Characterization of Muscle Low Calcium Requiring Form of Calcium Activated Protease

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Received June 11, 1987

A low calcium requiring form of calcium activated protease (LCAP) and a high calcium requiring form of calcium activated protease (HCAP), purified from porcine skeletal muscle, were studied. LCAP was most active at pH 8.2. The HCAP was rather unstable at high temperature or at alkaline pHs: The antiserum to purified LCAP did not make a precipitin line with HCAP. Those proteases hydrolyzed myofibril proteins to disassemble the structure of myofibrils. The electronmicrogram of the myofibril treated by LCAP resembled that of the muscle obtained from a vitamin E deficient rat. Both LCAP and HCAP hydrolyzed purified α-actinin, liver actin, and some other proteins which are not assigned. The microsome proteins were resistant to protease treatment, and only the 180 kd protein was hydrolyzed by LCAP.

Skeletal muscle is the largest organ in the human body and supplies amino acids as fuel under some physiological conditions, for example, fasting. The mechanism of production of amino acid from muscle proteins are not understood well. These are several different mechanisms in muscle, and one of these requires calcium. A protease, called calcium activated protease (CAP) is thought to be responsible for this pathway. There are at least two different type of CAP in muscle, one is the low calcium requiring form (LCAP) and the other is the high calcium requiring form (HCAP). LCAP was first reported by Mellgren2) and was purified from many tissues, but there are few reports on the skeletal muscle enzyme. The effects of the LCAP of muscle enzyme on myofibrils was described by Dayton et al., but they used a partially purified enzyme for the assay. Only the substrate specificity of heart muscle LCAP was described by Croall and Demartino4) using purified enzyme. They have shown that the purified tropomyosin, α-actin, and α-actinin were the substrate of LCAP and HCAP purified from bovine heart. However, it is not known whether the other components of the muscle are substrates.

There are many studies on LCAP and HCAP other than the skeletal one;5) the results were sometimes puzzling. Furthermore, the different roles of LCAP and HCAP are not explained well yet.

We purified LCAP from porcine heart muscle using mild conditions several years ago, using phenyl-Sepharose column chromatography and Matrex-blue column chromatography which were very effective for purification of the enzyme without denaturing or autolyzing it. In this paper we will describe the

Abbreviations: LCAP, low calcium requiring form of calcium activated protease; HCAP, high calcium requiring form of calcium activated protease; CAP, calcium activated protease; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; MCE, 2-mercaptoethanol; SDS, sodium dodecyl sulfate.
characterization and endogenous substrate of the LCAP.

MATERIALS AND METHODS

Animals. Porcine skeletal muscle was obtained at the slaughterhouse of Tottori City. The muscle of vitamin E deficient rats was prepared as described in a previous report.71

Chemicals. Freund's complete and incomplete adjuvant were purchased from Wako, Osaka, Japan. 3',5'-Cyclic AMP, 3',5'-cyclic GMP, and d-myo-inositol triphosphate were from Sigma. The other reagents were analytical grade.

Preparation of the purified LCAP and HCAP. The purified porcine skeletal muscle LCAP was prepared by the method described in a previous paper6 using ammonium sulfate fractionation, DEAE-cellulose column chromatography, phenyl-Sepharose column chromatography, and Matrex-blue column chromatography, with slight modifications. The elution of the proteins from the Matrex-blue column was done with decreasing KCl concentration gradually from 50mM to 0mM. The purified LCAP thus prepared was the purest enzyme preparation we had. The porcine skeletal muscle HCAP was prepared by the method described in Szpacenko et al.8) and further purified with DEAE-cellulose column chromatography. Chicken skeletal muscle HCAP was purified by the same method.

Preparation of myofibrils and glycerinated muscle. The myofibrils were prepared by the method of Solaro et al.9) Glycerinated muscle was prepared by the method of Huxley10) with slight modification as follows. The strip of freshly killed porcine muscle, approximately 4 mm x 2 mm in section and 10 cm in length, was dissected out, ties with wool onto a glass rod, and placed in a medium containing 50% glycerin in PBS AT -20°C was washed with cold PBS containing 1mM MgCl2 three times. The glycerinated muscle was then cut into 1 cm long pieces and a piece of the muscle was added to 5/A of 1 mMCE, 0.02mg of LCAP at 4°C. The muscle was stored in a deep freeze at -20°C.

