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PURIFICATION AND CHARACTERIZATION OF A MYOSIN-CLEAVING PROTEASE FROM RAT HEART MYOFIBRILS

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Summary

A proteolytic enzyme, which causes the limited degradation of cardiac myosin, was purified from rat heart myofibrils. The purified enzyme (a myosin-cleaving protease) was apparently homogeneous by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. Autolysis of the purified enzyme was observed at neutral pH without high concentration of $CaCl_2$. The molecular weight was estimated to be 26 000—27 000. The enzyme was active against casein, *N*-acetyl-L-tyrosine ethyl ester and *N*-glutaryl-L-phenylalanine-4-nitroanilide (Glu-Phe-NAn), but less active with *N*-benzoyl-DL-arginine-4-nitroanilide. Optimum pH values for the enzyme were 9.0 for casein and 8.4 for Glu-Phe-NAn. Caseinolytic activity of the enzyme was completely inhibited with phenylmethylsulfonyl fluoride and diisopropylphosphofluoride and partially inhibited with L-1-tosyl-L-phenylalanine chloromethyl ketone (Tos-PheCH_2Cl) and soybean trypsin inhibitor. Tos-LysCH_2Cl had no effect. Sulfhydryl reagents, metal-chelating agents and metal ions except for Zn^{2+} had little or no effect on the activity. Degradation of cardiac myosin with the enzyme produced two fragments having molecular weights of 130 000 and 94 000, accompanied by the disappearance of myosin heavy chain and light chain 2. Myosin degradation with the enzyme was more restrictive than with chymotrypsin.

Introduction

Muscle cathepsin had no effect on myosin and actin which are major myofibrillar proteins [1—4]. In addition to cathepsin, several proteases optimally

Abbreviations: Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; Glu-Phe-NAn, *N*-glutaryl-L-phenylalanine-4-nitroanilide; Bz-Arg-NAn, *N*-benzoyl-DL-arginine-4-nitroanilide; iPr_2P-F, diisopropylphosphofluoride; Tos-PheCH_2Cl, L-1-tosyl-L-phenylalanine chloromethyl ketone; Tos-LysCH_2Cl, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid.

active at neutral and alkaline pH have been found and purified from muscle [5—11]. Ca^{2+}-activated protease purified from pig skeletal muscle by Dayton et al. [11,12] degraded tropomyosin, troponin and C-protein but did not hydrolyze myosin and actin. More recently, Sanada et al. [13] presented evidence that an enzyme isolated from rat skeletal muscle hydrolyzed myosin and actin.

In a previous paper [14], we reported the degradation of rat cardiac myosin during a purification procedure and this degradation was completely inhibited using phenylmethylsulfonyl fluoride in all steps of preparation. These results suggested the existence of a seryl proteolytic enzyme in the myofibrillar fraction of rat cardiac muscle, which degrades myosin.

In this communication, we describe a new purification procedure for this proteolytic enzyme, a myosin-cleaving protease, from rat heart and some of its properties. The results show that the purified enzyme causes the limited degradation of cardiac myosin and resembles chymotrypsin in some of its properties.

Experimental

Materials. Materials were obtained from the following commercial sources: hydroxyapatite (Seikagaku Kogyo, Tokyo, Japan); bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, cytochrome *c* (Schwarz/Mann, Orangeburg, N.Y., U.S.A.); Sephadex G-200, CM-Sephadex C-50 (Pharmacia, Uppsala, Sweden); phosphorylase *a*, trypsin (Type III), chymotrypsin (Type II), soybean trypsin inhibitor, iPr_2P-F, phenylmethylsulfonyl fluoride, Bz-Arg-NAn (Sigma, St. Louis, Mo., U.S.A.); Hammarsten casein, Glu-Phe-NAn (Merck, Darmstadt, Germany); protamine sulfate, Tos-LysCH_2Cl, Tos-PheCH_2Cl, Ac-Tyr-OEt (Nakarai Chem., Kyoto, Japan). Other reagents were of reagent or biochemical research grade.

Wistar rats were used. Hearts were removed and immediately rinsed in ice-cold water and stored at −80°C.

