Comparison of the Properties of *m*-Calpain from Tilapia and Grass Shrimp Muscles†

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m-Calpains from tilapia and grass shrimp muscles were purified by Phenyl-Sepharose CL-4B, Sephacryl S-200, Q-Sepharose HP, and Superose 12 HR column chromatographies. The calpains from tilapia and grass shrimp muscles had the following characteristics: half-maximal activation, 0.23 and 2.4 mM of calcium; optimal temperature, 30 and 30 °C; optimal pH, 7.5 and 6.9; molecular weight: 110 000 and 160 000, respectively. *m*-Calpain obtained from tilapia muscle had two subunits of 80 000 and 28 000, while that from grass shrimp had two identical subunits of 80 000. Both proteases were activated by dithiothreitol, glutathione, and β-mercaptoethanol, inhibited by calpain inhibitor I, calpain inhibitor II, leupeptin, antipain, iodoacetic acid, and *p*-(chloromercuri)benzoate, but not affected by pepstatin A and *N*-ethylmaleimide. Both calpains were inhibited by Fe^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, or Hg^{2+} but activated by Ca^{2+}, Sr^{2+}, Ba^{2+}, or Mn^{2+}. The *m*-calpain from grass shrimp was activated, while that from tilapia was inhibited, by Na^+, K^+, or Mg^{2+} in the presence of 5 mM calcium. Co^{2+} activated the calpain from tilapia but inhibited that from grass shrimp. The thermal and frozen stability of calpain from tilapia was higher than that from grass shrimp. The pH stability of both calpains was almost the same.

INTRODUCTION

Calpains, intracellular cysteine endopeptidases, have been considered to participate in post-mortem muscle tenderization (Asghar and Bhatti, 1987; Asghar and Henrickson, 1982; Bird et al., 1980; Koohmaraie et al., 1988a,b). The activity of these enzymes is controlled by calcium, phospholipids, calpastatin, and activators (Mellgren, 1987; Suzuki et al., 1987; Murachi, 1983). On the basis of the calcium requirement, there are two types of calpains isolated from animal tissues, i.e., μ-calpain and *m*-calpain, which require 5–50 and 150–1000 μM Ca^{2+} for half-maximal activity, respectively (Imajoh-Ohmi et al., 1988; Goll et al., 1990). These two types ubiquitously exist in terrestrial animals and insects (Pinter and Friedrish, 1988) but not in plants (Goll et al., 1990). Only *m*-calpain is contained in carp muscle, eggs, and erythrocytes (Taneda et al., 1983; Toyohara et al., 1985) tilapia muscle (Jiang et al., 1991), lobster claw, and abdominal muscles (Mykles and Skinner, 1986).

Most μ- and *m*-calpains from vertebrate tissues have a distinct catalytic 80 000 subunit and an identical regulatory 30 000 subunit (Suzuki et al., 1987). However, there are two calpains with molecular weight (M_r) of 280 000 purified from *Drosophila* (Pinter and Friedrich, 1988) and four calpains with M_r of 310 000, 195 000, 125 000, and 59 000 from lobster (Mykles and Skinner, 1986).

Calpain from crustacean muscle could completely hydrolyze myofibrillar proteins, but that from vertebrate muscle could only selectively cleave myofibrillar proteins into fragments (Ishiura et al., 1982; Hara et al., 1983; Mykles and Skinner, 1982). Furthermore, calpain obtained from crustacean muscle appeared to be more stable than that from vertebrate muscles in the presence of calcium (Mykles and Skinner, 1982; Suzuki et al., 1987).

Calpastatin, a specific endogenous competitive inhibitor for calpain, coexists with calpain in the same cellular area. It possesses three or four repetitive inhibitory domains (Maki et al., 1987). Each domain may bind one calpain in the presence of calcium, but the inhibitory activity of the repetitive domains is not identical. The autolysis of calpain cannot be completely inhibited by the excess amount of calpastatin (Nakamura et al., 1989). Unautolyzed μ-calpain requires more calcium for half-maximal binding ability to calpastatin than that for half-maximal activity. However, other forms of calpain need more calcium for the half-maximal activity (Kapprell and Goll, 1989). According to Nakamura et al. (1989), calpain could degrade calpastatin into 15 000 fragments which still have inhibitory activity. However, the inhibitory mechanism of calpain by calpastatin is still unclear.

