Purification and Some Properties of Calpain II from Tilapia Muscle (*Tilapia nilotica* × *Tilapia aurea*)[†]

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Calpain II (EC 3.4.22.17), calcium-activated neutral proteinase, from tilapia muscle (*Tilapia nilotica* × *Tilapia aurea*) was purified by using hydrophobic chromatography with phenyl-Sepharose CL-4B and substrate affinity chromatography with α-casein agarose. Electrophoretic analysis and inhibitor specificity studies indicated that the calpain II was a monomer and purified to homogeneity. The molecular weight was electrophoretically determined to be 80 000. The requirement of Ca^{2+} concentration was 0.1 mM for half-maximal and 1 mM for maximum activation. This enzyme was fully activated by 10 mM 2-mercaptoethanol. The inhibitory ability of calpain inhibitor I was the highest, followed by leupeptin, calpain inhibitor II, antipain, iodoacetate, PCMB, and PMSF in decreasing order. Accordingly, the active site of this proteinase might contain cysteine and histidine for catalysis. The caseinolytic activity of this purified calpain was 480 units/mg at pH 7.5. The recovery was 35%.

INTRODUCTION

The intracellular regulatory functions of calcium ions have been widely investigated, since, except for calmodulin and Ca^{2+}-dependent phospholipid-activated protein kinase (C kinase), the Ca^{2+} proteases seem to play a wide variety of functions via calcium ions; these functions include muscle protein turnover (Reddy et al., 1975; Kameyama and Etlinger, 1979), proteolysis of the components of neurofilament and microtubules and other cytoskeletal proteins (Gilbert and Newby, 1975; Zimmerman and Schlaepher, 1982; Davies, et al., 1978; Klein et al., 1981; Nelson and Traub, 1982), transformation of receptors (Puca et al., 1977; Cassel and Glaser, 1982), and activation of various enzymes (Huston and Krebs, 1968; Drummond and Duncan, 1966, 1968; Inoue et al., 1977). According to Kishimoto et al. (1981), there are two classes of Ca^{2+} proteases: one with low and the other with high Ca^{2+} requirement. There are also endogenous inhibitor (Waxman and Krebs, 1978; Nishiura et al., 1978) and activator proteins (DeMartino and Blumenthal, 1982). All these proteins have been found mainly in the cytosolic fraction. These Ca^{2+}-dependent cysteine proteinases and inhibitor protein are ubiquitously distributed in mammalian and avian tissues. Generally, the term calpain I is for the enzyme with low Ca^{2+} requirement, calpain II that for the enzyme with high Ca^{2+} requirement, and calpastatin that for the inhibitor protein. Chronologically, calpain II was discovered in brain and skeletal muscle much earlier than calpain I (Guroff, 1964; Meyer et al., 1964; Kishimoto et al., 1981).

Recently, many studies have been performed to determine the mechanism of tenderization of muscle (Koohmaraie et al., 1986, 1988a,b). Although the tenderization is considered to be caused by the disappearance of Z-disks, dissociation of actomyosin complex, destruction of the connectin, and denaturation of collagen (Robbins et al., 1979; Hatori, 1986; Suyama and Konosu, 1987; Koohmaraie et al., 1986, 1988a), the mechanism of post-mortem tenderization is still unclear (Koohmaraie et al., 1986, 1988a,b). Many biological changes during post-mortem tenderness have generally been assumed to arise from the release of endogenous muscle proteases which are active at the post-mortem pH (Robbins et al., 1979). The proteases indigenous to skeletal muscle possibly include the Ca-dependent proteases and cathepsins (Asghar and Henrickson, 1982; Koohmaraie et al., 1988a,b). Many researchers recently reported that lysosomal enzymes were not involved in the tenderization process of bovine and lamb muscle, but the Ca-dependent proteases were (Furuno and Goldberg, 1986; Goodman, 1987; Koohmaraie et al., 1988a,b; Lowell et al., 1986). However, according to the previous study (Jiang et al., 1990), the pepstatin-sensitive proteases contributed to the degradation of post-mortem myofibrils at pH 5.5 and 6.0. This study was one of the author's endeavors to clarify the mechanism of tenderization of fish muscle and aimed to purify and characterize the calpains from tilapia muscle.