Preparation of acetone-dried powder. 200 g frozen heart tissue were thawed and cut into small pieces, then sheared twice for 5 s each (Sorvall Omni-mixer, 16 000 rev./min) in 4 vols. 0.05 M potassium phosphate buffer (pH 6.8)/1 mM EDTA/10 mM pyrophosphate/2 mM 2-mercaptoethanol at 4°C; this step was repeated three times. After each wash, the sheared tissue was centrifuged for 10 min at 10 000 rev./min at 4°C. The myofibrils obtained were sheared for 5 s in 5 vols. acetone at room temperature. The residue was collected on a Büchner funnel and washed twice by resuspending in 5 vols acetone. The filter cake was dried in a vacuum desiccator for 2 h at room temperature.

Purification of the enzyme. All steps were conducted at 4°C and centrifugation was carried out at 12 000 rev./min for 30 min throughout the purification procedure. Activity of each fraction was measured using casein as substrate.

Step 1: Acetone-dried powder (20 g) was suspended in 800 ml of 0.15 M potassium phosphate buffer (pH 7.5)/0.5 M KI/1 mM EDTA. The mixture was stirred for 6 h; stirring beyond this time produces an increase in soluble protein but no increase in soluble enzyme activity. The suspension was centrifuged and the residue was reextracted with 200 ml of the same buffer for 10 min and then centrifuged. Two supernatants were combined to yield 840 ml and used as crude enzyme solution.

Step 2: The crude enzyme solution was diluted with 0.6 vol. cold water. To this solution, 1% protamine sulfate (0.3 mg protamine sulfate/mg protein) was gradually added with stirring. After further stirring for 30 min in an ice bath, the precipitates were removed by centrifugation.

Step 3: Solid $(NH_4)_2SO_4$ was added to the supernatant obtained in step 2, and the fraction between 35 and 60% saturation was collected. The precipitate was dissolved in 0.15 M potassium phosphate buffer (pH 7.5)/0.5 M NaCl/1 mM EDTA and dialyzed against 0.3 M potassium phosphate buffer (pH 6.6).

Step 4: The dialyzed enzyme solution was applied to a hydroxyapatite column (1.8 × 10 cm) equilibrated with 0.3 M potassium phosphate buffer (pH 6.6). After the column was washed with 70 ml of the same buffer, stepwise elution was performed with 80 ml 0.5 M and 60 ml 1.0 M potassium phosphate buffer (pH 6.6). The enzyme activity was found in the fractions eluted with 1.0 M potassium phosphate buffer.

Step 5: The active fractions were applied to a Sephadex G-200 column (1.5 × 41 cm) equilibrated with 0.2 M sodium acetate buffer (pH 5.5)/0.6 M NaCl/5 mM $CaCl_2$. Elution was performed with the same buffer (flow rate 10 ml/h). The enzyme activity emerged as a symmetrical peak at an elution volume of 64 ml. The active fractions were pooled and dialyzed against 0.05 M Tris/acetate buffer (pH 5.8)/0.3 M NaCl/0.02 M $CaCl_2$.

Step 6: The dialyzed enzyme solution was applied to a CM-Sephadex C-50 column (1.8 × 7.5 cm) equilibrated with 0.05 M Tris/acetate buffer (pH 5.8)/ 0.3 M NaCl/0.02 M $CaCl_2$. After the column was washed with 70 ml of the same buffer, elution was performed with a linear gradient consisting of 150 ml each of the same buffer and the buffer with 0.7 M NaCl (flow rate 28 ml/h). The enzyme activity emerged at a NaCl concentration of about 0.54 M.

Step 7: The active fractions were concentrated to 10 ml by ultrafiltration using an Immersible Molecular Separator (Millipore Corp.) and applied to a Sephadex G-100 column (1.0 × 48.4 cm) equilibrated with 0.2 M sodium acetate buffer (pH 5.5)/0.6 M KCl/0.02 M $CaCl_2$. Elution was performed with the same buffer (flow rate 10 ml/h).

The purified enzyme obtained was quite stable in frozen and lyophilized conditions. No decrease of caseinolytic activity could be detected after a month at −20°C.

A summary of the purification procedure is presented in Table I. The yield of the enzyme activity was 13% with a 2100-fold purification.

Preparation of cardiac myosin. Cardiac myosin was isolated from rat heart by the procedure described previously [14].

Protein determination. Protein concentration was determined by the biuret method and the method of Lowry et al. [15] using crystalline bovine serum albumin as a standard. Protein concentration of cardiac myosin was determined by measuring the absorbance at 280 nm (assuming a factor of 0.5 $mg^{-1} \cdot ml$) with Shimadzu UV-200 double beam and Union SM-401 spectrophotometers.

