Purification and Characterisation of Calpastatin, μ- and m-calpains from Beef*

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Introduction

The calcium-dependent proteolytic system is considered to be one of the major cytosolic proteolytic system. Calpains have been considered to participate in muscle protein turnover and post-mortem muscle tenderization (Golf *et al.,* 1992; Koohmaraie, 1992). Their activities are controlled by calcium, calpastatin, phospholipids, and activators (Murachi, 1983; Mellgren, 1987; Suzuki *et al.,* 1987). Calpastatin, usually coexisted with calpain in the same cellular area, is a specific endogenous competitive inhibitor for calpain (Maki *et al.,* 1987).

On the basis of calcium requirement, there are two types of calpains isolated from animal tissues, i.e., M-calpain (calpain I) and m-calpain (calpain II) which require 5-50 and 150-1000 EM Ca2+ for half-maximal activity, respectively (Imajoh-Ohmi *et al.,* 1988; Goll *et al.,* 1990). These two types of proteinases ubiquitously exist in terrestrial animals and insects (Pinter and Friedrish, 1988). However, M-calpain was not found in fish and shrimp muscle (Wang *et al.,* 1993). The tenderization of marine animal muscles occur much faster than that of terrestrial animal muscles (Suyama and Konossu, 1989). In the previous studies (Jiang *et al.,* 1991, Wang and Jiang, 1991, Wang *et al.,* 1993), the proterties of calpain from fish and shrimp had been compared. For the further study on the crossinteraction among those proteinases from marine and terrestrial animals, the A- and m-calpain from bovine muscle were purified and characterised.

Materials and Methods

The *longissimus* muscle from Shorthorn (2 yrs old, 200kg/carcass) was excised at approximately 2 hr postmortem and used for proteinase purification. The protein dye reagent concentrate was obtained from Bio-Rad Laboratories (Richmond, CA). Bovine casein, ethylenediaminetetraacetic acid (EDTA), pmercaptoethanol (~-Me), calcium chloride, iodoacetic acid (IAA) were obtained from E. Merck (Darmstadt, Germany). DEAE-Sepharose Fast Flow, Q-Sepharose HP and Sephacryl S-300HR were the products of Pharmacia (Uppsala, Sweden). Tris, p(chloromercuri)benzoate (PCMB), N-ethylmaleimide (NEM) N,N-bis(2-hydroxyethyl)glycine (Bicine), 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS), propane 1,3bis[tris-(hydroxymethyl)methylamino]-propane (Bis-tris propane), leupeptin, antipain, calpain inhibitor I, calpain inhibitor II, pepstatin A and y-globulin were purchased from Sigma (St. Louis, MO).

Purification of Calpastatin, μ- and m-calpains

Calpastatin, μ- and m-calpain were extracted from bovine muscle by 3 volumes of 50 mM Tris-HC1 buffer, pH 8.3, containing 10 mM ,B-Me and 5 mM EDTA. A Pharmacia Hiload and FPLC systems were used for all column chromatographies. Calpastatin, ,u- and m-calpain were first isolated by DEAE-Sepharose Fast Flow(Figure 1). The proteins were futher purified through a series of Sephacryl S-300 HR, and Q-Sepharose HP columns chromatography.

Assay of Calpain Activity

The activity of calpain was determined using casein as substrate. The reaction mixture consisted of 4 mg/mL of casein, 50 mM Tris- HC1 (pH 7.5), 10 mM p-Me, 1 mM NaN3, 5 mM CaC12 and appropriate amount of calpain. After 20 min incubation at 25 C, equal volume of 10% TCA was added to the reaction mixture and centrifuged at 3000 rpm for 15 min. The absorbance of supernatants at 278 nm were measured using a Hitachi U2000 spectrophotometer (Hitachi, Tokyo, Japan). One unit of calpain activity was defined as the amount of enzyme that caused an increase of one absorbance unit at 278 nm after 20 min incubation at 25 C and corrected by subtracting the activity of blank, which used 5 mM EDTA instead of 5 mM CaC12.

Assay of Calpastatin activity

Calpastatin activity was determined on the basis of its inhibitory capability on mcalpain. One unit of inhibitory activity was defined as the amount of calpastatin which could inhibit one unit of m-calpain.

Determination of Protein Concentration

Protein concentration was determined by the protein-dye binding method (Bradford, 1976) using crystalline y-globulin as a standard.