MATERIALS AND METHODS

Materials. Tilapia (*Tilapia nilotica* × *T. aurea*, 300–400 g/fish), purchased from an aquatic farm in southern Taiwan, were kept alive and transported to the laboratory in oxygenated water.

Biochemistry grade casein, iodoacetic acid, 2-mercaptoethanol, and calcium chloride were obtained from E. Merck, Darmstadt, Germany. Phenyl-Sepharose CL-4B (40 μmol of ligand/mL of gel), α-casein agarose (5–10 mg of α-casein/mL of gel), and leupeptin were purchased from Sigma, St. Louis, MO. Calpain inhibitors I and II were obtained from Serva. The protein standards for electrophoretical molecular weight determination were the products of Pharmacia, Uppsala, Sweden. The protein-dye binding reagent was obtained from Bio-Rad, Richmond, CA.

Buffers. The following buffers were used in the chromatographic procedures for the purification of the calpain from tilapia muscle: 50 mM Tris-HCl buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA), 20 mM 2-mercaptoethanol, pH 7.5 (homogenizing buffer); 20 mM Tris-HCl containing 1 mM ethylene glycol tetraacetic acid (EGTA), 20 mM 2-mercaptoethanol, and 1 mM NaN_3, pH 7.5 (buffer A); 20 mM Tris-HCl containing 0.1 mM $CaCl_2$, 20 mM 2-mercaptoethanol, and 1 mM NaN_3, pH 7.5 (buffer B).

Purification of Calpain. *Preparation of the Crude Extract.* Tilapia dorsal muscle (390 g) was homogenized with 3 volumes

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of homogenizing buffer and centrifuged at 13000*g* for 30 min at 0 °C (Hitachi Centrifuger; rotor RPR 12-2). To the supernatant was added NaCl to a final concentration of 0.5 M.

Phenyl-Sepharose CL-4B Column Chromatography. The crude extract was applied to a phenyl-Sepharose CL-4B column (2.6 × 30 cm) which was previously equilibrated with buffer A containing 0.5 M NaCl. The column was washed with 20 bed volumes of equilibrating buffer to eliminate the unabsorbed proteins and was then eluted with buffer A. The fraction with caseinolytic activity was then rechromatographed on a phenyl-Sepharose CL-4B column (1.6 × 30 cm) with a linear gradient of 0.5–0.0 M NaCl in buffer A and then 0–50% ethylene glycol in buffer A. The flow rate was 12 mL/h. Fractions of 5 mL were collected.

α-Casein Agarose Column Chromatography. The final concentrations of $CaCl_2$ and leupeptin of the enzyme solution eluted from the phenyl-Sepharose column were made 8 and 20 mM, respectively. The resultant enzyme solution was then loaded onto the α-casein agarose column (1 × 4 cm) which was previously equilibrated by buffer B with 20 μM leupeptin. The column was washed with 10 bed volumes of buffer B with 20 μM leupeptin and repeated with 10 bed volumes of buffer B. The bound enzyme was then eluted by buffer B with 5 mM EGTA in place of $CaCl_2$. The flow rate was 10 mL/h. Fractions of 1.5 mL were collected.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). SDS–PAGE was performed on a 0.75 mm thick slab gel containing isotropic 10% acrylamide with a stacking gel of 3.75% acrylamide in the Laemmli buffer system (Laemmli and Favre, 1973). Silver staining and Coomassie blue staining were performed according to the procedures Rabilloud et al. (1988) and Neuhoff et al. (1988), respectively.

Determination of Molecular Weights. The molecular weight of the purified enzyme was determined by using SDS–PAGE. Phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean inhibitor (20 000), and α-lactalbumin (14 400) were used as standards.

Determination of Protein Concentration. Protein concentration was determined by the protein–dye binding method (Bradford, 1976) using crystalline bovine serum albumin as a standard.

