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Stability of Calcium-Autolyzed Calpain II from Tilapia Muscle (*Tilapia nilotica* × *Tilapia aurea*)†

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The inactivation of calpain II (EC 3.4.22.17) from tilapia muscle (*Tilapia nilotica* × *Tilapia aurea*) was investigated. The thermal stability was enhanced by decreasing the water activity with the addition of sucrose at temperatures below 67.8 °C (isokinetic temperature). Addition of 30% sucrose caused the increase of the transition temperature from 61.9 to 63.8 °C and of the activation energy from 70.0 to 104.6 kcal/mol. The optimal pH for the stability was 7.5. This proteinase was less stable at alkaline pH than at acidic pH. The molecular weight of this proteinase was substantially autolyzed from 80 000 to 61 000 and 65 000 in the presence of calcium; the activation energy was calculated to be 26.61 kcal/mol by second-order rate constant.

INTRODUCTION

Many studies have been conducted for understanding the mechanism of tenderization of muscle (Koohmaraie et al., 1986, 1988a,b). Muscle tenderization is recognized to result from 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). However, the mechanism of post-mortem tenderization is still unclear (Koohmaraie et al., 1986, 1988a,b). Many biochemical changes during post-mortem tenderness of muscle have generally been assumed to arise from the release of endogenous muscle protease which are active at the post-mortem pH (Robbins et al., 1979). The proteases indigenous to skeletal muscle possibly include the calpains and cathepsins (Asghar and Henrickson, 1982; Koohmaraie et al., 1988a,b). Calpain is an intracellular cysteine proteinase the activity of which is controlled by calcium ions and calpastatin. In most tissues, two types of calpains are well characterized: calpain I, with high calcium sensitivity, and calpain II, with low calcium sensitivity (Imajoh-Ohmi et al., 1988). It has been suggested that this enzyme was involved in post-mortem proteolysis resulting in increased muscle tenderness (Goll et al., 1983; Asghar and Bhatti, 1987). Since the duration of rigor mortis of fish is much shorter than that of mammalian animals (Siebert, 1962), the resistance of myofibrillar proteins against protease hydrolysis as well as the stability of proteases might be the major factors affecting the duration of rigor. The stability of calpain might be highly related to the post-mortem biochemical changes of muscle, but there are few studies on calpain stability. In previous studies (Jiang et al., 1991), only the calpain II from tilapia muscle was purified. This study aimed to investigate the stability of this enzyme and consequently speculate the possibility of this proteinase's involvement in muscle tenderization.

MATERIALS AND METHODS

Materials. Tilapia (*Tilapia nilotica* × *Tilapia aurea*, 300–400 g/fish), purchased from a commercial aquatic farm were kept alive and immediately transported to laboratory in oxygenated water.

Biochemistry grade casein, 2-mercaptoethanol, and calcium chloride were obtained from E. Merck, Darmstadt, Germany. Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine], CAPS [3-(cyclohexylamino)-1-propanesulfonic acid], Bis-Tris propane [1,3-bis[tris(hydroxymethyl)methylamino]propane], and sodium acetate were purchased from Sigma, St. Louis, MO. The protein standards for electrophoretic molecular weight determination were the products of Pharmacia, Uppsala, Sweden. The protein–dye binding reagent was obtained from Bio-Rad, Richmond, CA.

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Purification of Calpain. The calpain II was purified according to the previous study (Jiang et al., 1991). The purification procedure involved elution of calpain bound to a casein affinity column in the presence of Ca^{2+}, this procedure often results in some autolysis and loss of a 30-kDa subunit of the calpain. Because the Ca^{2+} concentration required for half-maximal proteolytic activity of the calpain II used in the present study was only 100 μM, it seems likely that this calpain was autolyzed.

Thermal Inactivation of Calpain. The calpain II (0.1 mg/mL) was incubated in 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM ethyl glycol tetraacetic acid (EGTA), 20 mM 2-mercaptoethanol, and 1 mM NaN_3 (buffer A) and buffer A with 30% sucrose (buffer B) at various temperatures. At definite time intervals (10-min intervals for that incubated at 45 and 50 °C; 5-, 1-, and 0.5-min intervals for that incubated at 55, 60, and 65 °C, respectively), the enzyme solutions were cooled at 0 °C for 5 min to stop the reaction. The resultant mixtures were allowed to stand at 25 °C for 5 min, and then the activity was determined. The rate constant (k_1) of the first-order reaction for the thermal inactivation of this enzyme was calculated according to the equation $\ln (A_t/A_0) = -k_1 t$ (A_0 is the activity before incubation and A_t the activity after t min of incubation).

