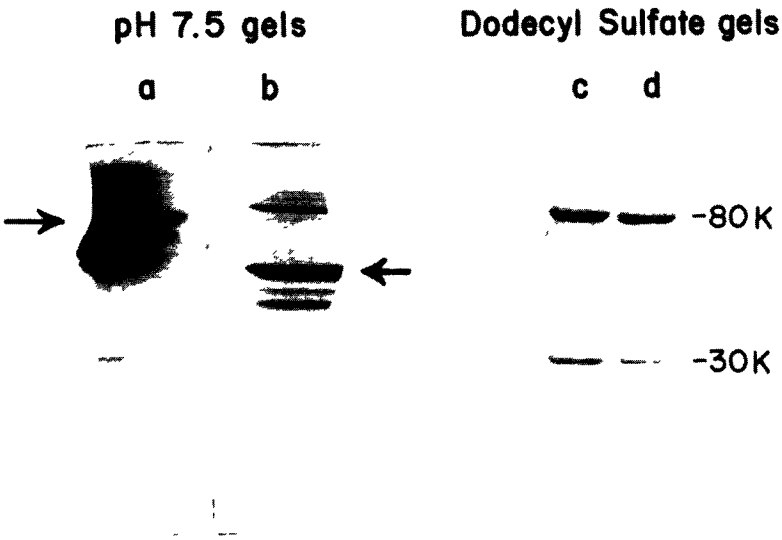

Fig 5 Purification of partially purified peak I and peak II proteases using preparative polyacrylamide slab-gel electrophoresis in pH 7.5 buffer (a) Gel strip cut from the edge of a nondenaturing, preparative gel run on the chromatographically-purified, peak I protease The arrow indicates the protein band containing Ca^{2+}-activated proteolytic activity (b) Gel strip cut from the edge of a nondenaturing, preparative gel run on the partially purified, peak II protease The arrow indicates the protein band containing Ca^{2+}-activated proteolytic activity (c) Banding pattern of 30 μg electrophoretically-purified peak I protease on an analytical SDS-polyacrylamide slab gel (d) Banding pattern of 30 μg electrophoretically-purified peak II protease on an analytical SDS-polyacrylamide slab gel All gels are 10% polyacrylamide

most highly purified preparation still contained several bands on a SDS-polyacrylamide gel. Consequently, this fraction was further purified by preparative electrophoresis in a nondenaturing buffer system. Fig. 5a and b shows gel strips cut from the side of nondenaturing, preparative gels run on the chromatographically-purified peak I and peak II proteolytic fractions, respectively. The arrows in Fig. 5a and b indicate the single band containing calcium-activated proteolytic activity in each fraction. Comparison of the gel strips in Fig. 5a and b shows clearly that the peak I and peak II proteases do not migrate similarly in the nondenaturing system used in this study. Thus, the peak I and peak II proteases appear to differ in size or in charge under the electrophoretic conditions used. In contrast, use of this gel system to compare the banding pattern of the peak II protease (Fig. 5b) and of the calcium-activated protease we originally purified from skeletal muscle [8] shows that these two proteases do co-migrate.

Examination of Fig. 5 (c and d) shows that, with the exception of minor contaminants, the SDS-polyacrylamide gel banding pattern of the electrophoretically-purified peak I protease is identical to that of the peak II protease (both banding patterns showing major bands at 80 000 and 30 000 daltons). Additionally, this banding pattern is identical to that of the calcium-activated protease that we have previously purified from porcine skeletal muscle [8]. Because the SDS-polyacrylamide gel banding patterns of the peak I and peak II proteases are identical, it is very likely that they are two different forms of

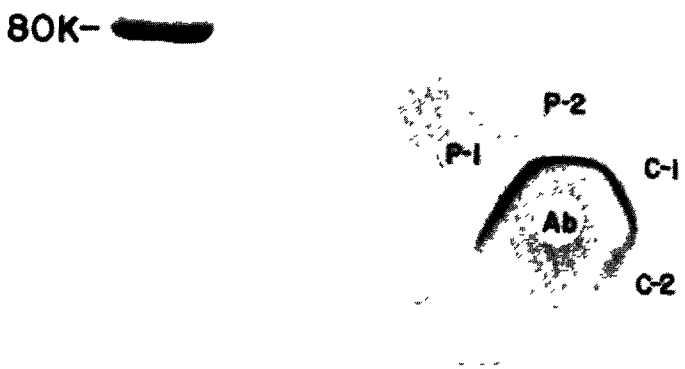

Fig 6 Banding pattern of 30 μg electrophoretically-purified 80 000-dalton subunit on a SDS-polyacrylamide slab gel The gel is 10% polyacrylamide