Preparation of skeletal a-actinin and liver actin. a-Actinin was purified from porcine skeletal myofibrils by the procedures of Suzuki et al.11) with slight modifications. The DEAE-cellulose was not repeated, and the a-actinin fractions from the first DEAE-cellulose column was further purified by ammonium sulfate fractionation. The a-actinin was salted out between 7g/100ml and 12g/100 ml. This fraction contained a-actinin and a small amount of a 43 kd protein which was assumed to be /?-actinin.12) Liver actin was prepared by the method of Nishida et al.13) using DNase-I affinity column chromatography.

Preparation of microsome of porcine skeletal muscle. Twenty-four and two-tenths grams of minced porcine skeletal muscle were homogenized with 200 ml of 0.25 m sucrose-1 mM EDTA-1 mM MgCl2-50 mM Tris-acetate (pH 7.5) buffer by a Waring blender for 30 sec. After centrifugation at 10,000 x g for 20 min to remove myofibrils, nuclei, and mitochondria, the supernatant was further centrifuged at 25,000 x g for 30 min to sediment lysosomes. The supernatant of this centrifugation was then centrifuged at 105,000 x g for 1 hr and the precipitate was dissolved in 5 ml of 1 mM EDTA-1 mM NaNO3-20 mM Tris-acetate (pH 7.5) buffer. This suspension was used as microsomes of porcine skeletal muscle.

Immunological method. The antiserum to the purified LCAP was prepared in a rabbit. The 50 /tg of purified LCAP was injected with 1 ml of Freund's complete adjuvant. Four weeks later, the same amount of the LCAP with incomplete adjuvant was injected. The serum was obtained 6 weeks after the first immunization. The reactivity of the serum to the LCAP was measured by the double immunodiffusion method of Ouchterlony.14)

Treatment of proteins with LCAP or HCAP. The myofibrils (1.5 mg) were treated with 0.04 mg of LCAP at 25°C for 2 hr in 2 ml of 8 mM Tris-acetate (pH 7.5)-0.5 mM CaCl2-0.4 mM ethylene glycol bis (/-aminoethyl ether)-N,N',N''-tetraacetic acid (EGTA)-0.5 mM NaN3-5 mM 2-mercaptoethanol (MCE)-115 mM KCl buffer. As a control, the myofibrils were treated in the buffer containing 5 mM EDTA instead of 0.5 mM CaCl2. The treatment with HCAP was done at 5 mM CaCl2 in the same buffer. The myofibrils thus treated was centrifuged at 3,000 rpm for 20 min and the precipitate was either electrophoresed on SDS-PAGE or observed by electron microscope. The liver actin and microsome were treated the same as the myofibrils. The glycerinated muscle that had been stored in 50% glycerin in PBS AT -20°C was washed with cold PBS containing 1 mM MgCl2 three times. The glycerinated muscle was then cut into 1 cm long pieces and a piece of the muscle was added to 5 /tg of MCE, 0.02 mg of the LCAP in 0.4 ml of 20 mM Tris-acetate (pH 7.5)-1 mM EGTA-1 mM NaN3 buffer, 50 /tg of 2 mM KCl, 5 /tg of 100 mM CaCl2, 10 /tg of 100 mM NaNO3, and 0.540 ml of water. After incubation at 25°C for 2 hr, the muscle was fixed with glutaraldehyde for electron microscopic observation. The volume of the CaCl2 solution was changed to 50 /tg for the incubation at higher concentrations of calcium for the LCAP. The 100 mM of EDTA was added instead of CaCl2 for the control sample. The treatment with HCAP was done in 5 mM CaCl2 under the same conditions.