Determination of Molecular Weights (Mr)

The Mr of purified proteases was estimated by Superose 12 HR gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Superose 12 HR (1 x 30 cm) was equilibrated and eluted with 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 10 mM ,B-Me, and 1 mM NaN3 at flow rate of 0.5 mL/min. Ribonuclease A (13,700), chymotrypsinogen (25,000), ovalbumin (43,000), albumin (67,000), aldolase (158,000), and catalase (232,000) were used as protein markers.

Effect of Metal Ions

Calpains in 50 mM Tris-HC1 buffer, pH 7.5, containing 10 mM p-Me, 0.5 mM NaN3, and 4 mg/mL of casein were incubated with 5 mM of various metal ions. After 20 min incubation at 25 C, the activity was measured as described above.

Thermal Inactivation of Calpain

Enzyme samples in 20 mM Tris-HCl buffer, pH 7.5, containing 10 mM ,B-Me, 0.5 mM EDTA, and 0.5 mM NaN3, were incubated at various temperatures (from 0 to 80 C) for 10 min. After being cooled to 0 C for 5 min. the remaining activity of samples was measured as described above.

pH Stability

Enzyme samples in 50 mM Good's buffer (Bicine, CAPS, Bis-tris propane, and sodium acetate) at pH 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, and containing 10 mM ,BMe, 0.5 mM EDTA, and 0.5 mM NaN3 were incubated at 25 C. After 20 min incubation, the pH of the reaction mixture was adjusted to 7.5 using 100 mM Tris-HC1 bufer. The residual activity was then measured.

Results and Discussion

Determination of Molecular Weights (Mr)

The Mr of both purified proteases from bovine muscle was estimated to be 110 kDa, using Superose 12 HR gel filtration, while that of purified calpain was 75 kDa. Both pand m-calpain consisted of 80 kDa and 30 kDa subunits.This result was the same as other vertebrate tissues (Suzuki et al., 1987) fish muscle (Wang *et al.,* 1993).

Inhibitor Study

Among the inhibitors shown in Table 1, calpain inhibitor I and II are specific inhibitors for calpain, IAA, a halogenated alkylating agent, essentially irreversibly reacted with SH groups, while PCMB and NEM are a sulPhydryl group blocking agents. The p- and m-calpains were inhibited by calpain inhibitor I and II, leupeptin, antipain, IAA, and PCMB, but not affected by Pepstatin A and NEM. Since leupeptin and antipain had similar structure to calpain inhibitor I and II, according to the data obtained in this study, the inhibitory capability of IAA seemed to be greater than that of other blocking agents (Table 1). This phenomenon was similar to the result of Wang *et al.,* 1993. On the inhibitory capacity of NEM with IAA, the lower inhibitory capacity of NEM might be due to the occurrence of the reaction with,B-Me during measurement.

Calcium requirement

As shown in Figure 2, the purified proteases were sharply and positively cooperated with calcium. The purified proteases were half-maximally activated by 0.035 and 0.35 mM calcium ions, respectively. They were fully activated by 0.1 and 1 mM calcium ions, respectively. According to the calcium requirement for full activities and inhibitor study, the purified proteases might be, μ- and m-calpains, respectively. The calcium sensitivity of μ-calpain

was ten fold higher than that of m-calpain. m-Calpain from bovine muscle needed slight by higher Ca2+ than that from tilapia and about 7-fold lower Ca2+ than that from grass shrimp (Wang *et al.*, 1993).

Effect of Metal Ions

Since the calcium was essential for calpain activity, the effect of some other metals was also investigated. μ- and m-Calpains purified from bovine muscle were activated by Ca2+ and Sr2+ (Table 2). μ-Calpain was activated, while m-calpain was not affected by Ba2+. Other metals did not affect the activity of μ- and m-calpains. In the previous study(Wang *et al.*, 1 993), calpains from tilapia and grass shrimp muscle were activated by Ca2+, Sr2+ and Ba2+, but highly inhibited by Fez+, Fe3+, Ni2+, Cu2+, Zn2+, Cd2+ and Hg2+.

Effect of Temperature and pH

The optimal temperature for μ- and m-calpains were 25 and 30 C, respectively (Fig 3), while the pH optimum was 8.0 (Fig 4).

pH and Thermal Stability

Temperatures for 50% inactivation of the μ- and m-calpains were 55 and 70 C, respectively (Fig 5). The thermal stability of μ-calpain was higher than m-calpain. The mcalpain had very broad stable pH range around 5-9. The m-calpain was more stable than μ-calpain at acidic pH (Fig 6).

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Fig 1.

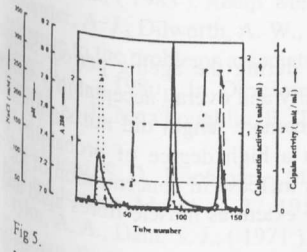


Fig 2.