Fig 7 Immunodiffusion of anti-80 000 IgG against peak I protease, peak II protease and two concentrations of the calcium-activated protease that was used to elicit production of the anti-80 000 IgG in rabbits Anti-80 000 IgG (75 μg) was placed in the center well (Ab) and diffused against 32 μg of the peak I (P-1) and peak II protease (P-2) and against 24 μg and 12 μg of the original antigen (C-1 and C-2, respectively)

the same basic enzyme. Additionally, the similarity in calcium requirement of the peak II protease and the calcium-activated protease we originally purified from porcine skeletal muscle, their co-migration on nondenaturing and SDS-polyacrylamide gels, and their identical behavior on DEAE-cellulose and Ultrogel AcA-34 columns suggest strongly that these proteases are identical

In order to further establish the identity of the peak I protease, the peak II protease and the calcium-activated protease we originally purified from skeletal muscle [8], we examined the ability of each of these enzymes to cross-react with an antibody directed against the 80 000-dalton subunit of high-calcium-requiring protease, we originally purified from porcine skeletal muscle [8]. The IgG fraction used in this study was prepared as described in Materials and Methods. Double immunodiffusion analysis showed that the anti-80 000 IgG gave a single precipitation line when reacted with calcium-activated protease [8] used as an antigen (Fig. 7). This result coupled with the homogeneity of the electrophoretically-purified antigen (Fig. 6) provides evidence that the anti-80 000 IgG reacts specifically with the porcine calcium-activated protease [8]. Double immunodiffusion assays (Fig. 7) show that the anti-80 000 fraction

also reacts with both the purified peak I and peak II proteases to form a single precipitin line. In addition, it is clear (Fig. 7) that when the peak I protease, the peak II protease and the calcium-activated protease we originally purified from skeletal muscle are placed in adjacent wells and diffused against the anti-80 000 IgG, precipitin lines form a single continuous line with no spur formation. Thus, all three calcium-activated proteases that we have isolated and purified from porcine skeletal muscle share common antigenic determinants and are consequently related, if not identical, molecules. Consequently, we believe that the peak I and peak II proteases are simply two forms of the same protease and that the peak II protease is identical to the calcium-activated protease we have previously purified [8].

Calcium requirement of purified peak I and peak II proteases

The concentration of calcium required to activate the peak I and the peak II proteases was examined using the caseinolytic assay. A standard assay containing 0.04 mM EDTA/0.04 mM calcium was used and additional calcium was added to give a final calcium concentration that exceeded the EDTA concentration by the amount shown on the graph in Fig. 8. In this assay system, the purified peak I protease has one-half maximal activity at approx. 45 μM calcium and has detectable activity as low as 0.005 mM calcium. In contrast, the peak II protease has one-half maximal activity at 0.74 mM calcium and no activity can be detected in the presence of calcium concentration less than 0.1 mM. The calcium requirement of the purified peak II protease is identical to that of the calcium-activated protease we have previously purified from porcine skeletal muscle [9].

In addition to activation by calcium, the peak I protease is also activated to varying degrees by several other divalent cations including Mg^{2+} and Mn^{2+}. Consequently, the peak I protease may be partially activated in vivo by Mg^{2+} or Mn^{2+} present in the cytoplasm. The dependency of peak I protease activity on

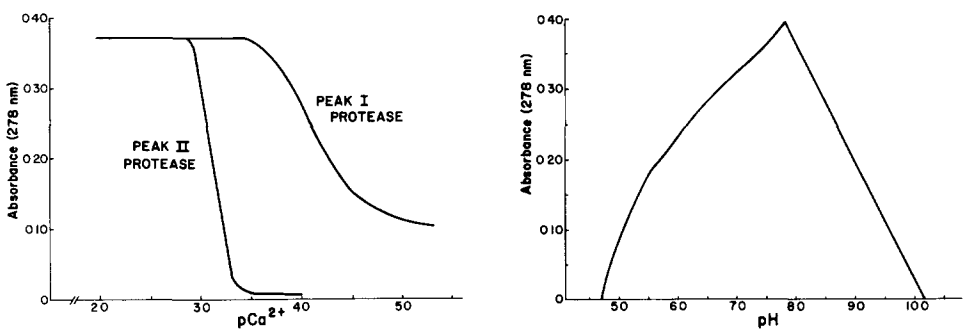

Fig 8 Effect of calcium concentration on rate of hydrolysis of casein by either the purified peak I protease or the purified peak II protease Assay condition 100 mM KCl/50 mM Tris-acetate pH 7 5/10 mM 2-mercaptoethanol/0.04 mM EDTA/5 mM NaN_3/5 0 mg/ml casein/$CaCl_2$ (in excess of EDTA) as indicated/10 μg purified calcium-activated (pCa^{2+}) protease (peak I or peak II)/ml, 30 min, 25°C