Assay of Calpain Activity. Calpain activity was determined by using casein as substrate. Each reaction mixture having a final volume of 1.0 mL contained 4 mg of casein, 50 mM imidazole hydrochloride buffer, pH 7.5, 10 mM 2-mercaptoethanol, 0.5 mM NaN_3, 0.05 M NaCl, and 5 mM $CaCl_2$. After an incubation at 25 °C for 60 min, the reaction was terminated by adding 0.5 mL of 10% chilled trichloroacetic acid (TCA). The content of TCA-soluble digestive products was determined by using the dye-binding method of Bradford (1976), for which 0.2 mL of the dye reagent concentrate was added to 0.8 mL of the TCA-soluble solution. After 5 min of incubation at 25 °C, the absorbance at 595 nm was read against the blank. One unit of calpain activity was defined as the amount of enzyme that caused an increase of 1 absorbance unit at 595 nm after 60 min of incubation at 25 °C and corrected by subtracting the activity of blank, which was in the presence of 5 mM EDTA.

RESULTS AND DISCUSSION

Purification of Calpain II. *Chromatography on Phenyl-Sepharose CL-4B.* In the preliminary studies, the recovery of enzyme activity was very low when the crude extract was applied to the phenyl-Sepharose column in the presence of 1 mM $CaCl_2$ and 20 μM leupeptin. This low recovery might be due to the rapid autolysis of calpain by Ca^{2+} when the calpain bound to phenyl-Sepharose. It might be also due to the calpain bound by calpastatin in the presence of Ca^{2+} and consequently lower binding ability of calpain to phenyl-Sepharose (Gopalakrishna and Head, 1985). Since the electrostatic potential surface of proteinase shrank in high ionic strength solution (Weber et al., 1989), NaCl (0.5 M) was added to crude extracts to increase the hydrophobic interaction between enzymes and the phenyl group of resins. In addition, calcium chloride can also induce conformational changes and, consequently, increase the hydrophobic surface regions of calpains, similar to other Ca^{2+}-binding proteins including calmodulin, S-100, and troponin C (Gopalakrishna and Barsky, 1985). This phenomenon enables the proteinases to bind to phenyl-Sepharose at low salt concentration. As indicated in Figure 1, the caseinolytic activity was concentrated on the protein peak; a 3.53-fold purification was achieved at this step (Table I). Fractions containing caseinolytic activity were collected and applied to an α-casein agarose column.

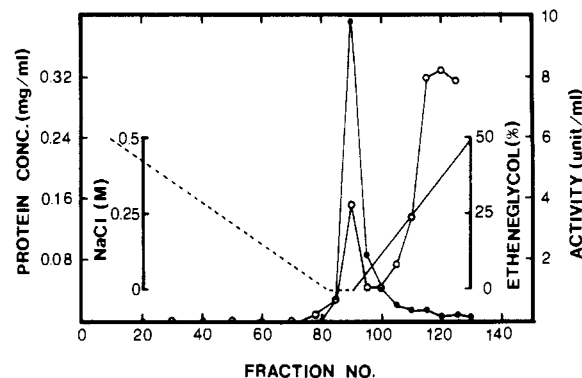

Figure 1. Column chromatography of calpain II on phenyl-Sepharose CL-4B [column: 1.6 × 30 cm; washed with 20 mM Tris-HCl containing 1 mM ethylene glycol tetraacetic acid (EGTA), 20 mM 2-mercaptoethanol, and 1 mM NaN_3, pH 7.5 (buffer A); eluted with a linear gradient from 0.5 to 0.0 M NaCl in buffer A and then from 0 to 50% of ethylene glycol in buffer A; flow rate; 12 mL/h; collection, 5 mL/tube]. (○) Protein concentration (mg/mL); (●) caseinolytic activity (units/mL).