Enzyme assay. Caseinolytic activity was measured by the method of Kunitz [16]. A stock solution of casein (1%, w/v) was prepared as described by Reimerdes and Klostermeyer [17]. The standard incubation mixture contained 0.5% casein, 0.1 M Tris · HCl buffer (pH 8.0) and enzyme in total volume of 1.0 ml. Reactions were allowed to proceed for 10—60 min at 37°C and were

TABLE I

PURIFICATION OF MYOSIN-CLEAVING PROTEASE

Specific activity was expressed as units/mg protein.

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purity (-fold)	Yield (%)
1. Extract	840	3100	2920	0.9	1	100
2. Protamine sulfate	1430	3570	3100	0.9	1	106
3. $(NH_4)_2SO_4$	61	1690	2440	1.4	1.6	84
4. Hydroxyapatite	11.8	40	2300	57.5	64	79
5. Sephadex G-200	15	11.4	1450	127	141	50
6. CM-Sephadex C-50	60	0.54	960	1777	1974	33
7. Sephadex G-100	10	0.20	390	1950	2167	13

terminated by the addition of 1.5 ml 5% trichloroacetic acid. After centrifugation for 5 min at 18 000 rev./min, the absorbance of the supernatant was measured at 280 nm. One unit of caseinolytic activity is defined as the amount of enzyme required to cause a change of 0.001 absorbance unit per min at 280 nm.

Esterase activity was measured using Ac-Tyr-OEt as substrate [18]. Incubation mixture contained 10 mM Ac-Tyr-OEt, 0.1 M Tris · HCl buffer (pH 8.0) and enzyme in total volume of 2.0 ml. Reactions were allowed to proceed for 0—30 min at 37°C. Residual ester was determined by formation of a ferric complex with hydroxamic ester. One unit of esterase activity is defined as the amount of enzyme that hydrolyzes 1 μmol Ac-Tyr-OEt per min.

Amidase activity was measured using Glu-Phe-NAn and Bz-Arg-NAn as substrate [19,20]. Reactions were carried out in the mixture containing 1 mM substrate, 0.1 M Tris · HCl buffer (pH 8.0) and enzyme in a total volume of 0.6 ml. The reaction was stopped by addition of 0.1 ml of 30% acetic acid. The release of 4-nitroaniline was followed spectrophotometrically at 410 nm using an extinction coefficient of 8800 $M^{-1} \cdot cm^{-1}$ [19]. One unit of amidase activity is defined as the amount of enzyme that releases 1 μmol 4-nitroaniline per min.

Phenylmethylsulfonyl fluoride, iPr_2P-F, Tos-PheCH_2Cl, Tos-LysCH_2Cl and soybean trypsin inhibitor were used to inhibit the caseinolytic activity of myosin-cleaving protease. Isopropyl alcohol solutions of iPr_2P-F and phenylmethylsulfonyl fluoride at 10 mM and methanol solution of Tos-PheCH_2Cl at 5 mM were freshly prepared. Before the addition of substrate, the enzyme was preincubated with inhibitor for 18 h at 4°C. For soybean trypsin inhibitor the enzyme was preincubated for 15 min at room temperature.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis at pH 4.3 was performed on 15% gel at 4°C [21]. Protein samples were dialyzed against 0.36% potassium acetate (pH 6.8)/10% glycerol/0.1 mM phenylmethylsulfonyl fluoride for 6 h at 4°C. The sample for the activity determination on a gel was dialyzed against the same buffer except for phenylmethylsulfonyl fluoride. The gels were run at a constant current of 5 mA per tube for 1 h. Pyronine G was used as the tracking dye.

SDS polyacrylamide gel electrophoresis at pH 8.8 was performed on 12%

gels [22]. Protein samples were dialyzed overnight against 0.0625 M Tris · HCl buffer (pH 6.8)/10% glycerol/0.1 mM phenylmethylsulfonyl fluoride. After dialysis, SDS and 2-mercaptoethanol were added to give final concentrations of 1 and 5%, respectively. The gels were run at a constant current of 2 mA per tube for 6 h. Bromophenol Blue was used as the tracking dye.

The slab gel (13 × 10 × 0.2 cm) was made from 12% acrylamide with 0.1% SDS [22]. Gel was run at a constant current of 24 mA for 6 h.

The gels were stained in 0.25% Coomassie Brilliant Blue overnight and destained in 5% methanol/7% acetic acid.