SDS-PAGE. SDS-PAGE was done by the method of Laemmli15) with a slab-gel electrophoresis system and stained with Coomassie brilliant blue by the method of Fairbanks et al.16)
Muscle Low Calcium Requiring Form of Calcium Activated Protease

Electron microscopic observation of myofibrils. The precipitate of myofibrils after treatment with the protease was fixed with glutaraldehyde for 2 hr and osmium tetra-acid for 1.5 hr. The fixed myofibrils were embedded in Spurr's resin17 and stained with uranyl acetate for 50 min and lead acetate for 30 min at room temperature. The glycinated muscle sample was prepared by the same procedures. The observation was done with a Hitachi electronmicroscope H-500.

Assay of the enzyme activity. The LCAP and HCAP activity was measured by the method described in a previous paper6 using casein as the substrate at 25°C.

Protein concentration. The protein concentration was measured by either the biuret method or a Protein Assay Kit (Bio-Rad, Japan Bio-Rad Laboratory, Japan) with bovine serum albumin as the standard.

RESULTS

Characterization of the purified enzymes

Some properties of the purified enzymes are summarized in Table I. The LCAP was most active at pH 8.2 which is much higher than previous reports.3,18 The HCAP was most active at pH 7.5. The LCAP and HCAP showed maximal activity at 32°C and 25°C, respectively. The stability of the enzymes were different; the LCAP was more stable at alkaline pH than the HCAP. The LCAP incubated at pH 8.9 still showed the same activity as the unincubated LCAP. The HCAP lost activity under these conditions. Both enzymes were unstable at pH 5.0. The LCAP was stable at 57°C; the HCAP lost activity at 57°C.

Table I. Some Properties of Purified Enzymes

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<thead>
<tr>
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<th>LCAP</th>
<th>HCAP</th>
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<tr>
<td>Optimal pH</td>
<td>8.2</td>
<td>7.5</td>
</tr>
<tr>
<td>pH stability</td>
<td>5.6~8.9</td>
<td>5.6~7.6</td>
</tr>
<tr>
<td>Optimal temperature</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Temp. stability</td>
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</tr>
<tr>
<td>Activator</td>
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<td>Ca</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>NEW, EDTA, NEM, EDTA</td>
<td></td>
</tr>
<tr>
<td>No effect</td>
<td>cAMP, cGMP, IPa</td>
<td>cAMP, cGMP, IPa</td>
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a At least 50% of the enzyme activity was left with this treatment.

b IP, myo-inositol triphosphate.

Fig. 1. Immunological Differences between LCAP and HCAP.

A double immunodiffusion test was done with 1.0% agar gel in 30 mM sodium barbital buffer (pH 8.6) containing 0.01% NaN3. Antiserum to purified LCAP was placed in the center well. Wells 1, 2, and 6: purified LCAP; Wells 3 and 5: purified HCAP which was composed of only the 80 K subunit; A well 4: the purified HCAP from chicken skeletal muscle which was composed of 80 K and 30 K subunits.

Fig. 2. Electron Microscopic Observation of Myofibrils Treated with LCAP.

The porcine skeletal myofibril was treated with purified LCAP in 8 mM Tris-acetate (pH 7.5)–5 mM MCE–0.5 mM NaN3–115 mM KCl–0.5 mM CaCl2–0.4 mM EGTA buffer. The LCAP to myofibril ratio was 1:37.5. After incubation at 25°C for 2 hours, the myofibril was centrifuged at 15000 rpm for 30 min. The precipitate was fixed with glutaraldehyde and osmium tetroacids. Uranyl acetate and lead acetate stain. Bar indicates 1 μm.
The immunological difference between the LCAP and the HCAP

The antiserum to the purified LCAP gave a precipitin line with purified LCAP. However, the antiserum did not make any precipitin line with the HCAP purified from porcine skeletal muscle or the HCAP purified from chicken skeletal muscle (Fig. 1).

