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STUDIES OF A MYOSIN-CLEAVING PROTEASE FROM DYSTROPHIC HAMSTER HEART

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SUMMARY

A myosin-cleaving protease was purified to homogeneity from the cardiac myofibrils of dystrophic hamsters. The biochemical properties of the enzyme was studied with both [^{3}H] acetyl-casein and purified myosin as substrates. Steadily increasing levels of the enzyme correlated to the development of cardiomyopathy.

INTRODUCTION

We have previously reported that the level of a myosin-cleaving enzyme from the cardiac myofibrils is elevated in dystrophic hamsters (1). We wish to report here: 1) a simple procedure to purify this enzyme to homogeneity and in high yield, 2) some properties of this enzyme as compared to a similar enzyme reported by others (4) and 3) the steady increase of the level of this enzyme with age, in correspondence with the development of ultrastructural abnormalities.

MATERIAL AND METHODS

Genetically myopathic hamsters Bio 53:58 (both male and female) were used. For enzyme purification, hearts from thirty 150-day old dystrophic hamsters were pooled, washed free of blood and stored in 50% glycerol at -70°C for further use. Protease assay and gel electrophoresis were as described by Bhan et al (2). One unit of protease activity was defined as the amount of enzyme that catalyzed a release of 10^6 cpm from the [^{3}H]-acetyl-casein in 1 hour. The specific activity of the protease was expressed as units per mg protein.

Human cardiac myosin were purified (3). For the digestion reaction, 1 mg of myosin was incubated with 0.2 units of protease for 1 h at 30°C pH 8.0. After precipitation by 10% TCA, the myosin pellet was redissolved in buffer for gel-electrophoresis.

EXPERIMENTAL

Purification of Cardiac Myofibrillar Protease - Hearts from thirty 150-day old dystrophic hamsters were homogenized for 1 min. in 0.05 M KCl/0.01M

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potassium phosphate/2mM EDTA, pH 6.8 (buffer A). The homogenate was centrifuged at 2000 g for 10 min. to collect the myofibrils. The crude washed myofibrils were stirred for 1 h with 10 volumes of a solution of 1% Triton X-100 in buffer A. The Triton-washed myofibrils were extracted with 10 volumes of a solution of 0.47 M KCl/0.02M sodium pyrophosphate pH 6.8 for 1 h. The extract was centrifuged at 15,000 g for 10 min. to remove insoluble materials. The supernatant was adjusted to pH 4.3 with 1 M acetic acid. The precipitated myosin was removed by centrifuging at 15,000 g for 20 min. The supernatant solution was then adjusted to pH 5.5 with 1 N NaOH and the protease precipitated by adding solid ammonium sulfate to 80% saturation. After centrifuging at 15,000 g for 20 min, the pellet was dissolved in a minimal volume of a solution of 0.05M Na-acetate/0.4 M KCl/5mM $CaCl_2$ pH 5.5 (buffer B). The protease sample was chromatographed on a column of sephacryl S-200, using buffer B as equilibration and elution buffer. The peak containing the protease activity was pooled and dialyzed against a solution of 0.05 M Na-acetate/5mM $CaCl_2$ pH 5.5 (solution C). The dialyzed enzyme solution was applied to a (1x10cm) CM-52 cellulose column equilibrated with solution C. Step-wise elution was performed with solution C containing first 0.1 M NaCl, then 0.3 M NaCl, finally 0.5 M NaCl where the enzyme activity emerged as a single peak.

RESULTS

The purification procedure is summerized in Table 1. The protease was purified 200 fold over the KCl-pyrophosphate extract or approximately 800 fold over the washed myofibrils. One mg of the enzyme was obtained from 30 g of cardiac tissue. The enzyme preparation gave a single band on SDS-gel electro phoresis (Fig 1).

Molecular weight determinations of both the native enzyme by a calibrated sephacryl S-200 column and of the denatured enzyme by SDS-gel electrophoresis (data not shown) gave identical value of 26,000 daltons. It is concluded that the native enzyme has only one subunit.