Effect of pH on the Stability. The calpain II (0.1 mg/mL) was incubated in 10 mM Good's buffer (Bicine, CAPS, Bis-Tris propane, and sodium acetate) with various pHs containing 10 mM 2-mercaptoethanol, 0.5 mM EGTA, and 0.5 mM NaN_3 at 25 °C. After 30 min of incubation, the pH of the reaction mixture was adjusted to 7.5 using 100 mM imidazole hydrochloride buffer, and then the calpain activity was measured.

The calpain II was incubated in 10 mM Good's buffer, pH 5.5, containing 10 mM 2-mercaptoethanol, 0.5 mM EGTA, and 0.5 mM NaN_3 at 25 °C. After 10, 20, 40, and 60 min of incubation, the pH of the reaction mixture was adjusted to 7.5 using 100 mM imidazole hydrochloride buffer, and then the calpain activity was measured.

Kinetic Study on the Autolysis of Calpain. The calpain II (0.1 mg/mL) was incubated in a 20 mM Tris-HCl buffer (pH 7.5) containing 20 mM 2-mercaptoethanol, 1 mM NaN_3, and 5 mM $CaCl_2$ at various temperatures. At definite time intervals, 10 μL of enzyme solutions was poured into 0.5 mL of chilled 100 mM imidazole hydrochloride buffer (pH 7.5) containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), 20 mM 2-mercaptoethanol, 1 mM NaN_3, 0.1 M NaCl, and 0.8% casein. After 5 min of incubation at 25 °C, the calpain activity was measured. The rate constants of the first (k_1) and second (k_2) order reactions for the autolytic inactivation of this enzyme were calculated.

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 (Hames, 1990). Silver and Coomassie blue staining were performed according to the methods of Rabilloud et al. (1988) and Neuhoff et al. (1988), respectively.

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

Assay of Calpain Activity. Calpain activity was determined 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, 50 mM NaCl, and 5 mM $CaCl_2$. After 60 min of incubation at 25 °C, the reaction was terminated by adding 0.5 mL of 10% chilled trichloroacetic acid (TCA). The TCA-soluble digestive products were determined using the dye-binding method (Bradford, 1976); i.e., 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 one absorbance unit after 60 min of incubation at 25 °C, corrected by subtracting the activity of blank (in the presence of 5 mM EDTA).

Calculation of Thermodynamic Parameters. The thermodynamic parameters were calculated according to Eyring's transition state theory (Dixon et al., 1979)

Table I. Rate Constant for Thermoinactivation of Calpain II at Various Temperatures

temp, °C	rate constant (1/min) × 10^3 A[a]	B[a]	temp, °C	rate constant (1/min) × 10^3 A[a]	B[a]
45	3.7	0.1	60	658	177
50	18	1	65	2188	1699
55	130	14			

[a] A, incubated in buffer A (20 mM Tris-HCl buffer, pH 7.5, 5 mM EGTA, 20 mM 2-mercaptoethanol, and 1 mM NaN_3). [b] B, incubated in buffer A containing 30% sucrose (buffer B).

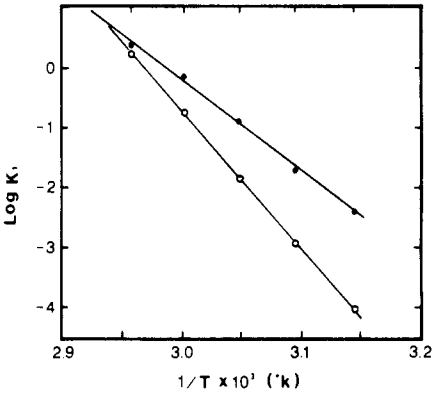

Figure 1. Arrhenius plots of the thermoinactivation rate constant of calpain II in buffers A (●) and B (○).

$$k = (K_B T/h)e^{-\Delta G/RT} \qquad \Delta G = \Delta H - T\Delta S$$
$$E_a = \Delta H + RT$$

where ΔG, ΔH, ΔS and E_a represent the free energy, enthalpy, entropy, and activation energy, respectively, for enzyme denaturation; K_B, h, T, and k are the Boltzmann constant (1.3087×10^{-23} J/K), Planck's constant (6.6262×10^{-34} JK/mol), absolute temperature (K), and inactivation rate constant, respectively.