Fig 9 Effect of pH on rate of release of soluble peptides from myofibrils by the peak I protease Assay conditions 100 mM KCl/100 mM Tris-acetate (pH varied from 4.5—10.0)/10 mM 2-mercaptoethanol/0 5 mM $CaCl_2$/5 mM NaN_3/5 0 mg myofibrillar protein/30 μg purified peak I protease, in final volume of 2 ml Assay was done for 30 min at 25°C

Mg^{2+} concentration is very similar to its dependency on calcium concentration, with maximum protease activity occurring at 0.5 mM Mg^{2+}. The maximum activity obtained in the presence of Mg^{2+}, however, is only about 34% of that obtained in the presence of calcium. Similarly, the peak I proteolytic activity is activated by Mn^{2+} with maximum activity (72% of that obtained using calcium as an activator) occurring at 0.5 mM.

pH dependency of peak I and peak II proteolytic activity

The pH dependency of the purified peak II protease was found to be identical to that of the calcium-activated protease we have previously purified from porcine skeletal muscle [9] Maximum proteolytic activity against a myofibril substrate was observed between pH 7.5 and 8.0, with very little activity below pH 6.0 or above pH 8.5. The purified peak I protease also exhibited maximum proteolytic activity between pH 7.5 and 8.0 (Fig. 9). The peak I protease, however, retains significant activity as low as pH 5.5 and as high as pH 8.5. Thus, the purified peak I protease appears to have a broader pH dependence than does the peak II protease.

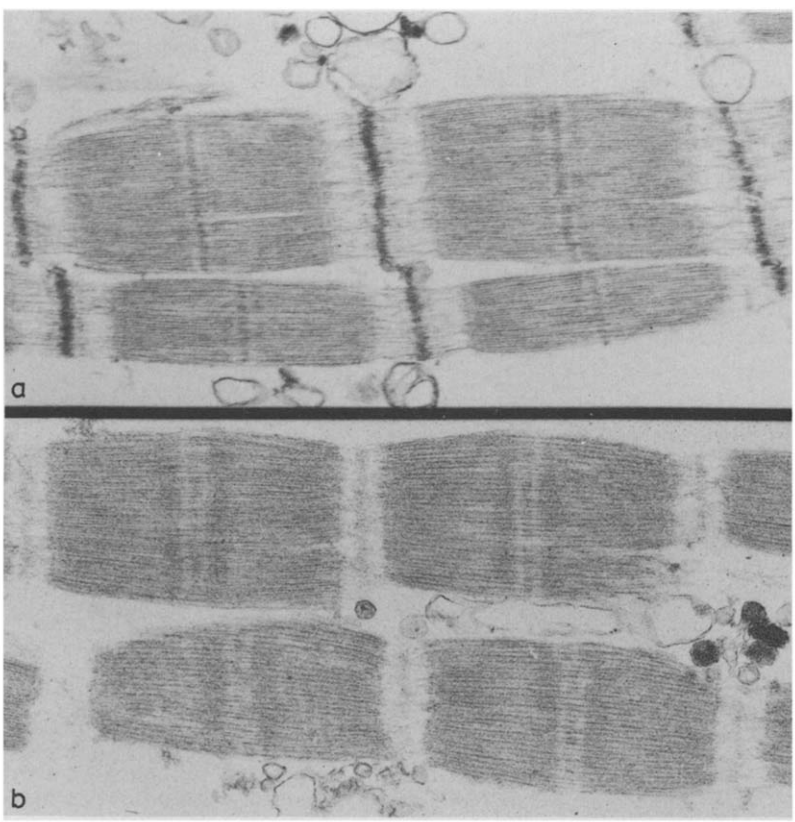

Fig 10 Electron micrograph showing the effect of the peak I protease on the ultrastructure of myofibrils in vitro (a) Control myofibril incubated in 100 mM KCl/50 mM Tris-acetate, pH 7 5/10 mM 2-mercaptoethanol/0 5 mM $CaCl_2$/5 mM NaN_3, for 30 min at 25°C Note that Z-disks and thin filaments appear to have normal ultrastructure (b) Myofibril incubated as in (a) but with addition of 30 μg of the peak I protease

Effect of peak I and of peak II proteases on the ultrastructure of myofibrils in vitro

The effect of the purified peak I or the purified peak II protease on the ultrastructure of purified porcine skeletal muscle myofibrils is identical (Fig. 10). Additionally, the effect of these proteases on myofibril ultrastructure is identical to that of the calcium-activated protease which we have previously purified [9]. Treatment of myofibrils with either the purified peak I or peak II protease in the presence of the appropriate amount of calcium, results in complete removal of the Z-disk (Fig. 10). Thin filaments present in protease-treated myofibrils appear to be disoriented compared to thin fillaments in non-treated myofibrils. This disorientation may simply be due to the loss of the Z-disk as an attachment point or it may be due to the effect of the protease on the thin filaments themselves.