Table I. Summary of the Purification of Tilapia Calpain II

procedure	total protein, mg	total act. unit	sp act.,[a] unit/mg	recovery, %	purifn, *x*-fold
phenyl-Sepharose CL-4B (1st)	31.20	1170.00[b]	37.50	100.00	1.00
phenyl-Sepharose CL-4B (2nd)	4.06	536.98	132.26	45.90	3.53
α-casein agarose	0.85	408.15	480.18	34.88	12.80

[a] One unit of calpain activity was defined as 1 mg of enzyme that increased the absorbance at 595 nm by 1.0 after 60 min of incubation at 25 °C, pH 7.5 (using casein as substrate). [b] Calpain activity in crude extract was only an apparent value, since the calpain coexisted with ihhibitor(s).

Chromatography on a Casein Agarose Column. Autolysis of calpain rapidly occurred in the presence of Ca^{2+}. This phenomenon was inhibited by the addition of leupeptin (Hathaway et al., 1982; Mellegren et al., 1982). Since the calpain is a Ca-activated proteinase, calcium chloride (8 mM) and leupeptin (20 mM) were added to the collected fraction (fraction 85–95) which had caseinolytic activity obtained from phenyl-Sepharose CL-4B column chromatography. The resultant solution was rechromatographed on α-casein agarose. A very sharp caseinolytic peak was obtained (Figure 2). At this step, calpain was analyzed with SDS–polyacrylamide gel electrophoresis and demonstrated to be of electrophoretical homogeneity (Figure 3). There was a small amount of 75-kDa protein band in the silver-stained gel but not in the Coomassie blue gel. This minor band might be the component resulted from the autolysis of calpain during purification. The presence of leupeptin in buffer B during purification did not affect the binding ability of the enzyme to α-casein agarose. This suggested that the leupeptin did not bind competitively to the binding site of substrate but might interact allosterically with calpain to inhibit the autolysis.

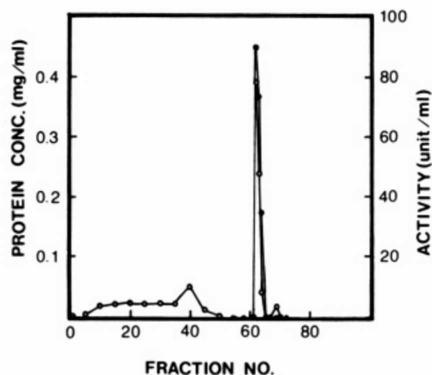

Figure 2. Column chromatography of calpain II on α-casein agarose [column: 1 × 4 cm; washed with 10 bed volumes of buffer B (20 mM Tris-HCl containing 0.1 mM $CaCl_2$, 20 mM 2-mercaptoethanol, and 1 mM NaN_3, pH 7.5) with 20 μM leupeptin and repeated with 10 bed volumes of buffer B; eluted with 20 mM Tris-HCl containing 5.0 mM EGTA, 20 mM 2-mercaptoethanol, and 1 mM NaN_3, pH 7.5; flow rate, 10 mL/h; collection, 1.5 mL/tube]. (○) Protein concentration (mg/mL); (●) caseinolytic activity (units/mL).

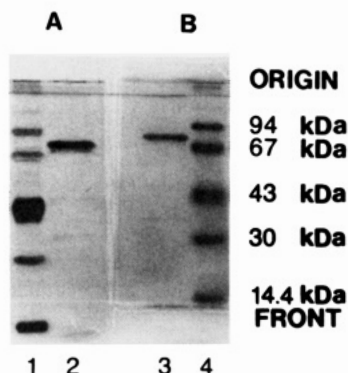

Figure 3. Disc gel electrophoretic pattern of the purified calpain II from tilapia muscle (SDS-PAGE was performed on a 0.75 mm thick slab gel containing isotropic 10% acrylamide with a stacking gel of 3.75% acrylamide). (A) Silver stained; (B) Coomassie blue stained.

The purification of calpain II (this proteinase is characterized as calpain II in the following section) from tilapia muscle is summarized in Table I. No calpain activity was detected in the crude extract. This might be due to the existence of calpastatin, which is a specific inhibitor for calpains (Toyohara et al., 1983). Therefore, the purification x-fold and yield were calculated on the basis of the value after the phenyl-Sepharose CL-4B chromatography. The final enzyme preparation was purified 12.8-fold over the phenyl-Sepharose CL-4B step with a total recovery of 35%.

