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PURIFICATION OF NEW CALCIUM ACTIVATED PROTEASE (LOW CALCIUM REQUIRING FORM) AND COMPARISON TO HIGH CALCIUM REQUIRING FORM

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SUMMARY A new calcium activated protease which requries low concentration of calcium was purified to almost homogeneity from porcine heart muscle. The protease was composed of two polypeptide chains of approximately 90 K and 30 K. The 90 K subunit was larger than the large subunit of the high calcium requring form of calcium activated protease, therefore we concluded that the low calcium requiring form is different from the high calcium requring form and its auto-digested protease. The low calcium reqiring form of calcium activated protease was also activated by manganese and balium, and was very stable even at pH 9.0

The recent studies on calcium activated protease have shown that there are two types of calcium activated protease (CAP) (1). One is activated by mM order of calcium concentration. This corresponds to the enzyme purified by Dayton et al. (2), and we call this type of the enzyme H-CAP, latter in this report. The other is activated by only 50 μM of calcium; therefore we call this as L-CAP. As calcium concentration is very low in cells, the L-CAP is thought to be the protease which is probably responsible for initial protein degradation in cells.

The H-CAP has already been purified by several investigators (2,3,4,5), however, the L-CAP has not been well purified (6). Furthermore, the recent study by Suzuki et al. (7) has shown that

ABBREVIATIONS: EDTA, Ethylenediaminetetraacetic Acid. CAP, Calcium acivated protease. L-CAP, Low calcium requiring form of CAP. H-CAP, High calcium requiring form of CAP. Buffer A, 20 mM Tris acetate (pH 7.5) buffer containing 1 mM EDTA and 1 mM Sodium azide.

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the auto-digested H-CAP changed its requirement for calcium to μM order concentration. This result may suggest that the L-CAP be the converted enzyme of the H-CAP by autolysis.

In the present paper, we describe the purification of the L-CAP and the difference of the L-CAP from the H-CAP and the auto-digested protease of the H-CAP. Especially, we show the results that the L-CAP is different from the auto-digested form of the H-CAP.

MATERIALS AND METHODS

DEAE cellulose(DE-52) was obtained from Whatman Co., Tokyo, Japan. Phenyl-Sepharose was obtained from Pharmacia Fine Chemicals AB Uppsala, Sweden. Matrex Gel Blue A was obtained from Amicon Corp., Lexington, Mass., USA. All other reagents are analytical grade.

Assay for proteolytic activity: CAP activity was measured using casien as substrate at final concentration of 0.5 %, in 1.0 ml of reaction mixture containing 10 mM 2-mercaptoethanol, 10 mM NaN3, 100 mM Tris acetate buffer (pH 7.5) and 5 mM CaCl2, and appropriate amounts of enzyme solution. The reaction was started by addition of CaCl2 solution. The mixture was incubated for 30 min at 25 C° and the reaction was stopped by addition of 1.0 ml of 5.0 % trichloroacetic acid. After the centrifugation, the absorbance of the supernatant at 280 nm was measured by Hitachi spectrophotometer model 220A. The CAP acivity was expressed by the increased absorbance values during the incubation. For the crude enzyme, the mixture containing 5 mM EDTA except 5 mM CaCl2 was used as control. One unit of the enzyme activity is defined as the amount of the enzyme which increases 1 unit of absorbance at 280 nm during the incubation at this condition.

Purification of H-CAP: The H-CAP was purified from porcine heart muscle by the method of Szpacenko et al.(8).

Concentration of protein: Protein was concentrated with the Minimojule NM-3 which was developed for macromolecule concentration by Aasahikasei Kogyo, Tokyo, Japan, at 4C°.

Disc gel electrophoresis: Na-dodecyl sulfate-polyacrylamide disc gel electrophoresis was performed by the method of Weber and Osborn(9). Polyacrylamide disc gel electrophoresis was performed using the method of Davis (10). Gels were stained overnight with Coomassie Brilliant Blue R-250 and destained with methanol:water:acetic acid, 50:875:75.