The tenderization of fish and shellfish muscle occurs faster than that of terrestrial animals (Suyama and Konosu, 1987). Among fish and shellfish, shrimps were considered to have no post-mortem rigor mortis. The incidence and duration of the rigor mortis and the mechanism of tenderization on post rigor seem to be sophisticated and need to be clarified. Investigations on the difference of calpain properties between fish and shrimp, therefore, might provide some clues to understand the differences in the biochemical changes of post-mortem muscle among these species.

MATERIALS AND METHODS

Materials. Tilapia (*Tilapia nilotica* × *T. aurea*, 500–600 g/fish) and grass shrimp (*Penaeus monodon*, 15–25 g/shrimp) were purchased from a commercial aquatic farm in northern Taiwan and immediately transported to the laboratory in oxygenated water.

Biochemistry grade casein, iodoacetic acid (IAA), β-mercaptoethanol (β-Me), and calcium chloride were obtained from E.

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Merck (Darmstadt, Germany). Tris, *N,N*-bis(2-hydroxyethyl)-glycine (Bicine), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), propane 1,3-bis[tris(hydroxymethyl)methylamino]-propane (Bis-tris propane), reactive brown 10 agarose, and leupeptin were purchased from Sigma (St. Louis, MO). Calpain inhibitors I and II were bought from Serva, Germany. Phenyl-Sepharose CL-4B, DEAE-Sepharose FF, Q-Sepharose HP, Superose 12 HR, standard protein kits for electrophoretical analysis, and gel filtration were the products of Pharmacia, Uppsala, Sweden. The protein–dye binding reagent was obtained from Bio-Rad (Richmond, CA).

Purification of Calpain. Grass shrimp or tilapia dorsal muscles were homogenized with 6 or 3 volumes of 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM ethylenediaminetetraacetic acid (EDTA), 20 mM β-Me, and 1 mM NaN_3 and then centrifuged at 13000*g*, 0 °C, for 30 min. NaCl was added to the supernatant and to adjust the final concentration of NaCl to 0.5 M (Jiang et al., 1991).

A Pharmacia Hiload system or FPLC system was used for all column chromatographies. The crude extract was purified on a Phenyl-Sepharose CL-4B column (5 × 20 cm) which was previously equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 20 mM β-Me, 1 mM NaN_3) containing 0.5 M NaCl. The column was then washed with 20 bed volumes of buffer A containing 0.5 M NaCl to eliminate the unabsorbed proteins (including calpastatin) and finally eluted with buffer A. The eluent flow rate was 120 mL/h, and 20-mL fractions were collected. Fractions with calpain activity were concentrated and then chromatographed on Sephacryl S-200 HR (2.6 × 90 cm) which was equilibrated with buffer B (20 mM imidazole–HCl, pH 6.5, 20 mM β-Me, 5 mM EDTA, 1 mM NaN_3). Samples were eluted with buffer B at a flow rate of 60 mL/h. Fractions of 10 mL were collected. The resulting samples were further purified using Q-Sepharose HP (2.6 × 10 cm) with a linear gradient of 0–0.5 M NaCl in buffer B. The flow rate was 60 mL/h. Fractions of 4 mL were collected.

Determination of Molecular Weights (M_r). The M_r values of the purified proteases were estimated by Superose 12 HR gel filtration and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Superose 12 HR (1 × 30 cm) was equilibrated and eluted with buffer A at a flow rate of 0.5 mL/min. Protein standards were ribonuclease A (13 700), chymotrypsinogen A (25 000), ovalbumin (43 000), albumin (67 000), aldolase (158 000), and catalase (232 000).

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 (Hames, 1990). Gels were stained by Coomassie Brilliant blue G-250 according to the procedure of Neuhoff et al. (1988). Protein standards were α-lactalbumin (14 400), soybean inhibitor (20 100), carbonic anhydrase (30 000), ovalbumin (43 000), bovine serum albumin (67 000), and phosphorylase *b* subunit (94 000).