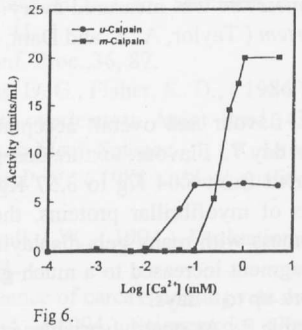
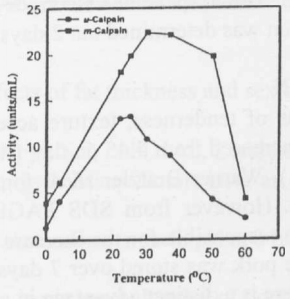


Fig 3.



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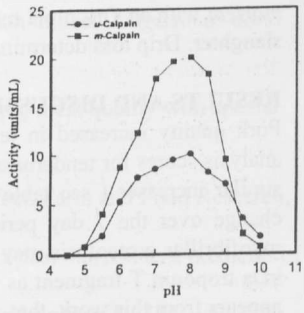

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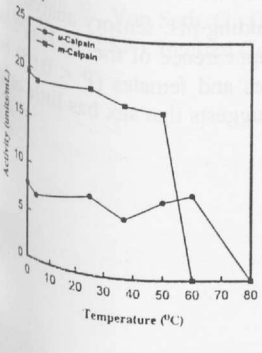

Fig 6.

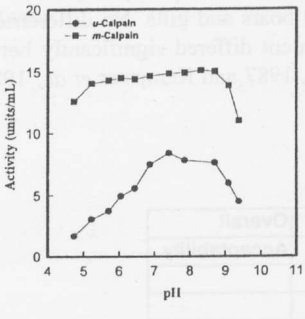

Table 1. Inhibitor effect on activities of *μ*- and *m*-calpains.

Inhibitors	Conc. (μM)	Activity μ-Calpain (units / mL)	Activity m-Calpain (units / mL)
None		9.40	22.80
Calpain inhibitor I	2	5.60	5.80
Calpain inhibitor II	2	0.00	0.00
Leupeptin	2	0.00	0.27
Antipain	2	2.80	4.53
Pepstatin A	2	10.33	22.00
Iodoacetic acid	1000	0.00	0.00
PCMB*	1000	3.13	9.73
NEM*	1000	9.66	22.46

*PCMB, *p*-(chloromercuri)benzoate; NEM, N-ethylmaleimide.

Table 2. Effect of metal ions on the activities of μ- and *m*-calpains.

Metal* (5 mM)	Activity μ-Calpain (units / mL)	Activity m-Calpain (units / mL)
$LiCl_2$	0.27	0.00
NaCl	0.53	0.00
KCl	0.00	0.00
NH_4Cl	0.00	0.00
$MgCl_2$	0.00	0.00
$CaCl_2$	8.27	19.07
$SrCl_2$	8.19	10.93
$BaCl_2$	7.86	0.00
$FeCl_2$	0.53	0.73
$FeCl_3$	0.00	0.00
$CoCl_2$	0.00	0.00
$NiCl_2$	0.00	0.00
$ZnCl_2$	0.00	0.00
$CdCl_2$	0.00	0.00
$CuCl_2$	0.20	0.00
$HgCl_2$	0.00	0.00
$AgCl_2$	0.00	0.00

*. The concentration of all metals was 5.0 mM.

Fig 1. Chromatography profile of calpains and calpastatin from bovine muscle on DEAE-Sepharose Fast Flow.

Fig 2. Calcium requirement for *μ*- and *m*-calpains.(Proteinases in 50 mM imidazole-HCl buffer, pH 7.5, were incubated with various concentrations of calcium chloride at 25°C for 20 min.)

Fig 3. Effect of temperature on the activities of μ- and *m*-calpains.(Proteinases in 50 mM imidazole-HCl buffer, pH 7.5, were incubated with casein at various temperatures for 20 min.)

Fig 4. Effect of pH on the activities of μ- and *m*-calpains.(Proteinases in 0.1 M Goods buffer with various pHs were incubated with casein at 25°C for 20 min.)

Fig 5. Thermal inactivation of calpains.(Proteinases in 20 mM Tris-HCl buffer, pH 7.5, were incubated at various temperatures for 10 min. After cooling to 0°C, the activity was measured.)

Fig 6. pH stability of calpains.(Proteinases in 50 mM Goods buffer with various pH were incubated at 25°C for 20 min, the activity was then measured.)