RESULTS

Purification of the L-CAP: Heart muscle was removed from the body immediately after the death, and was minced with meat

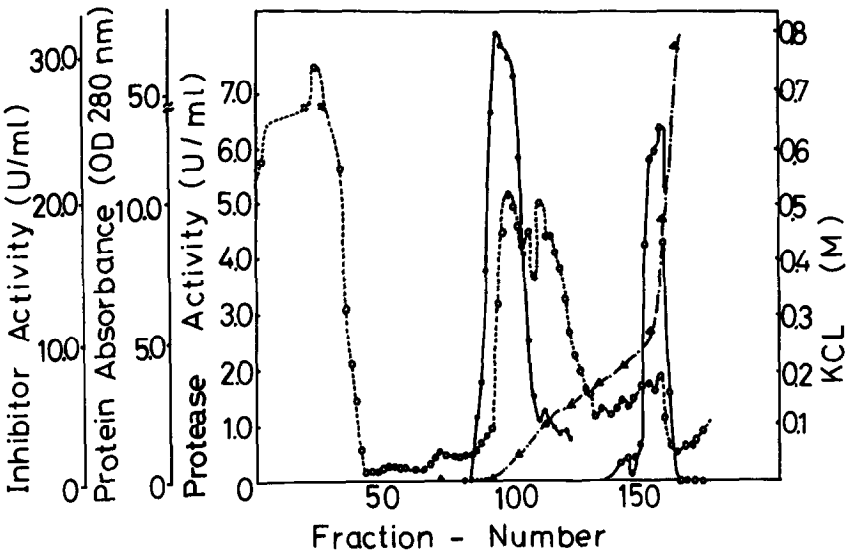

Fig.1. Elution profile of CAP and its inhibitor off a DEAE-cellulose column. The ammonium sulfate precipitated proteins (758 ml) were loaded onto a DEAE-cellulose column (4.4x42.6 cm) which had been equiribrated with 20 mM Tris acetate (pH7.5) buffer containing 1 mM EDTA and 1 mM NaN3 (Buffer A). After washing the column with Buffer A the proteins were eluted with increasing KCl concentration from 0 to 250 mM in the Buffer A. The column was washed with the Buffer A containing 500 mM KCl to elute the remaining proteins after this gradient elution. o—o CAP activity, ●—● inhibitor activity, ▲-·-▲ KCl concentration, o---o Protein.

grinder. To 1550 g of the minced muscle, 6200 ml of 50 mM Tris-acetate (pH 7.5) buffer containing 4 mM EDTA was added, and homogenized three times by a Wahring blender for 30 second each. The mixture was then centrifuged to obtain the crude extract. The crude extract was added ammonium sulfate to 65 % saturation, and was centrifuged. The precipitate was dissolved in small amount of 20 mM Tris acetate buffer (pH 7.5) containing 1 mM of EDTA and 1 mM NaN3 (Buffer A), and dialized against Buffer A overnight. During the dialysis, the buffer was changed at least 4 times. After the centrifugation at 15000 rpm for 30 min to remove insoluble materials, the dialyzate was loaded onto a DEAE-cellulose column (4.4 x 42.5 cm) which had been bufferized with Buffer A (Fig.1).