Properties of Calpain II. *Determination of Molecular Weight.* According to SDS-PAGE (Figure 4), the purified calpain II was a monomer (data not shown) with a molecular weight of 80 000, similar to that from carp (Toyohara et al., 1985).

Calcium Requirement. The purified calpain was half-maximally activated by 0.1 mM and fully activated by 1 mM calcium ions. Since the requirement of calcium concentration was at millimolar level, this enzyme was considered to be calpain II (Kishimoto et al., 1981). From the sigmoid curve of calcium requirement, this kind of enzyme sharply and positively cooperated with calcium (Figure 5).

Inhibitor Studies. The concentrations of calpain inhibitors I and II, leupeptin, antipain, iodoacetate, PCMB, and PMSF for inactivating 50% of the enzyme activity at

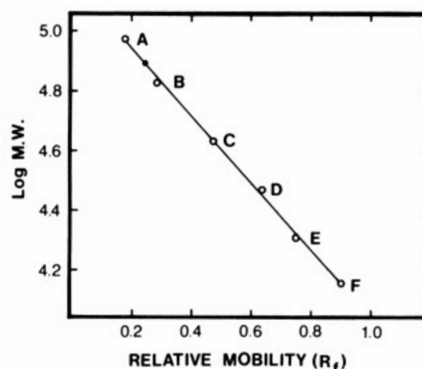

Figure 4. Calibration curve for the determination of molecular weight (using SDS-PAGE on 10% polyacrylamide gel). (A) Phosphorylase *b*, 94 000; (B) bovine serum albumin, 67 000; (C) ovalbumin, 43 000; (D) carbonic anhydrase, 30 000; (E) soybean inhibitor, 20 000; (F) α-lactalbumin, 14 400; (●) calpain II.

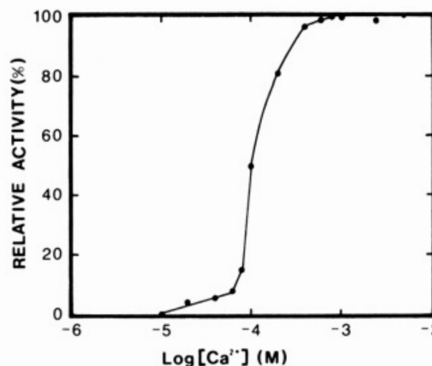

Figure 5. Calcium requirement of calpain II from tilapia muscle (substrate, casein; reaction mixture, casein 50 mM imidazole hydrochloride buffer, pH 7.5, 10 mM 2-mercaptoethanol, 0.5 mM NaN_3, 0.05 M NaCl, and various concentrations of $CaCl_2$; incubation at 25 °C for 60 min). The TCA-soluble digestive products were determined by using the dye-binding method; the relative activity was expressed as the percent ratio of the activity with various $CaCl_2$ concentrations to the maximal activity.

Table II. Effect of Various Inhibitors on Calpain II Activity

inhibitor	$I_{1/2}$[a]	inhibitor	$I_{1/2}$[a]
calpain inhibitor I	0.29 μM	iodoacetate	23.23 μM
calpain inhibitor II	1.45 μM	*p*-(chloromercuri)benzoate	1.69 mM
leupeptin	0.64 μM	phenylmethanesulfonyl fluoride	2.43 mM
antipain	2.81 μM		

[a] Concentration for inhibiting 50% activity of calpain II at 25 °C for 60 min.

25 °C for 60 min were 0.29, 1.45, 0.64, 2.81, 23.23, 1690, and 2430 μM, respectively (Table II). Among these inhibitors, calpain inhibitors I and II are specific inhibitors for calpain, iodoacetate is a halogenated alkylating agent that is essentially and irreversibly reacted with SH groups, PCMB, has high affinity for SH groups and can rapidly react with them, and PMSF sulfonylates the proteins exclusively at the active site of histidine.

From the inhibitor studies and calcium requirement, the purified proteinase was identified to be calpain II (high Ca^{2+} requiring form of calpain), according to the definition by Murachi et al. (1981). The active site of this enzyme might contain cysteine and histidine.