Discussion

Based on the preceding data, we believe that the calcium-activated proteolytic activities that we and others [3] have isolated from skeletal muscle are the result of two forms of the same proteolytic enzyme. One form of the enzyme (responsible for the proteolytic activity in peak I from the DEAE-cellulose column) is activated by calcium concentrations as low as 5 μM. The other form (responsible for the proteolytic activity in peak II from the DEAE-cellulose column and for the calcium-activated proteolytic activity we originally purified from porcine skeletal muscle) requires calcium concentrations of at least 0.1—0.2 mM for activation. The molecular modification responsible for the different calcium requirements exhibited by the two forms of the calcium-activated protease are not clear from our present data. Our studies do show that the modification does not involve any major changes in molecular size, subunit molecular weight or composition, or immunological cross-reactivity. The low-calcium-requiring form of the protease appears to be less negatively charged than the high-calcium-requiring form. This conclusion is based on the fact that the low-calcium-requiring form of the protease elutes from a DEAE-cellulose column at lower KCl concentration and has a lower mobility in non-denaturing polyacrylamide gel electrophoresis than the high-calcium-requiring form of the protease. Our current data thus suggests that conversion from the low-calcium-requiring form of the protease to the high-calcium-requiring form may involve addition of a relatively small, highly-charged, constituent to the low calcium-requiring protease. It is thus possible that the low-calcium-requiring form of the protease may be converted to the high-calcium-requiring form by phosphorylation. We are currently investigating this possibility.

The high-calcium-requiring form of the calcium-activated protease [8,9] has been shown to partially degrade myofibrils [12], and to hydrolyze filamin and intermediate (100 A) filaments [28,29]. In addition, treatment of cultured cells with the purified calcium-activated protease results in release of the cell-surface glycoprotein fibronectin, ultrastructural damage to the cell membrane and depolymerization of microfilaments which form part of the cytoskeleton of the cell (unpublished data). Even though the protease has been shown to have the above effects on specific cellular components, a major problem in

ascribing a physiological role to the calcium-activated proteolytic enzyme in skeletal muscle and in other tissues has been the inactivity of the enzyme in in vitro assays done at physiological calcium concentrations. Therefore, existence of a form of the calcium-activated protease that is active at calcium concentrations approaching those thought to be present in the cell is extremely important in understanding the conditions necessary to activate the protease in vivo. Moreover, in addition to activation by calcium, it appears that the low-calcium-requiring form of the enzyme is partially activated by other divalent ions found in the cytoplasm of the cell (e.g., Mg^{2+} and Mn^{2+}). It thus appears that the low-calcium-requiring protease can be activated under the conditions existing in muscle cells and is therefore the form of the protease that is active in vivo.

Our recent immunocytochemical localization of the calcium-activated protease in Z-disk of the myofibril [16] supports the theory that this protease plays a role in myofibril disassembly and degradation, since the Z-disk is a myofibrillar structure that is extremely susceptible to calcium-activated protease-catalyzed hydrolysis. Based on these findings it seems almost certain that the enzyme is involved in degradation of at least this myofibrillar structure. Presence of the calcium-activated protease in the Z-disk in normal muscle tissue makes it imperative that the proteolytic activity of the enzyme be stringently regulated, in order to prevent uncontrolled and complete degradation of Z-disks It is possible that the activity of the protease is controlled, at least in part, by conversion of the low-calcium-requiring form of the enzyme to the high-calcium-requiring form, thereby rendering it inactive at the free calcium concentrations normally found in muscle cells. Conversely, activation of the protease might involve conversion of the high-calcium-requiring form of the protease to the low-calcium-requiring form Additionally, an endogenous inhibitor of the calcium-activated protease has been isolated from several tissues, including skeletal muscle [30,31], and it is possible that this inhibitor may also help to control the activity of the protease. Our studies (unpublished data) have shown that the inhibitor inhibits both the high-calcium-requiring and the low-calcium-requiring forms of the protease.

In summary, we have isolated and purified a form of the calcium-activated muscle protease [8,9] that is active at physiological calcium concentrations The effect of the protease on myofibrillar ultrastructure is identical to that reported for the calcium-activated protease we previously purified from porcine skeletal muscle [8,9,12]. Existence of the low-calcium-requiring form of the protease provides an explanation for how the calcium-activated protease may be activated at the relatively low free calcium concentrations thought to be present in muscle cells and in other types of cells. Because the calcium-activated protease is present in muscle cells in structures that are affected early in the progression of many myopathies (i.e., the sarcolemma and Z-disk) [15,16], we believe the enzyme may play a significant role in degradation of these structures. Since interconversion of the low- and high-calcium-requiring forms of the calcium-activated protease (assuming such interconversion occurs) may play an important role in determining the level of protease activity in vivo and hence the rate of muscle protein degradation, we are currently studying the mechanism by which this interconversion might occur.

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