Properties of Calpain II from Tilapia Muscle (*Tilapia nilotica* × *Tilapia aurea*)

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The characteristics of calpain II (EC 3.4.22.17) were investigated in order to clarify the function of this enzyme in post-mortem fish muscle. Calpain II purified from tilapia muscle (*Tilapia nilotica* × *Tilapia aurea*) was activated by Ca, Sr, Ba, and Mn, but inhibited by Fe, Co., Ni, Cu, Zn, Cd and Hg ions. This proteinase was unaffected by Na, K and Mg ions. The calcium ion might induce conformational changes and consequently activate this proteinase. The optimal pH and temperature for the activity were 7.5 and 30°C, respectively. Water activity and the ionic strength of the reaction solution significantly affected the activity of calpain II. Increasing ethylene glycol and polyethylene glycol significantly decreased the activity of calpain II. However, no significant effect on calpain II activity was apparent from the addition of nucleotides and their related compounds.

The Ca^{2+} -activated proteases seem to play a wide variety of roles which include muscle protein turnover,^{1,2)} proteolysis of the neurofilament, microtubules and other cytoskeletal proteins,^{3,7)} transformation of receptors,^{8,9)} platelet aggregation,¹⁰⁾ hemoglobin degradation,¹¹⁾ glutamate reception in the hippocampus,^{12,13)} and activation of various enzymes.^{14–17)} These Ca^{2+} -dependent cysteine neutral proteinases are ubiquitously distributed in eukaryotic cells. The preparation and characterization of cytoplasmic Ca^{2+} -activated neutral proteinase from skeletal muscle had been accomplished by several researchers.^{18–21)} However, it has been difficult to ascribe the physiological functions of this proteinase because of the high Ca^{2+} requirement for optimum activity.¹⁸⁾ Another neutral proteinase, which required less than $100\ \mu\text{M}$ Ca^{2+} for optimum activity and therefore could function in muscle when the Ca^{2+} ions were released, has been purified from dog heart, and porcine and rabbit skeletal muscle.^{22–25)}

Recently, the mechanism for tenderizing muscle has attracted many researchers.^{26–28)}

Although tenderization is considered to be caused by the disappearance of Z-disks, dissociation of actomyosin complex, collapse of connectin, and denaturation of collagen,^{26,27,29–31)} the mechanism for post-mortem tenderization is still unclear.^{26–28)} Many biochemical changes associated with post-mortem tenderness have generally been assumed to arise from the release of endogenous muscle proteases which are active at the post-mortem pH values.²⁹⁾ The proteases indigenous to skeletal muscle possibly include the Ca-dependent proteases and cathepsins.^{27,28,32)} Many studies currently indicate that lysosomal enzymes are not involved in the tenderization process of bovine and lamb muscle, while the Ca-dependent proteases are.^{27,28,33–35)} In addition, the post-mortem release of Ca^{2+} in fish muscle has been recognized to occur when the functions of mitochondria and the sarcoplasmic reticulum were degraded, and when the concentration of Ca^{2+} was increased from 10^{-6} to $10^{-4}\ \text{M}$.³⁶⁾ This phenomenon advances the possibility of the Ca-dependent proteinases being involved

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in post-mortem muscle degradation. In general, most of the cysteine proteinases might be involved in the initial or terminal stages of extensive degradation of proteins. In the previous study,³⁷⁾ calpain II was purified from tilapia muscle. This study is an endeavor to clarify the mechanism for tenderization of fish muscle and is aimed at characterizing calpain II from tilapia muscle.

Materials and Methods

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

Purification of calpain. Calpain II was purified by phenyl Sepharose CL-4B and alpha-casein agarose column chromatography according to the previous study.³⁷⁾

Effect of pH and temperature on the activity. The reaction mixture was 10 mM 2-mercaptoethanol, 0.5 mM NaN_3 , 0.05 M NaCl, and 5 mM CaCl_2 in 10 mM Goods buffer (bicine, sodium acetate and bis-tris propane) at various pH values. On the basis of these pH conditions, the calpain activity was measured.

Effect of N-ethylmaleimide on the activity. N-Ethylmaleimide (NEM, 0.3 or 3.0 mM) with various concentrations of calcium chloride was added to an enzyme solution containing 20 mM Tris-HCl, 1 mM ethylglycol tetraacetic acid, 20 mM 2-mercaptoethanol and 1 mM NaN_3 at pH 7.5. After incubating at 25°C for 5 min, the NEM was neutralized by 10 mM 2-mercaptoethanol. The calcium concentration was then diluted to below 10 μM and the mixture allowed to stand at 25°C for 5 min, before the calpain activity was measured.