Effect of Reducing Agent. The reducing agent, 2-mercaptoethanol, revealed marked activating effect on the activity of this enzyme (Table III). Malik et al. (1983) observed that the rate of loss of calpain activity depended on the concentration of dithiothreitol added; the calpain activity was maintained for at least 1 year at 4 °C in the

Table III. Effect of 2-Mercaptoethanol on the Calpain II Activity

concn, mM	rel act.,[a] %	concn, mM	rel act.,[a] %
0.2	31.2	8.2	98.2
1.2	57.0	10.2	100.0
2.2	76.7	12.2	100.0
4.2	89.2		

[a] The relative activity was expressed as the percent ratio of the caseinolytic activity of calpain II with various concentrations of 2-mercaptoethanol to the maximum activity.

Table IV. Comparison of the Calcium Requirement for Half-Maximal Activity and Molecular Weight of Calpain from Tilapia with Those from Mammalian and Other Fish Species

species	calpain	MW	$A_{1/2}$,[a] mM	[Ca^{2+}], μg/g of tissue	ref[b]
tilapia skeletal muscle	II	80	0.1	36.7	
carp skeletal muscle	II	80	0.6–1.0		1, 2
rabbit skeletal muscle	II	73 + 30	1.2	48.4[c]	3
rabbit skeletal muscle	I	80 + 28	0.03	48.4[c]	4
	II	80 + 28	0.8		
rabbit skeletal muscle	I	76 + 28	0.05		5
procine skeletal muscle	I	80 + 30	0.045	41.4[c]	6
	II	80 + 30	0.74		
chicken skeletal muscle	II	81, 82	0.15, 0.26	38.9[c]	7, 8

[a] Calcium requirement for half-maximal activation of calpains. [b] 1, Toyohara et al. (1985); 2, Taneda et al. (1983); 3, Mellegren, et al. (1982); 4, Penny et al. (1985); 5, Inomata et al. (1983); 6, Dayton et al. (1981); 7, Suzuki and Ishiura (1983); 8, Kawashima et al. (1984). [c] Data were cited from Pearson and Young (1989).

presence of 10 mM 2-mercaptoethanol. This suggests that the formation of disulfide bonds plays an important role in the denaturation of the unique conformation which maintains the proper function of calpain II. This also suggests that calpain in the cytosol might exist in a reducing state (Darnell et al., 1986).

In this study, since the purification procedures did not involve dialysis and concentration procedures, the purification was very rapid and the recovery was high (Table I).

Generally, mammalian calpains are known to consist of two subunits, corresponding to 80 and 30 kDa (Murachi et al., 1981; Murachi, 1983). However, calpain II from tilapia muscle revealed a single band corresponding to 80 kDa in SDS–PAGE (Figure 3). This was almost the same as that from carp muscle (Toyohara et al., 1985). Calpain II from tilapia muscle required 1 mM Ca^{2+} for maximal activity, while that from carp muscle needed 3–5 mM Ca^{2+} for full activity (Murachi, 1983; Taneda et al., 1983). From a comparison of the calcium content of tilapia with that of mammalian muscles and other tissues, it seems likely that the higher the calcium content in the tissues, the higher the calcium requirement for maximal activity of calpains (Table IV). The requirement of the calcium was independent of the molecular weight of calpains.

Although calpain I, the low calcium requiring form, is known to be widely distributed together with calpain II in mammalian tissues, it was not detected in the tilapia muscle. According to Toyohara et al. (1985), there was only one type of calpain in the carp muscle. Currently, there is limited information regarding the existence of calpain I in fish muscle. Accordingly, further studies are necessary to clarify if calpain I exists in the fish muscle.

In summary, a calpain II was purified to homogeneity for the first time from tilapia muscle. The purification procedures were rapid and efficient, resulting in a high yield of the enzyme. The amount of this enzyme was apparently high in the tilapia muscle, because only a 12.8-fold purification made the enzyme homogeneous. This phenomenon suggests that this enzyme might play an important role in the post-mortem tenderization of fish muscle.

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