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ATPase activities and autolysis of kuruma prawn *Penaeus japonicus* muscle proteins

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Abstract

Ca^{2+}-, Mg^{2+}-, Mg^{2+},Ca^{2+}- and Mg^{2+}-EGTA-ATPase activities of natural actomyosin (NAM) from kuruma prawn (*Penaeus japonicus*) at different temperatures were determined. The maximal activities of all ATPase were observed at 35 °C and subsequently decreased with increasing temperatures. The loss in Ca^{2+}-sensitivity was noticeable as the temperatures increased, suggesting the denaturation of regulatory proteins. The autolysis of kuruma prawn muscle was also investigated at different temperatures. The maximal autolytic activity was found at 60 °C. NaCl at higher concentrations exhibited the greater inhibitory effect toward autolysis. Metalloproteinase inhibitors including 1,10-phenanthroline, EDTA, EGTA and pyrophosphate at the range of 1-10 mM could inhibit the autolysis of kuruma prawn muscle as evidenced by the lower trichloroacetic acid-soluble peptide content and more myosin heavy chain (MHC) band intensity retained. The result suggested that heat-activated metalloproteinases involved in the autolysis of kuruma prawn muscle at the elevated temperatures.

Keywords: ATPase, Natural actomyosin, Prawn, Muscle, Autolysis, Proteinase

Introduction

Myofibrillar proteins constitute as the major protein in the fish and shellfish muscle and play an essential role in functionality such as gelation, emulsifying property, etc. Myosin is the primary component responsible for gelation of fish protein (Sano et al. 1988; Visessaguan and An 2000). Furthermore, paramyosin was reported to affect the gel formation of invertebrate natural actomyosin (Ehara et al. 2004).

The property as well as stability of myofibrils was associated with many factors including the habitat temperature and spawning period. Post-mortem storage and spawning period were found to affect the ATPase

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activities of fish muscle proteins (Benjakul et al. 1997; Roura et al. 1990). Cold-water fish normally had higher myofibrillar ATPase activity at low temperature, while warm-water fish exhibited the optimal activity at higher temperature (Johnston et al. 1973). Myofibrillar thermal stability of American lobster was lower than that of Japanese spiny lobster. Such a difference was determined by their habitat temperature (Shimada et al. 2000).

Proteolytic degradation of myofibrillar proteins displays the detrimental effect on the functionality of muscle. Proteolysis can occur during postmortem storage of fish (Benjakul et al. 1997). Apart from fish muscle, squid mantle muscle underwent autolysis with the maximal rate at 25 °C (Konno et al. 2003). Grass prawn muscle contained the m-calpain involving in the post-mortem degradation of muscle (Wang et al. 1993). Artic and tropical shrimps also underwent the protein degradation during iced storage (Martinez et al. 2001). The proteolysis of muscle protein also takes place at the elevated temperature for some fish species (An et al. 1996; Visessanguan and An 2000). Heat activated protease actively involve in the softening of fish muscle gel (Lin and Lanier 1980). However, no information regarding the ATPase activities as well as autolysis of prawn muscle at elevated temperatures has been reported. The objective of this investigation was to study the ATPase activities and autolysis of kuruma prawn muscle as influenced by various temperatures.

Materials and methods

Chemicals

Adenosine 5'-triphosphate (disodium salt) was purchased from Sigma Aldrich Co. (St. Louis, MO). Ammonium molybdate, ρ-methylaminophenol sulfate, trichloroacetic acid, potassium chloride, calcium chloride, magnesium chloride, 1,10-phenanthroline monohydrate and ethylenediamine-N,N,N',N'-tetraacetic acid disodium salt dihydrate (EDTA) were obtained from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). O,O'-Bis (2-aminoethyl) ethyleneglycol-N-N,N',N'-tetraacetic acid (EGTA) was procured from Dojindo Laboratories (Kumamoto, Japan). Sodium pyrophosphate was procured from Alfa Aesar (Lancs, UK).