RESULTS AND DISCUSSION

Thermal Inactivation. The thermal inactivation followed first-order kinetics as calculated. The rate constants for thermal inactivation of calpain II gradually increased with the increase of incubation temperatures (Table I). The half-life periods of this enzyme at 45, 50, 55, 60, and 65 °C were 94, 38, 5.3, 1.1, and 0.3 min in buffer A and 6931, 578, 49, 4, and 0.4 min in buffer B (calculated from Table I), respectively. The thermal stability of this calpain II was higher than that from rabbit skeletal muscle (Mellgren et al., 1982) but lower than that from carp muscle (Taneda et al., 1983). The Arrhenius plots for the thermal inactivation rate constants of calpain II in buffer A and buffer B intersected at isokinetic temperature (critical temperature, T_c) of 67.8 °C (Figure 1). This phenomenon indicated that addition of 30% sucrose substantially decreased the rate constant for thermal inactivation of this proteinase when the environmental temperatures were below 67.8 °C. The transition temperature (T_m), a temperature at which the thermal inactivation rate constant (k) is equal to 1, is shown in Table II. Addition of 30% sucrose significantly raised the T_m of this enzyme by about 2 °C, as compared with that without sucrose. The changes in free energy (ΔG), enthalpy (ΔH), and entropy (ΔS) at T_m are listed in Table II. This enzyme had greater changes in entropy and enthalpy in buffer A with 30% sucrose (buffer B) than in that without sucrose (buffer A). Activation energies obtained from the enzyme in buffer A and buffer B were 70.0 and 104.6 kcal/mol, respectively (Table II). These data suggested that sucrose per se and/or lowering of the water activity stabilized the calpain II

Table II. Thermodynamic Parameters[a] of Thermal Denaturation of Calpain II in Sucrose–Water Mixtures

sucrose, % w/v	E_a	T_m	ΔG	ΔH	ΔS
0	70.0	61.9	19.7	69.3	148.2
30	104.6	63.8	19.8	103.9	249.7

[a] E_a, activation energy (kcal/mol). T_m, transition temperature (°C). ΔG, free energy change at T_m (kcal/mol). ΔH, enthalpy change at T_m (kcal/mol). ΔS, entropy change at T_m (kcal/(mol K)).

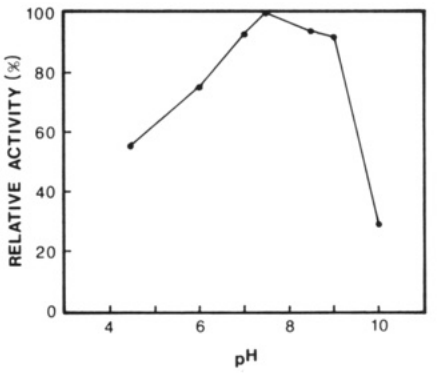

Figure 2. pH stability of calpain II from tilapia muscle.

against thermal denaturation. At present, however, it is still not possible to offer a definitive explanation on the molecular level for the inactivation of this enzyme. Gekko and Timasheff (1981) reported that glycerol increased the thermostability of proteins. The current data and the results obtained by Gekko and Timasheff (1981) suggested that both sucrose and glycol might have the same stabilizing effect on solvent ordering around protein molecules.

Effect of pH on the Stability of Calpain II. The calpain II had a very broad stable pH range from 7.0 to 9.0 and was the most stable at pH 7.5 (Figure 2). This curve was similar to the optimum pH curve (Wang and Jiang, 1991). A very sharp activity decline was observed at pH >9.0, while the activity gradually decreased with the decrease of pH from 7.0 to 4.5 (Figure 2). These data suggested that the enzyme conformation was stable at pH 7.5 and less stable at alkaline than at acidic pH. Rapid decrease in stability at pH >9.0 might be due to the oxidation during incubation, since the oxidation rate of cysteine was very rapid at alkaline pH (Dawson et al., 1986).