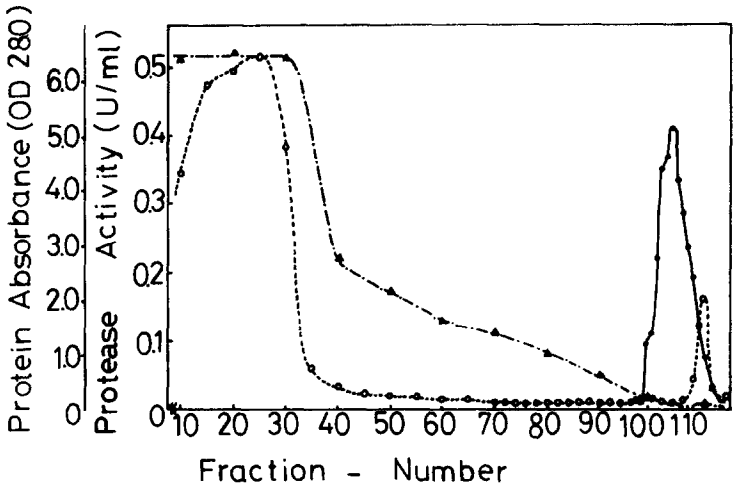

Fig.2. Elution profile of L-CAP off a Phenyl-Sepharose column . The L-CAP fractions off the DEAE-cellulose column was added KCl to 250 mM and was loaded onto a Phenyl-Sepharose column (2.5x 24.0 cm) that had been bufferized with Buffer A containing 250 mM KCl. The L-CAP was eluted with decreasing KCl concentration to 0 mM and increasing ethylen glycol to 20% in Buffer A. In order to elute the remaining proteins, the column was washed with 1 mM Tris EDTA (pH 7.0) buffer containig 1 mM NaN3: O---O Protein, ●——● L-CAP activity, ▲-·-▲ KCl concentration.

The proteins were eluted with increasing KCl concentration from 0 to 250 mM in Buffer A. The eluate was collected 20 ml each and assayed CAP activity and its inhibitor activity. The CAP activity was found in the tubes about 155. The KCl concentration of these fractions was the same as the KCl concentration of the fractions where the H-CAP was eluted (1,2) . However, the CAP activity was not found on the tubes at which KCl concentration the L-CAP was usualy eluted as several investigators have reported (1,8). The activity of endogenous inhibitor of the CAP was found on the tubes just before the tubes where the L-CAP usually was eluted. Because of the high activity of the inhibitor, we concluded that the L-CAP was overlapped by the inhibitor and no activity was detected in these tubes. Accordingly, the tubes from 101 to 124 where the L-CAP was probably eluted, were collected.

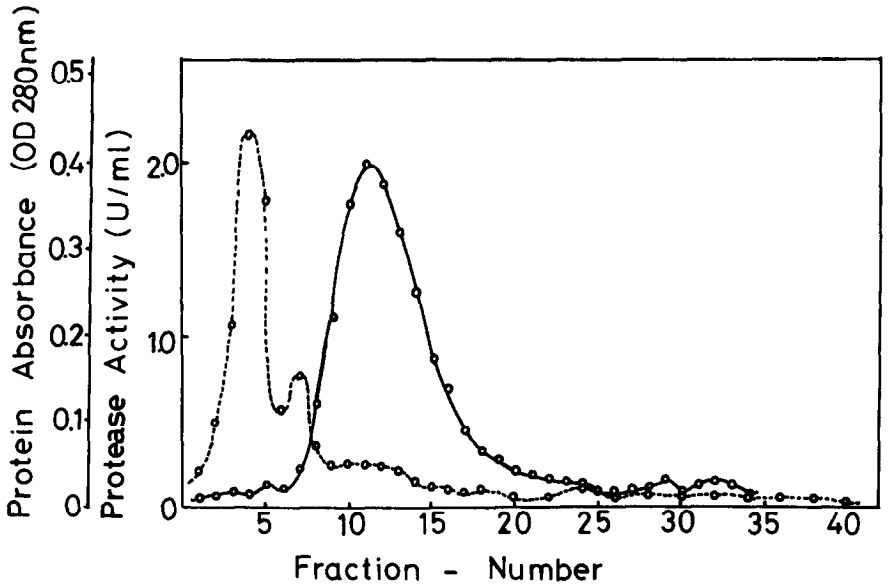

Fig.3. Elution profile of L-CAP off a Matrex Blue A column. The L-CAP (120 ml) off the Phenyl-Sepharose column was concentrated to 3 ml with a Minimojuor. This sample was loaded onto a Matrex Blue A column (0.95 x 21.2 cm) previously equiribrated with 5 mM Tris acetate (pH 7.5) buffer containing 1 mM EDTA and 1 mM NaN3 . The proteins were eluted with the same buffer: O···O protein, O——O L- CAP activity.

The collected solution was added KCl to 250 mM concentration then loaded on to a Pheny-Sepharose column (2.5x24.5cm) which had been equiribrated with 250 mM KCl in Buffer A. The proteins were eluted with decreasing KCl concentration in Buffer A (Fig.2). The L-CAP activity was found in the tubes from 100 to 105 at this column chromatography, because the L-CAP was separated from its endogenous inhibitor by this column chromatography. The active fractions were combined and concentrated by a Minimojule to 3.0 ml for further purification. The recovery of the L-CAP through this concentration step was about 72 %.