Determination of molecular weight. The molecular weight of the purified myosin-cleaving protease was estimated from the mobility on SDS polyacrylamide gels. It was also determined by gel filtration [23] on a Sephadex G-100 column (1.0 × 48.4 cm), equilibrated and eluted with 0.2 M sodium acetate buffer (pH 5.5)/0.6 M KCl/0.02 M $CaCl_2$ (flow rate 10 ml/h at 4°C). The following proteins were used as standards: ovalbumin, M_r = 43 000; chymotrypsinogen, M_r = 25 700; soybean trypsin inhibitor, M_r = 21 500; myoglobin, M_r = 17 200; cytochrome *c*, M_r = 12 400.

Proteolytic digestion of myosin. Cardiac myosin was dialyzed overnight against 0.02 M potassium phosphate buffer (pH 7.0)/0.5 M KCl/1 mM EDTA at 4°C and digested at 20°C with myosin-cleaving protease and also with chymotrypsin. At given time, digestion was terminated by adding phenylmethylsulfonyl fluoride to a final concentration of 0.3 mM. Digestion products of cardiac myosin were diluted with 4 volumes of 0.1 M Tris · HCl (pH 6.8)/14% glycerol/2% SDS/7% 2-mercaptoethanol and subjected to SDS polyacrylamide slab gel electrophoresis.

Results

Homogeneity of the purified enzyme. In the last purification step, molecular sieve chromatography, the elution pattern of the activity, as well as protein, was symmetrical (Fig. 1). The specific activity of the enzyme eluted was constant; 1950 units per mg of protein.

To test the homogeneity of the purified enzyme, polyacrylamide gel electrophoresis was performed (Fig. 2a). The electrophoretogram obtained shows a single band of protein and the enzyme activity having the same mobility was detected, judged by extracting and assaying slices of a parallel unstained gel. An electrophoretogram of the enzyme on SDS polyacrylamide gel also shows a single protein band (Fig. 2b). Fig. 2c shows an electrophoretogram of the autolyzed enzyme on SDS polyacrylamide gel, which was preserved in 0.3 M potassium phosphate buffer (pH 6.6) for 16 h at 4°C.

Absorption spectrum. The ultraviolet absorption spectrum of the purified enzyme in 0.2 M sodium acetate buffer (pH 5.5), 0.6 M KCl, 0.02 M $CaCl_2$ had a maximum at 277 nm and a minimum at 250 nm. The ratio of A_{280nm}/A_{260nm} of the enzyme was 1.85, indicating freedom from nucleic acid [24]. The extinction coefficient, $E^{1\%}_{1cm}$, was calculated to be 3.2 at 280 nm.

Molecular weight. Molecular weight of the purified enzyme estimated by gel filtration on Sephadex G-100 was 26 000. An electrophoretogram of the reduced and denatured enzyme on SDS polyacrylamide gel showed a single pro-