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

Assay of Calpain Activity. Calpain activity was determined using casein as substrate. Each reaction mixture in a final volume of 1.0 mL contained 4 mg of casein, 50 mM imidazole–HCl buffer, pH 7.5, 10 mM β-Me, 0.5 mM NaN_3, 0.05 M NaCl, and 5 mM $CaCl_2$. After a 60-min incubation at 25 °C, the reaction was terminated by the addition of 0.5 mL of 10% chilled trichloroacetic acid (TCA; 4 °C). The content of TCA-soluble digestive products was measured using the dye-binding method of Bradford (1976); i.e., 0.2 mL of protein-dye binding reagent was added to 0.8 mL of TCA-soluble solution. After a 5-min 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 one absorbance unit at 595 nm after a 60-min incubation at 25 °C and corrected by subtracting the activity of a blank, which was in the presence of 5 mM EDTA.

Effects of Metal Ions on the Activity of Calpain. Calpains in 50 mM imidazole–HCl buffer, pH 7.5, containing 10 mM β-Me, 0.5 mM NaN_3, 0.05 M NaCl, and 4 mg/mL of casein with or without 5 mM $CaCl_2$ were incubated with 5 mM of various metal ions. After a 60-min incubation at 25 °C, the activity was measured as described above. The activity was expressed as a percent ratio of the enzyme activity of samples with various metals to that of sample with 5 mM calcium.

Effect of Reductants. Calpains in 50 mM imidazole–HCl buffer, pH 7.5, containing 1 mM β-Me, 0.5 mM NaN_3, 0.05 M NaCl, and 4 mg/mL of casein were incubated with 1 mM of DTT and glutathione (GSH) or 1, 5, and 20 mM of β-Me. After a 60-min incubation at 25 °C, the activity was measured as described above. The activity was expressed as a percent ratio of the enzyme activity of samples with various reductants to that of sample with 1 mM β-Me.

Thermal Inactivation of Calpain. Enzyme samples in buffer A were incubated at various temperatures (from 0 to 80 °C). At definite time intervals, samples were cooled in ice-water for 5 min to stop the reaction. The resulted mixtures were then placed on a water bath at 25 °C for 5 min. The activity was then determined. The rate constant (k_1) of the first-order reaction for thermal inactivation of calpain was calculated according to the equation $\ln(A_t/A_0) = -k_1$, where A_0 is the activity before incubation and A_t is the activity after t min of incubation) (Wang et al., 1992).

Effects of Frozen Storage on Calpain. Enzyme samples in buffer A were stored at −20 °C for 2 months. At definite time intervals (tilapia—0, 8, 16, 28, 43, and 49 days; grass shrimp—0, 1, 2, 4, and 12 days), samples were defrosted at 25 °C for 5 min. The activity was then determined.

Effects of pH on 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 10 containing 10 mM β-Me, 2.5 mM EDTA, and 0.5 mM NaN_3 were incubated at 25 °C. After a 30-min incubation, the pH of the reaction mixture was adjusted to 7.5 using 100 mM imidazole–HCl buffer. The residual activity was then measured.

RESULTS AND DISCUSSION

Determination of Molecular Weights (M_r). The M_r values of the purified proteases from tilapia and grass shrimp muscles were estimated to be 110 000 and 160 000, respectively, using Superose 12 HR gel filtration. According to the SDS–PAGE analysis (Figure 1), the protease obtained from tilapia appeared to have two subunits with M_r of 80 000 and 28 000, while that from shrimp muscle had a single subunit with M_r of 80 000. Accordingly, it was considered that both purified proteases were dimers, and that from shrimp muscle had two identical subunits of 80 000.