The last step of the purification was a kind of affinity chromatography using a Matrex Blue A column shown in Fig.3. The concentrated L-CAP was loaded onto a Matrex Blue A column (0.95x21.2cm) which had been equiribrated with 5mM tris acetate (pH7.5) buffer containing 1 mM EDTA and 1 mM NaN3. The L-CAP was

Table 1. Summary of purification of L-CAP from porcine heart muscle.

Step	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Recovery (%)
Crude Extract	5800.0		35.4	0.00083 (1)	100
Ammonium Sulfate	758.0	-	143.8	0.001 (1.3)	(79)
DEAE-cellulose	508.0	-	35.3	0.0047(5.7)	(49)
Phenyl-sepharose	120.0	0.49	0.29	1.701(2048)	(34)
Matrex Blue A	19.6	1.49	0.115	12.96(15614)	(17)

eluted with the same buffer (Fig.3). It seems that the enzyme has weak affinity to the Matrex Blue A at this condition. The L-CAP was eluted after almost of the proteins had been eluted. The active fractions were collected as purified enzyme for further investigation.

Table 1 shows the results of the purification. Because the L-CAP activity was not found until Phenyl-Sepharose step, it is difficult to calculate the recovery and the purification fold. However, we estimate these figures using the parameter of 70% of the recovery for each step of the purification. Through the purification, as estimated like this, the L-CAP was purified about 15600 fold with 17% recovery.

Polyacrylamide disc gel electrophoresis: The purified L-CAP was run on polyacrylamide disc gel electrophoresis. After the electrophoresis, one of the gels was stained with Coomassie Briliant Blue R-250; the other was cut into pieces of 2.5 mm long from top of the gel. These pieces were added casein substrate solution and $CaCl_2$, and assayed its CAP activity as usual. (Fig.4). There are two bands on the gel of purified enzyme, one major band corresponds to the activity. As judged from the densitometer pattern, the enzyme is 90 % pure. To compare the molecular weight of the L-CAP with the H-CAP, two enzymes were subjected to electrophoresis on Na-dodecyl sulfate polyacrylamide slab gel (Fig.5). The large subunit of the L-CAP was obviously

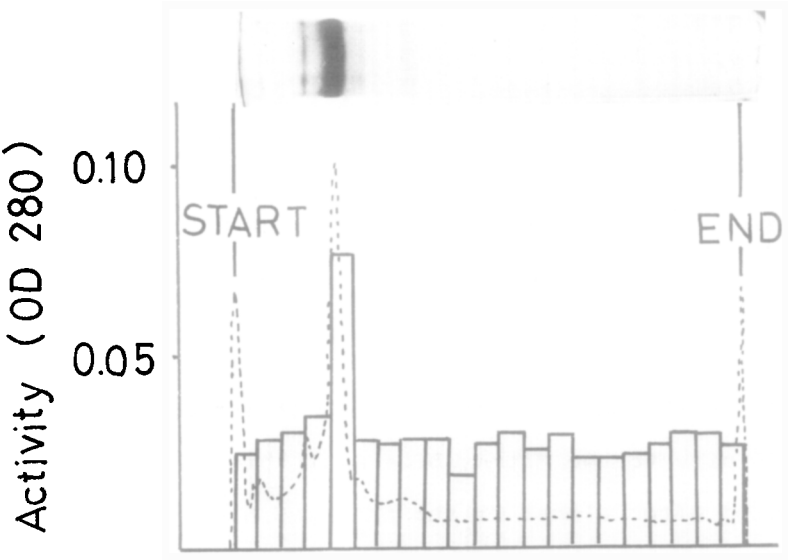

Fig.4. Assay of the enzyme activity on a polyacryl amide disc gel electrophoresis gel. 20 microgram of the purified enzyme was run on polyacryl amide disc gel electrophoresis. After the run, one of the gels was stained with Coomasie Briliant Blue, and the other was sliced sidewards into 2.5 mm pieces. The acivities of these pieces was assayed: ····· densitometer scaning of the stained gel, ——— enzyme activity.

larger than the large subunit of the H-CAP. There was no difference in smaller subunit of both enzyme. For this reason, we concluded that the L-CAP is not the same as the auto-digested protease of the H-CAP. The molecular weight of these two subunits of the L-CAP were estimated 90,000 and 30,000 using myofibrils as standard proteins.