The ultrastructure of the myofibrils treated with LCAP

The myofibrils treated with the LCAP are shown in Fig. 2. The myofibrils were destroyed at the Z-line when they were treated with LCAP in 100 μM CaCl₂ for 2 hr. The thick filament and M-line were not changed by LCAP treatment. Some of the myofibrils were degraded further (Fig. 2, arrow). The structure of the sarcomeres was destroyed and a filamentous structure that was the disorganized thick and thin filaments was observed. The thin filaments also were scattered in the upper part of the electronmicrogram. We tried to see if there are the same structure in atrophied muscle. For this purpose, we checked the muscle obtained from a vitamin E deficient rat (Fig. 3). We could see the same structure in the vitamin E deficient rat as the myofibrils treated with LCAP. The HCAP had the same effects on myofibrils in the presence of 5 mM CaCl₂ (data not shown).

Electron microscopic observation of glycerinated muscle treated with the protease

The glycerinated muscle was put in buffer containing 100 μM of free calcium and LCAP for 2 hr and then observed with the electronmicroscope (Fig. 4). The Z-line was completely destroyed but the M-line, thick filaments, and thin filament were intact. The glycerinated muscle that was treated with LCAP or HCAP in 5 mM CaCl₂ had the same features. The glycerinated muscle that was treated with LCAP in 5 mM EDTA (Fig. 4) were not different from the electronmicrogram of the original muscle fixed in situ. The Z-line and M-line were clearly observed.

Treatment of myofibrils with LCAP or HCAP

The myofibrils treated with LCAP in the
Fig. 4. Electronmicrogram of the LCAP-treated Glycerinated Muscle.

A piece of glycerinated muscle was cut to 5 mm long, and was treated with LCAP as in Fig. 2. The upper photograph is the muscle which was treated with LCAP in buffer containing 100 μM free calcium, and the lower is the muscle treated in 5 mM EDTA. Uranyl acetate and lead acetate stain. Bar indicates 1 μm.
Porcine skeletal muscle myofibrils were treated with LCAP (B, C, b, c) or HCAP (D, E, d, e) in the presence of calcium (c, e, C, E) or in the absence of calcium (B, D, b, d) at 25°C for 2 hr. The reaction mixture was then centrifuged at 3000 rpm for 30 min. The precipitate (A to E) or supernatant (a to e) was analyzed by SDS-PAGE. Approximately 100 μg of protein was put on the gel (A to E). The LCAP and HCAP to myofibril ratio was 1 : 37.5 and 1 : 27.5, respectively. Calcium concentration was 100 μM for LCAP treatment and 5 mM for HCAP. Lane a and A were the myofibrils incubated without enzyme in 100 μM CaCl₂. The numbers on the right indicate the position of the following proteins: 2, 10, 12, 14, myosin; 4, α-actinin; 5, desmin; 6, actin; 7, 11, 13, troponin; 8, tropomyosin.

Buffer containing 100 μM free calcium were analyzed by SDS-PAGE (Fig. 5, lane C). Bands 1, 3, 4, 5, 9, 10, 11, 12, 13, and 14 disappeared. Band 4, with a molecular weight of about 100 kd, was assumed to be α-actinin. Band 5 was assumed to be desmin, and 9, 10, and 11 were myosin light chain, troponin-N, and myosin light chain, respectively. Several new bands were observed. The new bands in lane C that appeared around the α-actinin band were probably the degradation materials of the some high molecular weight proateins (bands 2 and 3) which may be titin, nebulin, mysin, or α-actinin. One of the bands was also observed in the supernatant of the LCAP-treated myofibrils (lane c). The myofibrils treated with HCAP in the buffer containing 5 mM CaCl₂ (lane E or e) showed almost the same pattern as the gel of lane C or c. Figure 5 shows that neither protease affected actin (band 6), and also shows that the LCAP and HCAP treatment increased the amount of protein solubilized from the myofibrils. There were no protein bands at 100 kd and 50 kd in the SDS-PAGE of supernatant fractions. This fact suggest that α-actinin and desmin were hydrolyzed.

Treatment of α-actinin and liver actin with LCAP or HCAP

These results suggest that the LCAP destroyed α-actinin from myofibrils resulting in myofibril destruction. The results of Suzuki et al. have shown that the HCAP did not hydrolyze α-actinin, but released α-actinin from myofibrils. Therefore, the α-actinin was treated with LCAP to see if the α-actinin was hydrolyzed by the LCAP (Fig. 6). The α-actinin was hydrolyzed by LCAP and HCAP. The α-actinin fraction contained small amount of a 43 kd protein assumed to be β-actinin. This protein was also hydrolyzed. We also
Muscle Low Calcium Requiring Form of Calcium Activated Protease

Fig. 6. Treatment of α-Actinin.