In the previous study (Jiang et al., 1991), the *m*-calpain with M_r of 80 000 purified from tilapia muscle was identified. However, the *m*-calpain with M_r of 110 000 was also purified from tilapia muscle in this study. The data, obtained from the previous study (Jiang et al., 1991) and the present study, indicated that the calpain from tilapia muscle purified by casein–agarose affinity chromatography seemed to lack the M_r 30 000 subunit, which is an important subunit for calpain from other species (Dayton et al., 1981; Hathaway et al., 1982; Mellgren et al., 1982). However, the calpain with M_r 80 000 purified in the previous study (Jiang et al., 1991) was similar to the other calpains reported by other researchers (Ishiura et al., 1978; Suzuki et al., 1981; Croall and DeMartino, 1983). The subunit composition of calpain was affected by the purification procedures (DeMartino and Croall, 1983). Autolysis of calpain rapidly occurred in the presence of calcium. In the purification, 20 μM leupeptin, which could inhibit the autolysis of protease, was added. Furthermore, casein can serve protective role in the autolysis. Thus, the subunits of calpain might be dissociated during chromatography on an α-casein–agarose column, and only the 80 000 subunit might be purified.

Calcium Requirement. The purified proteases from tilapia and grass shrimp were half-maximally activated by 0.23 and 2.4 mM calcium ions, respectively (Figure 2).

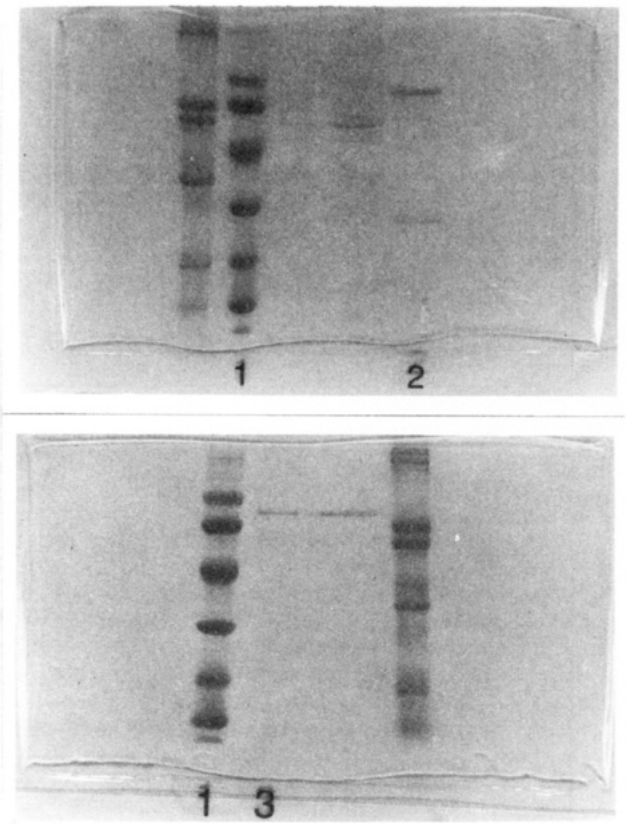

Figure 1. Disc SDS–PAGE of purified calpains from tilapia and grass shrimp muscles. (Lane 1) Protein standards; (lanes 2 and 3) purified calpain from tilapia and grass shrimp, respectively.

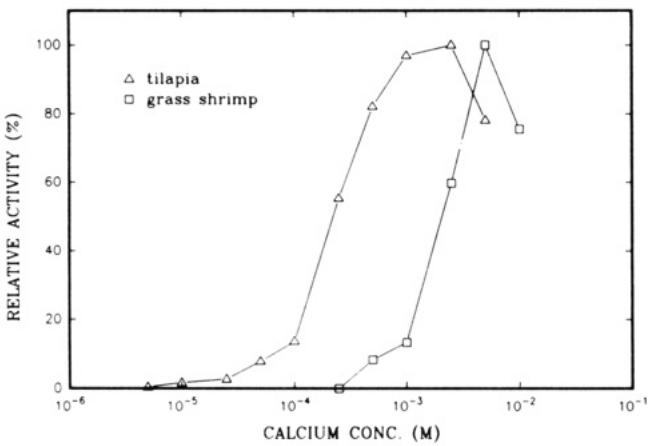

Figure 2. Calcium requirement of calpains from tilapia and grass shrimp muscles. The relative activity was expressed as percent ratio of the activity with various concentrations to that with maximal activity.