Effect of ionic strength and water activity on the activity. The reaction mixture was 50 mM imidazole-HCl, 10 mM 2-mercaptoethanol, 0.5 mM NaN_3 , 0.05 M NaCl and 5 mM CaCl_2 at pH 7.5 (buffer A). The ionic strength of the enzyme solution was adjusted by NaCl or KCl, while the water activity was varied by adding sucrose. The calpain activity in these reaction mixtures was then determined.

Effect of ethylene glycol and polyethylene glycol (PEG) on the activity. Various concentrations of ethylene glycol, PEG-4000 (average M.W. 4000), PEG-10000 (average M.W. 10000) and PEG-20000 (average M.W. 20000) were added to the enzyme in buffer A. The calpain activity in these reaction mixtures was then measured.

Effect of ATP and its related compounds on the activity. To the enzyme dissolved in buffer A, 1.0 or 5.0 mM of ATP, ADP, AMP, IMP, HxR, Hx or uric acid was added. The calpain activity in each of these reaction mixtures was measured.

Determination of protein concentration. Protein concentration was determined by the protein-dye binding method,³⁸⁾ using crystalline bovine serum albumin as a standard.

Assay of calpain activity. A 4.0 mg quantity of casein was added to 1.0 ml of each reaction mixtures, which was then incubated 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,³⁸⁾ 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 is 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 is corrected by subtracting the activity of the blank, which was in the presence of 5 mM EDTA.

Results and Discussion

Effect of pH and temperature on the calpain II activity

The optimum pH value for calpain II was very broad, maximum activity occurring at pH 7.5 (Fig. 1). Almost no activity was detected when the pH of the reaction mixture was lower

Fig. 1. Effect of pH on Calpain II Activity.

The reaction mixture was 10 mM 2-mercaptoethanol, 0.5 mM NaN_3 , 0.05 M NaCl and 5 mM CaCl_2 in 10 mM Goods buffer (bicine, sodium acetate and bis-tris propane) at pH 7.5.

than 4.5 or higher than 10. A very sharp activity decline was observed at pH values higher than 8.0, while the activity gradually decreased with decreasing pH from 7.0 to 4.5. It is recognized that all the calpains from different sources have a similar optimum pH around 7.5.³⁹⁾ However, the curves for calpain activity as a function of pH from different organisms were slightly different. This suggested that the pKa values for the -SH and imidazole groups were slightly different. Accordingly, the catalytic groups in the active site of the corresponding proteinases from different sources might be cysteine and histidine residues, although the environment around the two residues was not similar. Since the isoelectric zone of casein is at around pH 4.7, this might also have affected the pH profile for calpain II.

As indicated in Fig. 2, the optimum temperature for calpain II from tilapia was at 30°C, which is almost the same as that from carp.⁴⁰⁾ There was a rapid activity decrease at reaction temperatures higher than 30°C. However, the calpain II activity linearly increased with increasing reaction temperature from 4 to 30°C. Since most fish are poikilothermic, their metabolic rate, growth and feed intake are mainly regulated by the water temperature.⁴¹⁾ Both tilapia (as used in this study) and carp⁴²⁾ have an optimum growth rate at a temperature of 30°C. This suggested that the optimal temperature for

calpain activity might be related to the temperature of the habitat. It is well known that the pH of fish muscle gradually decreases to an ultimate level of 5.5–5.8, and that the muscle enters into full rigor mortis during the post-mortem change.³¹⁾ As indicated in Fig. 1, calpain II still had 65% of its activity at pH 5.5 and 25°C. This suggested that calpain II might be involved in the degradation of myofibrillar protein during the tenderization process. In addition, since calpain II still had 20% of its activity at 0°C and pH 7.5 (Fig. 2), muscle tenderness might continuously proceed during chilling.

Effect of various metal ions on the calpain II activity

Since the Ca ion was essential for calpain-activity, the effect of some other metal ions with and without the Ca ion was also investigated under physiological conditions. In the absence of the calcium ion, the Sr ion almost fully activated the calpain II activity, while the

Fig. 2. Effect of Temperature on Calpain II Activity.

The reaction mixture was 10mM 2-mercaptoethanol, 0.5mM NaN₃, 0.05M NaCl and 5mM CaCl₂ in 50mM imidazole-HCl buffer at pH 7.5.