The character of the L-CAP: There were no difference between the L-CAP and the H-CAP in optimum pH and optimum temperature, though the several characters were different. The calcium concentrations for half maximal activity were 50 μM for the L-CAP and 0.5 mM for the H-CAP as same as the results showed by many investigators (1,6,8). The L-CAP was activated not only by calcium but by manganese and by barium, on the other hand the H-CAP was not activated by those metal ions. Suzuki et al. (7) repoted that the auto-digested form of the H-CAP which was

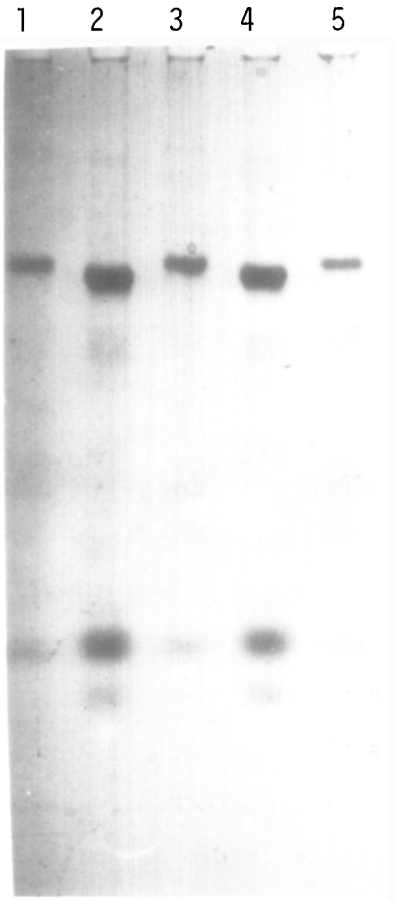

Fig.5. Difference of subunit molecular weight between L-CAP and H-CAP. Both of the enzymes were run on the same Na-dodecyl polyacrylamide slab gel and stained with Coomassie Brilliant Blue: 1,3,5 are the L-CAP, and 2,4 are the H-CAP.

activated by μM order of calcium was very unstable and became inactive during DEAE-cellulose chromatography. In contrast, the L-CAP which we described here was very stable. For example, the results of the enzyme stability at different pH showed that the L-CAP was more stable at pH 9.0 than the H-CAP (not shown in Fig.).

DISCUSSION

Many of proteases have been purified from muscle homogenate, however, the studies during last several years have shown that

many of these proteases are located in non muscle cells such as mast cells which are usualy contained in muscle homogenate (11, 12). It is now shown that cathepsins A,B,C,D,H,L, and lysosomal carboxypeptidase B and CAP exist inside of skeletal muscle cells (13,14,15). Therefore, the proteases which are responsible to protein turn over are those listed here. The cathepsins and lysosomal carboxypeptidase B are located in lysosme and are active at rather acidic pH than phisiological pH in cells. It is probable that the lysosomal enzymes can not hydrolyze the cytosol proteins until those proteins are engulfed into lysosome. For this reason, it is reasonable to say that the CAP is a key enzyme of intracellular protein degradation pathway, because only the CAP is coexist with intracellular proteins such as myofibrillar proteins and cytosol proteins.

Even though the CAP has been shown to have the ability of degrading those intracellular proteins, a major problem is the inactivity of the enzyme at physiological concentration of calcium. Therefore, the exsistence of the L-CAP is very important to understand the mechanism of intracellular protein degradation.

There are several reports which have purified the L-CAP , however, the purified protein in those reports was composed of two subunits of 80,000 and 30,000 (6,8,16). The difference between the subunit molecular weights of 90,000 and 30,000 which we described in this report and those molecular weights of 80,000 and 30,000 is unclear. The results on the auto-digestion of the H-CAP might suggest that the L-CAP be the auto-digested protease of the H-CAP. But, the results of the subunits molecular weights and the stability of the L-CAP clearly shows that the L-CAP is not the same as the auto-digested protease of the H-CAP.

The substrate specificity, especialy the effects on myofibrill proteins of the purifed L-CAP are very important for

disclosing intracellular protein degradation mechanism. Therefore, we are now investigating the action of the purified L-CAP on myofibrilar proteins.

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