The optimal concentrations of calcium were 1–2.5 and 5 mM for tilapia and grass shrimp calpain, respectively. Since the requirement of calcium was at the millimolar level, these proteases were considered to be *m*-calpain (Kishimoto et al., 1981). From the curves of calcium requirement of both calpains, these enzymes sharply and positively cooperated with calcium (Figure 2). *m*-Calpain from grass shrimp muscle needed about 10-fold higher Ca^{2+} for activation than that from tilapia (Figure 2).

Inhibitor Study. As shown in Table I, the purified proteases from tilapia and grass shrimp were inhibited by calpain inhibitor I, calpain inhibitor II, leupeptin, antipain, IAA, and *p*-(chloromercuri)benzoate (PCMB). Pepstatin A and *N*-ethylmaleimide (NEM) did not affect either protease. The substrate specificities of *m*-calpain from tilapia and grass shrimp muscles were similar (Table I).

Table I. Effect of Various Inhibitors on Calpain Activity

inhibitor	concn, μM	rel activity, % tilapia	rel activity, % grass
none		100.0	100.0
calpain inhibitor I	2	10.0	5.3
calpain inhibitor II	2	6.8	32.7
leupeptin	2	6.8	0.0
antipain	2	12.7	7.9
pepstatin A	2	97.7	100.0
IAA[a]	1000	1.4	0.0
PCMB[b]	1000	58.4	44.2
NEM[c]	1000	94.4	100.0

[a] IAA, iodoacetic acid. [b] PCMB, *p*-(chloromercuri)benzoate. [c] NEM, *N*-ethylmaleimide.

Table II. Effect of Reductants on Calpain Activity

reductant	concn, mM	rel activity, % tilapia	rel activity, % grass
GSH[a] + β-Me[b]	1 + 1	124.6	113.3
DTT[c] + β-Me	1 + 1	112.3	166.7
β-Me	1	100.0	100.0
	5	208.8	169.3
	20	256.1	230.7

[a] GSH, glutathione. [b] β-Me; β-mercaptoethanol. [c] DTT, dithiothreitol.

Among these inhibitors, calpain inhibitors I and II are specific inhibitors for calpain and IAA is a halogenated alkylating agent that is essentially and irreversibly reacted with SH groups. PCMB and NEM are well-known to be sulfhydryl group blocking agents. They have high affinity for SH groups and can rapidly react with them. According to the data obtained in this study, the inhibitory capability of IAA seemed to be greater than that of other blocking agents (Table I). This phenomenon suggested that some functional groups neighboring SH groups on these proteases can either enhance or suppress the reactivity of SH groups with IAA (Means and Feeney, 1964). From a comparison of the inhibitory capacity of NEM with IAA, the lower inhibitory capacity of NEM might be due to the occurrence of the reaction with β-Me during measurement.

According to the M_r, calcium requirement, and inhibitor studies, both purified proteases were identified as *m*-calpain, according to the definition by Murachi et al. (1981). The active site of these proteases might contain cysteine.

Effects of Reducing Agents. Among the reductants used in this study, DTT, GSH, and β-Me revealed a marked activating effect on the activity of both purified proteases (Table II). Since the proteases were suspended in buffer A which contained 20 mM β-Me, the reaction mixtures contained at least 1 mM β-Me. Reductants were necessary for calpain activity and stability. According to the study by Malik et al. (1983), the decreasing rate of calpain activity depended on the concentration of DTT added. They also found that the calpain activity could be maintained for at least 1 year at 4 °C in the presence of 10 mM β-Me. From the reductant study and the observations by Malik et al. (1983), the formation of disulfide bonds might play an important role in the denaturation of the unique conformation which maintains the proper function of calpain. These data also suggest that the calpain in the cytosol might exist in a reducing state (Darnell et al., 1986).