Table I. EFFECT OF METAL IONS ON CALPAIN II ACTIVITY

Metal ion ^a (5 mM)	A ^b	B ^b	C ^b	D ^b	E ^b
Na ⁺	0 ^c	100.0	0.95	0.9	4
K ⁺	0	100.0	1.33	0.8	4
Mg ²⁺	0	100.6	0.65	1.2	6
Ca ²⁺	100.0	100.0	0.99	1.0	6
Sr ²⁺	91.0	100.1	1.13	1.0	6
Ba ²⁺	85.4	95.8	1.35	0.9	6
Mn ²⁺	73.6	93.2	0.82	1.4	6
Fe ²⁺	0	30.1	0.76	1.7	6
Fe ³⁺	0	36.7	0.64	1.8	6
Co ²⁺	0	0	0.74	1.7	6
Ni ²⁺	0	25.2	0.73	1.8	4
Cu ²⁺	0	0	0.72	1.8	4
Zn ²⁺	0	0	0.74	1.5	4
Cd ²⁺	0	0	0.96	1.5	4
Hg ²⁺	0	0	>0.96	—	—

^a The chloride ion was the counter ion of all metal ions.

^b A, without the addition of Ca²⁺; B, with 5mM Ca²⁺ added; C, ionic radius (nm); D, electronegativity; E, coordination number. The data for C, D and E were taken from ref. 51.

^c Values in this table are expressed as a percentage relative to that measured at 5mM CaCl₂.

Ba and Mn ions partially activated it. The activation ability of the Ca ion was the highest, followed by the Sr, Ba and Mn ions in this decreasing order; no calpain activity was detected in reaction mixtures with 5 mM of Na, K, Mg, Fe, Co, Ni, Cu, Zn, Cd or Hg ions (Table I). This result is similar to that from rabbit and chicken skeletal muscle,⁴³⁾ calf brain,⁴⁴⁾ and rat kidney.⁴⁵⁾ Among the metal ions tested in this study, metals with a coordination number (CN) of 4, or with a CN of 6 and ionic radius of less than 0.08 nm did not activate calpain II, while those with a CN of 6 and ionic radius of more than 0.8 nm fully or partially activated it in the absence of Ca (Table I). These specific properties are reflected in the sensitivity of the metals to calpain. The addition of Na, K or Mg ions into reaction mixtures in the presence and absence of Ca did not affect the calpain II activity, suggesting that these metals could not bind to the proteinase. The data obtained in this experiment indicate that none of these metals was better than Ca for activating calpain II. Generally, the Ca ion is favored to form a complex with an octahedral configuration. This phenomenon indicates that other configurations might be not suitable for activating this enzyme.

In the presence of 5 mM Ca, the calpain II activity was little affected by the addition of Sr, Ba or Mn ions, while the Fe, Co, Ni, Cu, Zn, Cd or Hg ions fully or partially inhibited the calpain activity. In spite of the coordination number and ionic radius, all the metals with an electronegativity of more than 1.5 substantially inhibited calpain II (Table I). Metals such as Zn, Cd and Hg have been reported to bind to -SH groups of the target enzyme and to subsequently inhibit enzymatic activity.⁴⁶⁾ Accordingly, the inhibition of calpain II activity might be the result of these metals binding at non-specific sites on calpain.

Effect of N-ethylmaleimide (NEM) on the calpain II activity

The addition of 3.0 mM of NEM highly inhibited the calpain activity in the presence and absence of Ca, while 0.3 mM of NEM only

Table II. EFFECT OF *N*-ETHYLMALIMIDE (NEM) ON THE CALPAIN II RELATIVE ACTIVITY IN THE PRESENCE OF CaCl₂

CaCl ₂ (mM)	NEM (mM)	
	0.3	3.0
Control ^a	100.0	100.0
0.0	93.5	4.2
0.1	73.6	3.1
1.0	72.3	1.3
5.0	70.2	0.0

^a The activity was measured with 5 mM CaCl₂ and without *N*-ethylmaleimide.

Fig. 3. Effect of Ionic Strength on Calpain II Activity. Refer to the legend to Fig. 2 for the reaction mixture.

partially inhibited it (Table II). It is considered that Ca²⁺ might have induced conformational changes which caused exposure of the masked binding site containing -SH groups, and consequently activated the enzyme. NEM is a maleimide alkylating reagent which essentially and irreversibly reacts with the -SH groups of proteins. The calpain activity was lower in the mixture with 3 mM NEM than with 0.3 mM NEM in the absence of calcium (Table II). This suggests that the active site might have been the -SH groups in the surface region.