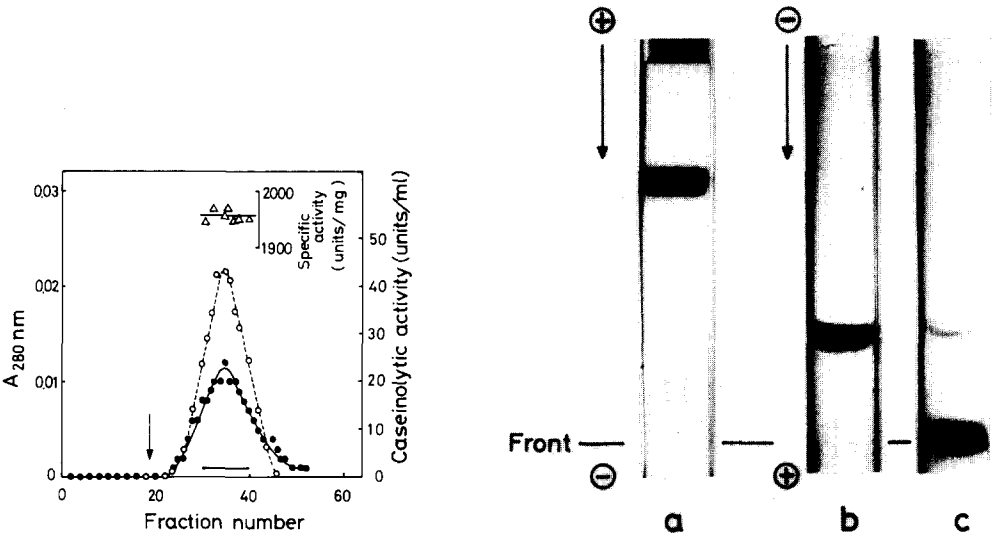

Fig. 1. Gel filtration on a Sephadex G-100 column. Active fractions (0.54 mg) obtained from a CM-Sephadex C-50 column were concentrated and applied to a Sephadex G-100 column (1.0 × 48.4 cm) equilibrated and eluted at pH 5.5. 1-ml fractions were collected. ●———●, absorbance at 280 nm; ○- - - - -○, caseinolytic activity. Specific activity was expressed as units per mg protein. The fractions indicated under a bar were combined and designated the purified enzyme. An arrow indicates void volume.

Fig. 2. Polyacrylamide gel electrophoresis of the purified myosin-cleaving protease. (a) Electrophoretogram of the enzyme on 15% polyacrylamide gel. (b) Electrophoretogram of the reduced and denatured enzyme on 12% polyacrylamide gel with 0.1% SDS. (c) Electrophoretogram of the autolyzed enzyme on 12% polyacrylamide gel with 0.1% SDS. The 10—15 μg protein were applied to each gel.

tein band corresponding to a molecular weight of approx. 27 000 (Fig. 2b).

Substrate specificity. Three different synthetic substrates were used to test the substrate specificity (Table II). The enzyme was active toward Ac-Tyr-OEt and Glu-Phe-NAn (which are substrates for chymotrypsin) but less active toward Bz-Arg-NAn (which is a substrate for trypsin).

Effects of inhibitors. The effects of various inhibitors on the activity of the enzyme are shown in Table III. Phenylmethylsulfonyl fluoride and iPr_2P-F completely inhibited the caseinolytic activity. The enzyme was also moderately

TABLE II

SPECIFIC ACTIVITIES OF MYOSIN-CLEAVING PROTEASE, CHYMOTRYPSIN AND TRYPSIN

Specific activity was expressed as units per mg protein.

Substrate	Specific activity (units/mg)		
	Myosin-cleaving protease	Chymotrypsin	Trypsin
Casein	$1.95 \cdot 10^3$	$6.98 \cdot 10^3$	$3.42 \cdot 10^3$
Ac-Tyr-OEt	83.4	288	12
Glu-Phe-NAn	$4.0 \cdot 10^{-2}$	$7.4 \cdot 10^{-2}$	$1.5 \cdot 10^{-3}$
Bz-Arg-NAn	$5.0 \cdot 10^{-3}$	0	$6.9 \cdot 10^{-1}$

TABLE III

EFFECT OF INHIBITORS ON MYOSIN-CLEAVING PROTEASE

3 μg enzyme was preincubated with inhibitor for 18 h at 4°C, and then caseinolytic activity was measured. Preincubation with soybean trypsin inhibitor, however, was carried out for 15 min at room temperature.

Inhibitor	Concentration	Relative activity (%)
iPr_2P-F	1.0 mM	0
Phenylmethylsulfonyl fluoride	1.0 mM	0
	0.1 mM	0
Tos-PheCH_2Cl	0.5 mM	51
	0.1 mM	78
Tos-LysCH_2Cl	1.0 mM	100
Soybean trypsin inhibitor	0.1 mg/ml	26

sensitive to soybean trypsin inhibitor and Tos-PheCH_2Cl, resembling chymotrypsin. Tos-LysCH_2Cl had no effect.

Effects of metal ions and various reagents. As shown in Table IV, the enzyme activity was not affected by the presence of the following metal ions at 1 mM; Ca^{2+}, Mg^{2+} and Mn^{2+}, whereas Zn^{2+} and Co^{2+} inhibited partially the activity. Metal-chelating agents (including EGTA) and the thiol reagents had little or no effect on the activity, indicating that the thiol group is not involved in the active site of the enzyme.

The anionic detergent, SDS, lowered the activity and a non-ionic detergent, Triton X-100, gave 69% stimulation. KCl at 1 M decreased activity by 28%, suggesting a difference between this enzyme and a chymotrypsin-like protease of mast-cell origin [25—27].

TABLE IV

EFFECTS OF METAL IONS AND VARIOUS REAGENTS ON CASEINOLYTIC ACTIVITY

2.5 μg/ml enzyme was preincubated with effector for 10 min at pH 7.5 and room temperature, and then caseinolytic activity was measured. Activity without effector was taken as 100%.