Effect of Metal Ions on Calpain Activity. Since the calcium ion was essential for calpain activity, the effect of some other metal ions with and without the calcium ion was also investigated. All calpains were highly inhibited by Fe^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, or Hg^{2+} but activated by Ca^{2+}, Sr^{2+}, Ba^{2+}, or Mn^{2+} (Table III). The calpain

Table III. Effect of Metal Ions on Calpain Activity

	tilapia		grass	
metal ion[a] (5 mM)	A[b]	B[b]	A	B
Na^+	0.0[c]	65.4	0.0	102.6
K^+	0.0	65.4	1.5	110.8
Mg^{2+}	0.0	64.4	1.0	107.2
Ca^{2+}	100.0	86.0	100.0	78.5
Sr^{2+}	85.5	87.7	25.1	111.8
Ba^{2+}	50.3	73.2	17.9	132.3
Mn^{2+}	16.8	50.8	1.5	112.8
Fe^{2+}	0.0	21.8	0.0	10.3
Fe^{3+}	11.7	46.7	4.1	23.6
Co^{2+}	63.3	96.7	0.0	4.1
Ni^{2+}	1.6	23.0	0.0	15.4
Cu^{2+}	12.5	35.5	9.7	0.0
Zn^{2+}	0.0	0.0	0.0	0.0
Cd^{2+}	0.0	0.0	0.0	0.0
Hg^{2+}	0.0	0.0	0.0	0.0

[a] Chloride ion was the counterion of all metal ions. [b] A, without the addition of Ca^{2+}; B, with 5 mM Ca^{2+} added. [c] Values in this table are expressed as percentage ratio relative to that measured at 5 mM $CaCl_2$.

obtained from grass shrimp muscle was activated, while that from tilapia was inhibited, by Na^+, K^+, or Mg^{2+} in the presence of 5 mM calcium. Co^{2+} activated the tilapia calpain but inhibited the grass shrimp calpain (Table III). Hg^{2+} has been reported to bind to SH groups of the target enzyme and to subsequently inhibit the enzymatic activity (Klee, 1988). Inhibition of tilapia and shrimp calpain by IAA, PCMB (Table I), and Hg^{2+} (Table III) suggested that the active site of these calpains might contain cysteine. It is recognized that the catalytic groups of all calpains from different sources might contain cysteine and histidine and have an optimum pH around 7.5. This suggested that the pK_a values for the SH and imidazole groups were slightly different and the environments around these two residues were not similar.

According to the data obtained in this study, none of the metals used in this experiment activated calpain better than Ca^{2+} (Table III). In the absence of calcium, the activation ability of Sr^{2+}, Ba^{2+}, and Mn^{2+} was better on the calpain from tilapia than on that from grass shrimp (Table III). Comparing the calcium requirement, calpain from grass shrimp muscle needed 10-fold higher Ca^{2+} for activation than that from tilapia (Figure 2). Therefore, 5 mM Sr^{2+}, Ba^{2+}, or Mn^{2+} was not enough for the activation of calpain from grass shrimp muscle. Since shrimps have to undergo a sequential atrophy and restoration during each molting cycle, the calcium content in the shrimp muscle should be higher than that in tilapia muscle.

Effect of Temperature and pH on Calpain Activity. The optimal temperatures for both calpains were the same, 30 °C (Figure 3). As indicated in Figure 4, the optimal pH values for calpains of tilapia and grass shrimp were broad, and the maximal activity occurred at pH 7.5 and 6.9, respectively.

Effect of Temperature on Stability of Calpain. The first-order kinetics of thermal inactivation was calculated. The rate constants for thermal inactivation of tilapia *m*-calpain gradually increased with the increase of incubation temperatures. The Arrhenius plots for the thermal inactivation rate constants of this *m*-calpain are shown in Figure 5. The thermal stability of this *m*-calpain was higher than that from previously purified 80 000 calpain (Jiang et al., 1991). The activation energy for denaturing the 110 000 calpain was 88.7 kcal/mol. This suggested that the existence of 30 000 subunit in calpain increased the molecular stability. Temperatures for 50% inactivation of the calpains from grass shrimp and tilapia within