α-Actinin (14μg) in 150μl of 1 mM NaHCO₃ was incubated with 0.4μg of LCAP (lane A, B, C, D) in 50μl of 4 mM Tris-acetate (pH 7.5), 20 mM MCE, 3 mM CaCl₂ (lane A, B) 2 mM NaN₃ and 0.2 mM EDTA buffer. After 1 min (lane A, C, E, G) and 2 hr (lane B, D, F, H), 50μl of the reaction mixture was added by 25μl of SDS-PAGE sample buffer and run the SDS-PAGE. Lane C and D shows the α-actinin which were incubated with LCAP in the buffer containing no calcium. The α-actinin was also treated by 0.4μg of HCAP under the same conditions (lane E and F with calcium, lane G and H with EDTA). Numbers on the right show: 1, α-actinin; 2, β-actinin.

We tried to hydrolyze another actin which we purified from porcine liver (Fig. 7). The liver actin and the proteins which bound to DNase I affinity column, probably a kind of actin binding protein, were hydrolyzed very quickly.

Fig. 7. Treatment of Liver Actin and Actin Binding Protein.

Thirty μg of liver actin purified by DNase-1 affinity chromatography was incubated with 0.8μg of LCAP in 200 μl of 8 mM tris acetate (pH 7.5), 2 mM MCE, 0.3 mM CaCl₂, 0.75 mM MgCl₂, 0.075 mM ATP, 0.2 mM NaN₃ buffer at 25°C for 2 hr (lane H). The sample was added to 100μl of SDS-PAGE sample buffer and run on SDS-PAGE. Lane G is the sample incubated for 1 min. Lane E and F are the samples incubated with EDTA for 1 min and 2 hr respectively. The liver actin was also treated by 1μg of HCAP under the same conditions but the CaCl₂ concentration was 3 mM (lane A, 1 min with EDTA; lane B, 2 hr with EDTA; lane C, 1 min with calcium; lane D, 2 hr with calcium). The lane Mf shows the standard myofibrils, lane S shows control liver actin.

DISCUSSION

The purified LCAP had strong activity at pH 8.5 which was almost the same activity at the optimal pH of the LCAP. This result was different from previous works on porcine skeletal muscle LCAP,3) rat kidney,18) calf brain,21) rabbit muscle,22) and rabbit liver.23) The partial purified porcine skeletal muscle LCAP3) had a lower optimal pH than this. The optimal pH of the purified rat kidney calpain I (LCAP) was pH 7.0~7.5, and this is lower than the optimal pH of calpain II (HCAP). The reason for the difference between these

Treatment of microsome proteins with LCAP or HCAP

We tried to find another substrate of the LCAP. Microsomes of porcine skeletal muscle were prepared (Fig. 8). The proteins in the microsome fraction were rather stable against LCAP treatment. Only the 180 kd protein (arrow) disappeared from the gel upon treatment with LCAP in 100 μM calcium. This protein also disappeared from the gel upon HCAP treatment. Furthermore, this band became faint when the myofibril was incubated in the buffer containing calcium without pro-
Fig. 8. Treatment of Muscle Microsomes.

Two hundred μg of microsomes were treated with 0.5 μg of LCAP (lane E, F, G, H) in 50 μl of 4 mM Tris-acetate (pH 7.5), 20 mM MCE, 3 mM CaCl₂ (lane E, F) 2 mM NaN₃ and 0.2 mM EDTA buffer. After 1 min (lane A, C, E, G, I, K) and 2 hr (lane B, D, F, H, J, L), 50 μl of the reaction mixture was added to 25 μl of SDS-PAGE sample buffer and run on SDS-PAGE. The lane G and H shows the microsomes incubated with LCAP in the buffer containing no calcium. The microsomes were also treated by 0.4 μg of HCAP under the same conditions (lane I and J with calcium, lane K and L with EDTA). As a control, microsomes were incubated under the same conditions without enzyme (lane A, 1 min with calcium; lane B, 2 hr with calcium; lane C, 1 min without calcium; lane D, 2 hr without calcium).