Compound	Concentration	Activity (%)
None		100
Mg^{2+}	1 mM	94
Ca^{2+}	1 mM	100
Mn^{2+}	1 mM	95
Co^{2+}	1 mM	85
Zn^{2+}	1 mM	63
EDTA	1 mM	98
EDTA	10 mM	95
2-Mercaptoethanol *	7 mM	97
P-Chloromercuribenzoate *	1 mM	98
KCl *	1 M	72
Triton X-100	0.1%	169
SDS	0.1%	28

* 0.1 M potassium phosphate buffer (pH 7.5).

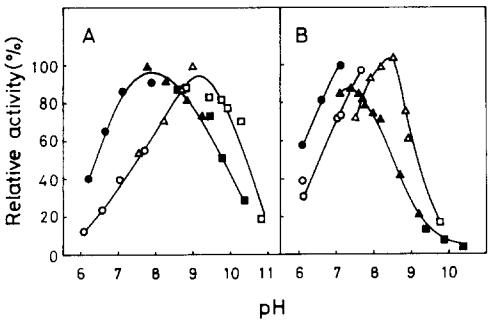

Fig. 3. pH versus activity profiles for hydrolysis of casein (A) and Glu-Phe-NAn (B) by myosin-cleaving protease (open symbols) and by chymotrypsin (closed symbols). The buffers used were; 0.1 M potassium phosphate (○, ●), 0.1 M Tris/acetate (△, ▲) and 0.1 M borate (□, ■). Activity at the optimum pH was taken as 100%.

pH effect. The effect of pH variation on the activity of myosin-cleaving protease using casein as substrate is shown in Fig. 3A, the effect on chymotrypsin was taken for comparative purposes. The pH optima for casinolysis by the enzyme and chymotrypsin were 9.0 and 7.9, respectively. The effect of pH variation using synthetic substrate, Glu-Phe-NAn, is shown in Fig. 3B. The pH optima were 8.4 for the enzyme and 7.4 for chymotrypsin.

Limited degradation of cardiac myosin. The purified cardiac myosin from rat heart was degraded with myosin-cleaving protease; electrophoretograms of products of myosin degraded for various times are shown in Fig. 4, those of myo-

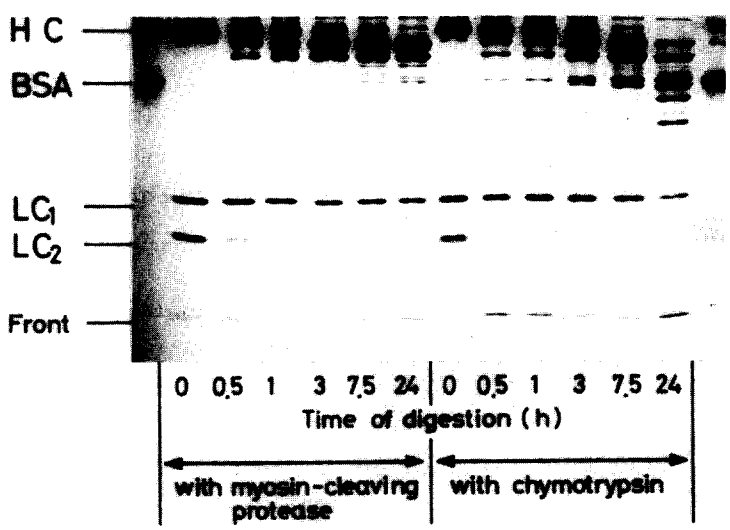

Fig. 4. Electrophoretograms of the products of cardiac myosin degraded with myosin-cleaving protease and with chymotrypsin after various times. 1 mg cardiac myosin was digested in 0.6 ml 0.02 M potassium phosphate buffer (pH 7.0)/0.5 M KCl, 1 mM EDTA at 20°C. 3 units of each enzyme (caseinolytic activity) were used. An aliquot of the digestion product (corresponding to 10 μg myosin) was applied to SDS polyacrylamide slab gel. Other conditions were as described in the text. BSA, bovine serum albumin; HC, myosin heavy chain; LC_1, light chain 1, LC_2; light chain 2.

sin degraded with chymotrypsin were taken for comparative purposes. As the time of digestion increases, two bands corresponding to myosin heavy chain and light chain 2 disappeared almost completely and two bands appeared, corresponding to new fragments having molecular weights of 130 000 and 94 000. Over the period of 24 h, three bands were predominant. A band corresponding to light chain 1 remained intact, judged by densitometer tracing.