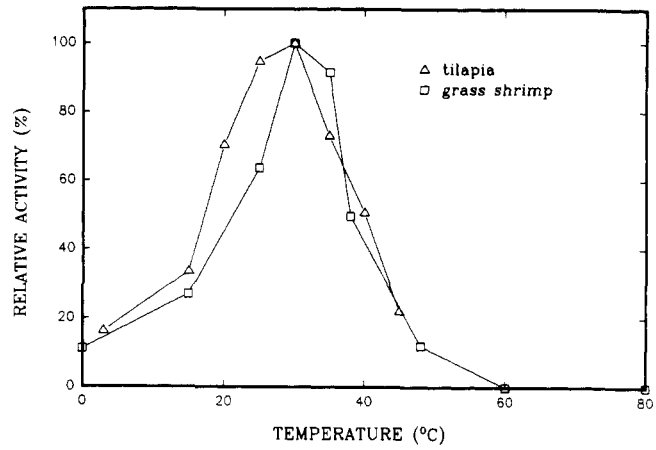

Figure 3. Effect of temperature on calpain activity. Proteinase in imidazole–HCl buffer, pH 7.5, was incubated with casein at various temperatures for 1 h.

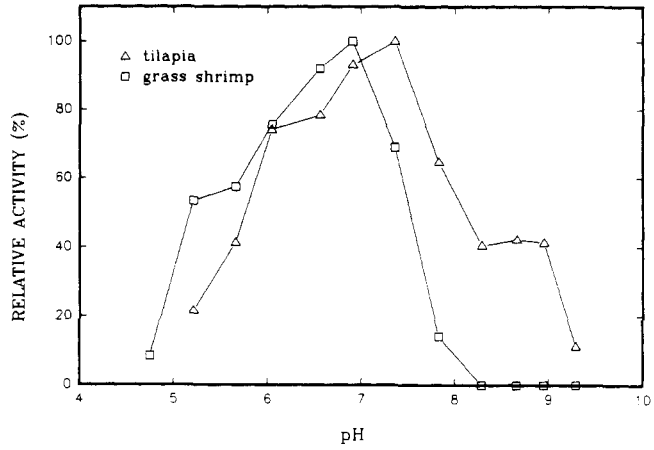

Figure 4. Effect of pH on calpain activity. Proteinase in Good's buffer with various pHs was incubated with casein at 25 °C for 1 h.

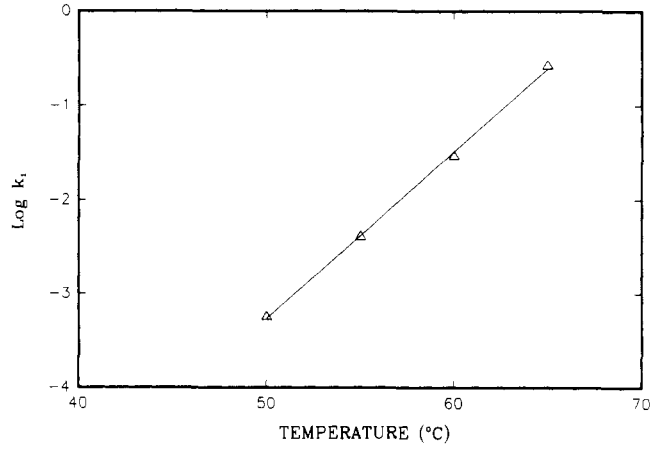

Figure 5. Arrhenius plots of the thermal inactivation rate constant of tilapia calpain.

10 min were 48.5 and 61 °C, respectively (Figure 6). Although both calpains appeared to have same optimal temperatures, the thermal stability of calpain from tilapia was higher than that from grass shrimp muscle. After 8 days of frozen storage at −20 °C, the activity of tilapia calpain had no change and was completely lost after 2 months of storage, while grass shrimp calpain had 30% activity remaining (Figure 7). The frozen denaturation of calpain from grass shrimp was greater than that from tilapia.