Effect of Calcium on the Stability of Calpain II. Since the thermal inactivation rate constants of calpain II were very low at temperatures below 30 °C, the loss of enzymatic activity during preincubation from 9.5 to 30 °C with 5 mM Ca^{2+} was considered to be primarily due to Ca-induced autolysis. As shown in Table III, this autolysis might be an intermolecular reaction because the data had a higher correlation coefficient in the second-order reaction. The half-life period of Ca autolysis was 23 min at 25 °C (Table IV), and the activation energy was 26.61 kcal/mol (calculated from Table III). The calpain II from tilapia muscle had lower autolytic inactivation rate than that of carp muscle (Taneda et al., 1983), chicken gizzard muscle (Crawford et al., 1987), rabbit skeletal muscle (Mellgren et al., 1982), and rat heart (Croall and DeMartino, 1983). From the SDS–PAGE analysis, after 10, 20, 30, and 40 min of incubation at 25 °C in the presence of 5 mM Ca^{2+}, the molecular weight of calpain II decreased from 80 000 to 61 000 and 65 000 (Figure 3). These two major components still had the caseinolytic activity. This result was different from that of chicken gizzard smooth muscle which was autolyzed into a 78-kDa fragment and further

Table III. Specific Velocity Constant of the First- and Second-Order Reaction of Autolysis at Various Temperatures

temp, °C	k_1,[a] min^{-1}	k_2,[a] mL/(min μg)	temp, °C	k_1,[a] min^{-1}	k_2,[a] mL/(min μg)
9.5	3.2×10^{-3} (−0.993)[b]	3.5×10^{-5} (0.995)	25	3.2×10^{-2} (−0.987)	4.4×10^{-4} (0.993)
15	5.3×10^{-3} (−0.990)	5.9×10^{-5} (0.993)	30	6.1×10^{-2} (−0.927)	7.1×10^{-4} (0.940)
20	1.7×10^{-2} (−0.989)	2.1×10^{-4} (0.993)			

[a] $k_1 = -\ln (A_t/A_0)/t$ and $k_2 = (A_0 - A_t/A_0A_t)/t$, where A_0 is the activity of calpain II before incubation (100%), and A_t is the activity of calpain II relative to the A_0 after t min of incubation. [b] Values in parentheses are correlation coefficients.

Table IV. Comparison of the Half-Life Periods for Inactivation of Calpain II at 25 °C

conditions[a]	half-life	conditions[a]	half-life
pH 7.5 (buffer A)	215 days	autolysis	23 min
pH 5.5 (Good's buffer)	58 min		

[a] Detailed experimental conditions are given in the text.

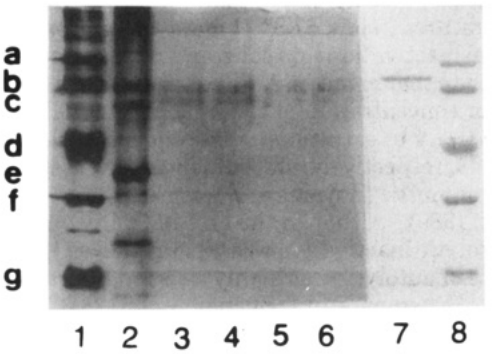

Figure 3. Analysis of autolyzed calpain II on SDS–PAGE. Calpain II (1.4 μg) was autolyzed for 0 (lane 7), 10, 20, 30, and 40 min (lanes 3–6) in the 20 mM Tris-HCl buffer containing 20 mM 2-mercaptoethanol, 1 mM NaN_3, and 5 mM Ca^{2+}, pH 7.5 at 25 °C. Lanes 1, 2, and 8 were protein standards. (a) Phosphorylase *b* (94 kDa); (b) bovine serum albumin (67 kDa); (c) catalase (60 kDa); (d) ovalbumin (43 kDa); (e) lactate dehydrogenase (36 kDa); (f) carbonic anhydrase (30 kDa); (g) α-lactalbumin (14.4 kDa).