The immunological identity between LCAP and HCAP has been also described by several investigators, but our results were different. Dayton et al.3) has shown that the antiserum to the HCAP 80 kd subunit made a precipitin line with LCAP. Croall and DeMartino4) described a lack of cross reactivity between the LCAP and the anti-serum to the HCAP 80 K subunit. The antiserum to purified LCAP which we made, did not react with the HCAP. The HCAP which we used for this experiment lacked the small subunit, therefore, the anti-serum to LCAP did not cross react with the HCAP, because the homology of the large subunit between LCAP and HCAP is reported as 55.3% by Emori et al.24)

Even though the LCAP and HCAP are thought to be responsible for the proteolytic system in cells, there were many difference between the LCAP and the HCAP. The LCAP was rather stable at high pHs and high temperatures. For what ever reason, it is clear that the LCAP is different from HCAP in many points, and this may suggest that the LCAP has a different role from that of HCAP. Therefore, it is necessary to find the natural substrate of LCAP. There is only one report which describes the substrate of skeletal muscle LCAP. Dayton et al.3) have shown that the partial purified LCAP weakened myofibrils at the Z-line. Croall and DeMartino4) have shown that the purified heart muscle LCAP degraded α-actin, tropomyosin, and α-actinin. Our result was that many skeletal muscle myofibril proteins were degraded by LCAP but microsome proteins were rather stable except for the 180 kd protein. The purified α- and β-actinin and liver actin were also degraded by LCAP. The product of the LCAP and HCAP treatment was not the same when α-actinin was used as the substrate, but we do not have enough data to show the difference of substrate specificity between LCAP and HCAP. We observed Z-line removal as did Dayton et al.,3) and we further observed scattered thin and thick filaments which lost tertiary structure. The loss of thin filament structure may be due to the loss of the Z-line. On the other hand, the loss of thick filament
structure may be due to the degradation of the protein which links thick filaments. The important characteristic of the LCAP on the myofibril or glycerinated muscle is that the LCAP destroyed the Z-line but did not affect the M-line the same as HCAP. Because cathepsin L, which is a lysosomal protease, removed the M-line with the Z-line destruction, these characteristics may be useful to understand the protein degradation pathway in muscle cells. If we observe a single sarcomere of which the M-line is intact, it will show that the muscle is degraded by LCAP and/or HCAP but not by the lysosomal proteolytic system, because only LCAP and HCAP are proteases which degrade myofibrils at the Z-line without removing the M-line. We can see those electronmicrographs in several case of diseased muscle of Duchenne muscular dystrophy, ischemia, and prune berry syndrome, even in a macrophage. These photographs of diseased muscle, therefore, show that the muscle was degraded by the calcium activated protease, not by lysosome. The other characteristic structure of the enzyme reaction is pointed out by the arrow in Fig. 2. We can also see this kind of structure in many electronmicrographs of vitamin E deficient rat muscle (Fig. 3) and some diseased muscle. This disassembled muscle is thought to be the first step of the myofibril degradation in muscle. The LCAP was thought to be the key enzyme as described. It is important to discover the regulatory mechanism of the LCAP. We have purified an endogenous inhibitor of the HCAP, and the LCAP was also inhibited by this inhibitor (data was not shown).

Acknowledgments. We thank Ms. Yukari Kondo for her skillful technical assistance with the electron microscope. We also thank Dr. Susumu Maeda, Zoeken Research Institute, U.S.A., and Dr. Kunihiro Matsuamoto, DNAX Institute, U.S.A., for their encouragement in this work. We also thank to Miss. Junko Aoki for her assistance in the experiments. Part of this work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and also by grants for research from the Mishimakaiun Fund, Ito Memorial Fund, Japanese Agriculture Chemistry Promotion, and Muscular Dystrophy Association of America.

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