Table 1

Purification of Myosin-Cleaving Protease

	Step	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)
1.	KCl-Pyrophosphate extract	1600	920	0.5
2.	PH 4.3 Supernatant	192	920	4.8
3.	Ammonium Sulfate	110	624	5.7
4.	Sephacryl S-200	25	610	24.4
5.	CM-52 Cellulose	0.96	100	100

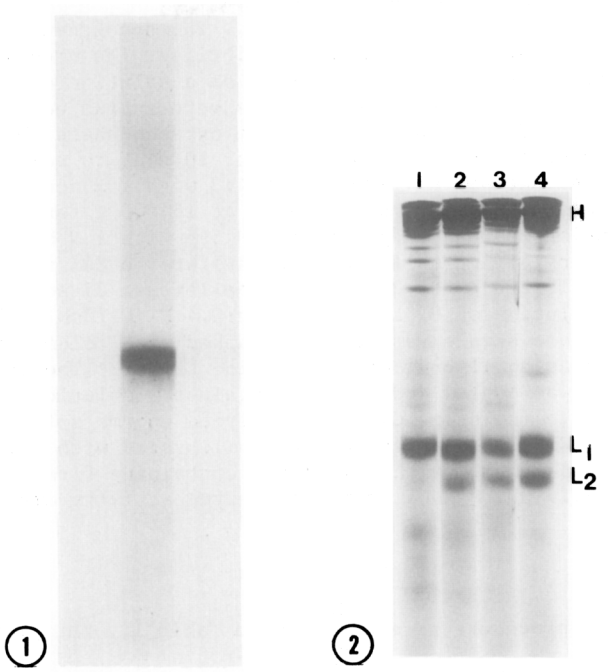

Figure 1. SDS-gel electrophoresis of the purified myosin-cleaving protease. The gel contained 10% polyacrylamide with 0.1% SDS.

Figure 2. SDS-gel electrophoretic pattern of human cardiac myosin after incubation with and without purified protease. 1) 5 mM EDTA 2) 5 mM Ca^{2+} 3) 5 mM Mg^{2+} 4) No protease H=myosin heavy chain L_1= light chain 1 L_2=light chain 2.

Table 2

Effect of Various Reagents on Caseinolytic Activity

Addition	Concentration	Activity (%)
None		100
Ca^{2+}	5 mM	40
Mg^{2+}	5 mM	73
EDTA or EDTA	5 mM	100
N-ethylmaleimide	5 mM	100
KCl	0.5-1 M	99
PhenylMethyl-Sulfonyl fluoride	1 mM	5
Trypsin Inhibitor	0.1 mg/ml	43

7.5 µg/ml enzyme was preincubated with various reagents for 10 min at room temperature before the start of the assay. Activity without effector was taken as 100%.

Table 3.
Specific Activity of the Myosin-cleaving Enzyme vs. Age of Hamster

Age (mos)	Control	Dystrophic	Dystrophic / Control
1	22.2 ± 0.5	49.1 ± 4	2.2
2	34.9 ± 3	125.9 ± 2	3.6
3	20.0 ± 1.5	102.5 ± 9	5.1
4	19.0 ± 1.0	102.5 ± 6	5.4

Values are expressed as mean ± S.D of 8 studies. Specific activity is expressed as μg casein hydrolyzed/mg protein/hour.

As shown in Table 2, the enzyme activity was not affected by the presence of chelating agents, EGTA or EDTA, indicating that divalent cations are not required for enzyme activity; in fact, with Ca^{2+} or Mg^{2+} present at 5 mM concentration, there was partial inhibition of activity. The sulfhydryl reagent N-ethylmaleimide had no effect on the enzyme, suggesting that free-SH groups are not required for activity. Phenylmethylsulfonyl fluoride completely inhibited the caseinolytic activity of the enzyme whereas soybean trypsin inhibitor was only moderately effective. In contrast to reports by others (4-7) the enzyme was neither stimulated nor inhibited by KCl at 1 M.