In the previous study,³⁷⁾ calpain II was substantially hydrolyzed during purification procedures in the presence of Ca²⁺. However, when the calpain existed with substrates such as casein or with inhibitors such as leupeptin, this hydrolysis was significantly retarded. From the evidence of the inhibitory effect of NEM

on calpain II activity in the presence of Ca^{2+} (Table II), the inhibitor and substrate retarded the autolysis of calpain II coexisting with Ca^{2+} , which might have been due to the protective effects against autolysis.

Effect of ionic strength on the calpain II activity

The activity of calpain II was gradually decreased with increasing NaCl or KCl concentration (Fig. 3). This data indicates that NaCl and KCl had the same effect on the calpain II activity. It is recognized that the electrostatic potential of a proteinase surface shrinks and the configuration changes in a high-ionic-strength solution. This would increase the hydrophobicity and consequently make the enzymes aggregate. The calpain II activity was, therefore, inhibited in a high concentration of NaCl or KCl solution. On the other hand, the high ionic strength of the solution would interfere with the interaction of the Ca ion with calpain II, which decreased the stability of the calcium-calpain complex.

Effect of water activity on the calpain II activity

The addition of sucrose decreased the water activity of the reaction mixture and substantially decreased the calpain II activity (Fig. 4). However, it was surprising that calpain II was more sensitive to the water activity of the reaction mixture than were other enzymes.⁴⁷⁾ Since the hydrolysis at 25°C started from the point at which the sorption curve for the mixture departed from the linear region at an A_w of 0.966, this water activity is considered to be necessary for initiating enzymatic reactions. Since calpain II is a hydrolytic proteinase, water is another substrate for its activity. The sucrose in the reaction mixture would have competed with the calpain to bind water and increase the viscosity, which consequently decreased the activity of calpain II.

Effect of ethylene glycol and polyethylene glycol (PEG) on the calpain II activity

As shown in Table III, when the concentra-

Fig. 4. Effect of the Water Activity of the Reaction Mixture on Calpain II Activity.

Refer to the legend to Fig. 2 for the reaction mixture: —●—, calpain activity; —, sorption isotherm.

Table III. EFFECT OF ETHYLENE GLYCOL AND POLYETHYLENE GLYCOL ON CALPAIN II ACTIVITY

Concentration (% w/v)	A ^a	B ^a	C ^a	D ^a
0.00	100.0	100.0	100.0	100.0
0.05	100.0	99.5	100.0	98.6
0.50	100.0	101.6	95.1	95.9
1.00	99.6	90.5	80.7	79.6
5.00	88.2	60.8	36.7	37.0
10.00	68.5	44.5	5.2	7.1
15.00	42.8	—	—	—
20.00	25.6	—	—	—
25.00	18.5	—	—	—
30.00	4.5	—	—	—

^a A, ethylene glycol; B, polyethylene glycol 4000; C, polyethylene glycol 10000; D, polyethylene glycol 20000.

tion of the added ethylene glycol, PEG-4000, PEG-10000 or PEG-20000 was below 0.50%, no significant effect on the activity of calpain II was observed. However, the activity was inhibited at concentrations higher than 0.50%, and the inhibitory effect was enhanced by the increasing concentration of these added compounds. The inhibitory ability of PEG-10000 and PEG-20000 was the highest, and then of PEG-4000 and ethylene glycol. EG and PEG lowered the water activity and increased the viscosity of the reaction mixture. They had the same inhibitory effects as sucrose on the

calpain II activity. However, Malik *et al.*⁴⁸⁾ have reported that the calpain I activity from calf brain was enhanced by PEG-8000. The difference in the effects of ethylene glycol and PEG on the activity might have been due to the type of calpain.

Effect of ATP and its related compounds on the calpain II activity

There are a number of enzyme systems in muscle that require ATP as an energy source to carry out cellular functions. In post-mortem muscle, ATP is degraded into ADP, AMP, IMP, HxR, Hx and uric acid. The effects of these components on the calpain activity were also investigated. The activity of calpain II was not affected by these compounds (data not shown). According to Ducastaing *et al.*,⁴⁹⁾ calpain activity was gradually lost during the rigor mortis process. This might have been due to the autolysis of this enzyme at high ionic strengths, low water activity, and low pH, since an increase in the intracellular free Ca content and ionic strength, and a decrease in the water-holding capacity and pH occurred in the post-mortem muscle. Furthermore, organisms continuously undergo a variety of irreversible processes such as development, growth, aging, death and post-mortem changes. Imajoh-Ohmi *et al.*⁵⁰⁾ have reported that the calpain-calpastatin system is an irreversible process which might play a key role in the regulation related to these irreversible changes in living or post-mortem muscle. In order to control the tenderization process of fish muscle, further studies on the regulatory functions of this enzyme in post-mortem muscle are definitely necessary.

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