into 54- and 37-kDa fragments (Crawford et al., 1987), rabbit skeletal muscle which was autolyzed into 54- and 43-kDa fragments (Mellgren et al., 1982; Nakamura et al., 1989), and bovine skeletal muscle which was autolyzed into a 76-kDa fragment and further into 55- and 34-kDa fragments (Nishimura and Goll, 1991). Calcium ion was an important factor for activation and autolytic inactivation of this enzyme. The autolytic inactivation of calpain was an irreversible process which might be important for its function in the cell. According to the law of mass action, the rate of autolysis of many proteinases should be second-order reaction. However, according to Mellgren et al. (1982) and Suzuki et al. (1981), the autolytic reactions of calpains from rabbit and chicken were independent of the enzyme concentration in the presence of Ca^{2+}, and they concluded that the autolytic rate of calpain should be first-order reaction. The reasons for this discrepancy were still not clear but might be because of the difference in experimental procedures. Inomata et al. (1988) and Nakamura et al. (1989) suggested that the autolysis of calpain molecules involved intermolecular and intramolecular events. From these facts, the autolysis of calpains might involve first- and second-order reactions at different stages. According to the stainability (extent of staining or coloring of proteins) of proteins (Heukeshoven and Dernick, 1985),

the staining sensitivity of this enzyme was decreased after autolysis. This phenomenon reflected the differences in structure and redox properties of the 80-, 61-, and 65-kDa fragments. Further studies on the properties of the 61- and 65-kDa components will be interesting and engaging.

Calpain has been shown to degrade myofibrillar proteins, which would consequently promote the splitting of myofibrils and increase fragility of muscle (Goll et al., 1983; Penny et al., 1984; Koohmaraie et al., 1986; Zeece et al., 1986). Although the thermal stability and autolytic resistibility of calpain I was higher than that of calpain II in vitro (Murachi, 1983), calpain I and calpastatin of beef muscle lost their activity quite rapidly on post-mortem storage (Ducastaing et al., 1985; Koohmaraie et al., 1987). In addition, post-mortem storage had very little effect on the activity of calpain II (Ducastaing et al., 1985; Koohmaraie et al., 1987). Accordingly, the calpain II might play an important role in the degradation of post-mortem muscle.

The water activity in the muscle cell was lower than 1, because the water content of muscle is about 70–80% (Pearson and Young, 1989). The addition of 30% sucrose caused the increase of thermal stability of calpain II at temperatures below 67.8° (Figure 1). Accordingly, the thermal inactivation rate constant in muscle cell might be lower than that in buffer A. The pH and intracellular free calcium concentration of fish muscle gradually changed from 7.0–7.4 to an ultimate pH of 5.5–5.8 and from 10^{-6} to 10^{-4} M, respectively, and consequently entered into the full rigor mortis (Suyama and Konosu, 1987; Pearson and Young, 1989). Although the half-life period of calpain II for autolytic inactivation was 23 min at 25 °C (Table IV), the rate of autolysis was highly affected by concentration of Ca, pH, temperature (Suzuki et al., 1981), substrates, and calpastatin (Inomata et al., 1988). When the calpain existed with substrates such as casein, the hydrolysis was significantly retarded (Wang and Jiang, 1991). Inomata et al. (1988) reported that high concentration of substrate inhibited the autolysis of calpain but not in the case of the low concentration of substrate. They also demonstrated that calpains were autolyzed very slowly in the presence of the calpastatin but the calpastatin could not completely inhibit the autolysis (Inomata et al., 1988). In addition, the calcium concentration and pH of the post-mortem muscle were lower than those in experimental conditions. Accordingly, the autolytic rate constant in muscle cell might be lower than that in our experimental conditions, and the half-life periods for thermal inactivation and autolytic inactivation at any storage temperatures (usually below 10 °C) should be long enough for muscle tenderization. Therefore, although the calpain II lost about 50% activity at pH 5.5 after 58 min of incubation at 25 °C (Table IV), calpain II might be involved in the initial stage of fish muscle tenderization. Furthermore, from the data obtained by Ducastaing et al. (1985) and Koohmaraie et al. (1987), the activity of calpain I decreased very rapidly when the pH of beef muscle sharply dropped. Thus, the post-mortem pH of muscle might be the main factor affecting the calpain activity and stability. The calpain I might lose its activity more rapidly in fish muscle than in beef muscle, since the biochemical degradation in fish muscle was faster than that in beef. This might be the reason we could not obtain calpain I from tilapia muscle.

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Registry No. Calpain II, 78990-62-2; calcium, 7440-70-2.