Incubation of purified myosin from hearts of control hamsters, from human, or from skeletal muscle of the rabbit with purified protease gave similar results as illustrated in Fig 2. When human cardiac myosin was incubated with protease for 1 h in the presence of 5 mM EDTA, there was complete loss of the 18000 dalton light chain (L_2) and partial breakdown of the heavy chain into 3 or 4 major peptides of molecular weight in the range of 100,000-180,000 daltons. This digestion of L_2 was completely blocked by the presence of divalent cations, Ca^{2+} or Mg^{2+}.

The steady increase of PMSF inhibitable protease activity with age is shown in Table 3. The hearts of control animals varied little with increasing age whereas hearts of myopathic animals showed higher protease activity than control animals at all ages: (2.2 fold at 1 month and 5.4 fold at 4 months).

DISCUSSION

The isolation of non-lysosomal enzymes (8-11) offers an alternative mechanism for intracellular protein degradation besides the classical lysosomal mechanism (12). In the case of cardiac and skeletal muscles, a myofibril associated protease(s) with alkaline pH optimum has been reported by several laboratories. The activity of this protease is elevated under conditions of increased muscle protein turnover (10,13). A 13% yield after 2100 fold purification was achieved by Murakami and Uchida from normal rat heart (4).

In our previous studies of genetically dystrophic hamsters, we have consistently observed a reduction or complete loss of the 18000 dalton light chain (L_2) during purification of cardiac myosin (1). The loss of L_2 in myopathic cardiac myosin was found to be due to elevated levels of protease activity co-purifying with myosin. This then led us to the partial purification of a myosin-cleaving enzyme from the cardiac myofibrils of the dystrophic hamsters (2). Subsequent work showed that this preparation contains multiple proteins. Further purification described in this paper resulted in approximately 10% yield of an enzyme preparation that is purified 800 fold over the washed myofibrils and that gives a single sharp band on SDS-gel electrophoresis. The yield in weight was 1 mg from 30 g of cardiac muscle, as compared to 0.2 mg from 200 g rat heart by Murakami and Uchida.

The two enzymes, from hamster and from rat, have many similarities: molecular weight of 26,000 daltons, pH optimum at 9 with casein as substrate (data not shown), insensitivity towards metal chelating agents and sulfhydryl reagents, association with myofibrils and ability to degrade both light (L_2) and heavy chains of myosin. There are however also differences in properties. The myopathic hamster enzyme is not inhibited by 1 M KCl whereas the rat enzyme is inhibited by 28%. The relative degradative activities towards the light and heavy chain of myosin is somewhat different. Finally, the enzyme reported here degrades the light chain (L_2) of myosin at a significantly reduced rate when 5 mM Ca^{2+} or Mg^{2+} is present. The rat enzyme was reported to be unaffected

by 1 mM Ca^{2+} or Mg^{2+} with casein as the substrate. Whether it's activity towards myosin is affected by Ca^{2+} and Mg^{2+} is not known.

Although a role of the enzyme in cardiomyopathy has not been established definitively, there are strong reasons to believe that it may be an important one. Early findings of myocardial disease appear by the age of two months and cardiac failure occurs at 7-8 months in the myopathic hamster. Ultrastructurally, there is degeneration of the contractile elements at one month of age, prior to noticeable gross abnormality of the heart. At this stage, lysosomes are seen only as dense bodies primarily in the perinuclear region. More extensive ultrastructural damage occurs at a later stage, as reported by Paterson et al (4) and confirmed by us. (unpublished results). The results shown in Table 3 thus are consistent with the premise that initial tissue damage is primarily due to the myosin-cleaving enzyme whereas the action of lysosomes may be involved in the later tissue destruction. The role, if any, of the Ca^{2+}-activated protease isolated from pig skeletal muscle (8), capable of degrading Z disk, tropomyosin and troponin but not myosin or actin, is unknown.

It should be mentioned that a serine protease of similar properties has been shown in mast cells (15). In our studies, since there is already appreciable increase (2.2 fold) of the myosin-cleaving enzyme in one month old animals whose hearts showed no or very little inflammation, and since the enzyme is associated with myosin in early steps of purification, it seems reasonable to conclude that this enzyme is of muscle cell origin. At later stages when hypertrophy and/or inflammation are present, the contribution from mast cells may be significant.

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