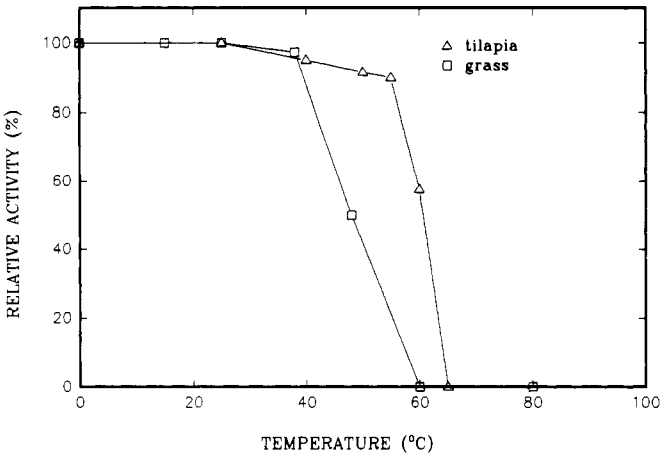

Figure 6. Thermal inactivation of calpain at various temperature. Proteinase in imidazole–HCl buffer, pH 7.5, was incubated at various temperatures for 10 min. After the proteinase had cooled to 0 °C, the activity was measured.

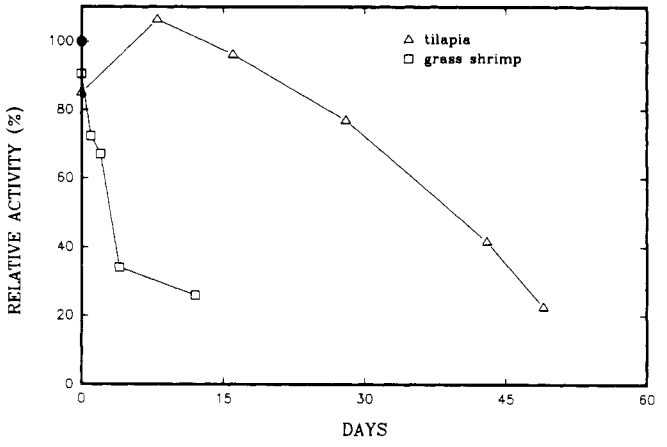

Figure 7. Stability of calpains obtained from tilapia and grass shrimp muscles at −20 °C.

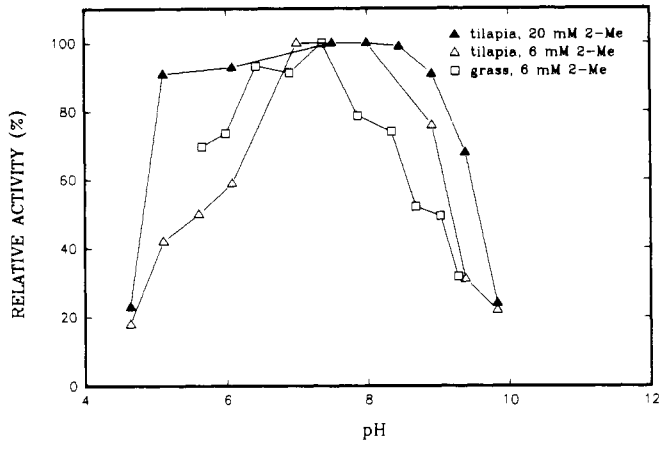

Figure 8. pH stability of calpain. Proteinase in Good's buffer with various pHs was incubated at 25 °C for 30 min, and then the activity was assayed.

Effect of pH on Stability of Calpain. The calpain of tilapia had a very broad stable pH range from 5.0 to 9.0 in the presence of 20 mM β-mercaptoethanol (Figure 8). Calpain from tilapia was stable at pH 6–9, while that from grass shrimp was more stable than that from tilapia at acidic pH but more labile than that from tilapia at alkaline pH. The pH stability curve was similar to the optimum pH curve (Figure 4). These data suggested that the conformation of the purified calpains was stable at pH 6–7.5 and less stable at alkaline than at acidic pH. The rapid decrease in stability at alkaline pH might be due to oxidation during incubation, since the oxidation rate of cysteine was very rapid at alkaline pH (Dawson et al., 1986).

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