Unlike myosin-cleaving protease, chymotryptic digestion leads to a rapid and slow disappearance of the bands corresponding to light chain 2 and light chain 1, respectively. There was also further digestion of the 130 000 and 94 000 dalton bands accompanied by the appearance of four bands whose mobilities correspond to molecular weights between 70 000 and 50 000.

These results should show that digestion of myosin with myosin-cleaving protease is more restrictive than that with chymotrypsin.

Actin in cardiac actomyosin extracted from rat heart was not digested with myosin-cleaving protease (unpublished data).

Discussion

In the present study, an endogenous protease capable of degrading myosin was purified from myofibrillar fraction of rat heart to the stage of homogeneity as judged by gel filtration and gel electrophoresis. The purified enzyme is unstable at neutral and alkaline pH without high Ca^{2+} concentration. Under these conditions the enzyme undergoes autolysis, preserving its caseinolytic activity. Ca^{2+} is not required for the enzyme activity, but it seems to protect the enzyme from autolysis, similar to other serine proteases. A small increase of the activity was observed after protamine sulfate treatment. The increase may be due to the elimination of an inhibitor [6,9] and/or polyanions, nucleic acid and heparin. In our laboratory, an inhibitor was found to be in the supernatant fraction of heart muscle homogenates (unpublished data). The enzyme, having molecular weight of 26 000—27 000, was more active toward substrates for chymotrypsin than those for trypsin. In addition, the typical inhibitors for chymotrypsin-like serine protease were effective. However, the susceptibility to metal ions and the optimum pH of the activity were clearly different from those of chymotrypsin. These results, together with the results in which the sulfhydryl reagents and metal-chelating reagents have little or no effect, suggest that the enzyme is a serine protease.

Huston and Krebs [7] purified a protease from rabbit skeletal muscle, whose activity was Ca^{2+} dependent. Proteolytic activity, reported by Koszalka and Miller [5,28], was located in the supernatant of rat muscle homogenates. Goldspink et al. [9] have observed that proteolytic activity found in rat skeletal muscle was partially inhibited with 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. Brush [8] found an insulin-degrading protease in the supernatant of muscle homogenates whose activity is unaffected with 1 mM phenylmethylsulfonyl fluoride. Dayton et al. [11,12] have reported the purification of Ca^{2+}-activated protease from pig skeletal muscle, which removed z-disks from myofibrils and degraded tropomyosin, troponin and C-protein but not myosin and actin. Recently, Katunuma et al. [10,29] have reported new serine proteases in crystalline form from rat skeletal and intestinal muscle, having

molecular weights of 13 000 and 24 000, respectively. They also presented the degradation of myosin and actin with a protease from skeletal muscle [13]. Noguchi and Kandatsu [6,30] have purified a protease from rat skeletal muscle without clear information on molecular weight and on hydrolysis of myosin. Their protease was active at pH 9.5—10.5 and inhibited with iPr_2P-F. They also reported the remarkable activation effect of KCl on the autolysis of myofibrillar fraction and little or no influence of divalent metal ions, EDTA, *p*-chloromercuribenzoate and iodoacetamide. There are some differences between the properties of myosin-cleaving protease and the proteases reported previously. Identity of the enzyme with the proteases described above should not be determined yet.

It was suggested that an alkaline protease from rat skeletal muscle might be a chymotrypsin-like protease of mast-cell origin [25—27]. The prominent properties of the protease are the lower solubility at low concentration of salt and the stimulation of the activity with salt. The result in which caseinolytic activity of myosin-cleaving protease was not stimulated with KCl in various concentrations but inhibited with 1 M KCl may not agree with a possibility that it is mast-cell origin.

Yagi and Kuwayama [31] have reported that an apparent homogeneous subfragment 1 was obtained from pig cardiac myosin by chymotryptic digestion. They also suggested a possibility of overdigestion of subfragment 1. We obtained previously a preparation of subfragment 1 from rat cardiac myosin, which is homogeneous on SDS-polyacrylamide gel [14]. Myosin-cleaving protease made a limited degradation of myosin, suggesting that it may be suitable to use for preparation of homogeneous subfragment 1. Although the physiological role of this enzyme is not yet known, the enzyme may be involved in metabolic turnover of myosin in myofibrils.

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