Prawn sample and preparation

Fresh kuruma prawns (Panaeus japonicus) with the size of 50 prawns/kg were obtained from the market in Tokyo, Japan. The prawns kept in ice with the sample/ice ratio of 1:2 (w/w) were transported to the Department of Food Science and Technology, Tokyo University of Marine Science and Technology, Japan within 30 min. Upon arrival, the prawns were deheaded, peeled, deveined and the meat was collected. The meat was then finely chopped and used for natural actomyosin preparation.

Preparation of natural actomyosin (NAM)

NAM from kuruma prawn muscle was prepared according to the method of Benjakul et al. (1997) with a slight modification. Prawn meat (50 g) was homogenized in 10 volumes of chilled 0.6 M KCl, pH 7.5 every 10 s with a 10 s rest interval for totally 1.5 min using a blender (National MX-X103, Tokyo, Japan).

The homogenate was stirred gradually for 10 min in ice to allow the complete solubilization. The extract was centrifuged at 5,000 xg for 30 min at 4 °C using a refrigerated centrifuge (Tomy CX250, Tokyo, Japan). Ten volumes of chilled deionized water were added to precipitate NAM. NAM was then collected by centrifuging at 5,000 xg for 20 min at 4 °C. NAM pellet was then dissolved in 20 mM Tris-HCl containing 0.6 M KCl (pH 7.5).

Effect of different temperatures on ATPase activities of kuruma prawn NAM

Ca^{2+}-, Mg^{2+}-, Mg^{2+},Ca^{2+}- and Mg^{2+}-EGTA-ATPase activities of kuruma prawn NAM were assayed at different temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60 and 65 °C). The assay reaction was terminated using 15% trichloroacetic acid (TCA) after 10 min. ATPase activities and Ca^{2+}-sensitivity were then calculated.

Determination of ATPase activities and Ca^{2+}-sensitivity

ATPase activities were determined as described by Benjakul et al. (1997). To 1 ml of NAM solution (3.5-4mg protein/ml), 0.6 ml of 0.5 M Tris-maleate (pH 7.0) was added. To the mixture, the following chemicals were added to obtain the designated concentrations with the total volume of 9.5 ml: 10 mM $CaCl_2$ for Ca^{2+}-ATPase; 2 mM $MgCl_2$ for Mg^{2+}-ATPase; 0.1 mM $CaCl_2$ and 2 mM $MgCl_2$ for Mg^{2+},Ca^{2+}-ATPase and 2 mM $MgCl_2$ and 0.5 mM EGTA for Mg^{2+}-EGTA-ATPase.

To each assay solution, 0.5 ml of 20 mM ATP was added to initiate the reaction. The reaction was conducted for 10 min and terminated by addition of 5 ml chilled 15% (w/v) TCA. The reaction mixture was subjected to centrifugation at 3,000 xg using a high speed refrigerated micro centrifuge (Tomy MRX-152, Tokyo, Japan) for 5 min. The inorganic phosphate released in the supernatant was measured by the method of Feske and Subbarow (1925). Specific activity was expressed as μmoles inorganic phosphate (Pi) released/mg protein/min. A blank was performed by adding the chilled TCA prior to the addition of ATP. Ca^{2+}-sensitity was calculated according to Seki and Narita (1980) as follows:

Ca^{2+}-sensitivity = [1-(Mg^{2+}-EGTA-ATPase/ Mg^{2+},Ca^{2+}-ATPase)]x100.

Preparation of muscle homogenate

Prawn meat was added with 4 volumes of cold 40 mM Tris-maleate (pH 7.0). The mixture was homogenized every 10 s with a 10 s rest interval for totally 1.5 min using a blender (National MX-X103, Tokyo, Japan). To remove the connective tissue, the homogenate was filtered through three layers of cheesecloth. The filtrate used as the muscle homogenate was kept in ice prior to autolysis study.

Effect of different temperatures on autolysis of kuruma prawn muscle homogenate

Homogenate was mixed with deionized water or 1 M NaCl at 1:1 ratio. The mixture (4 ml) was incubated at different temperatures (20-75 °C) for 30 and 60 min. The autolysis was terminated by addition of either 12.5% SDS solution (85 °C) or 12.5% TCA. The assay mixture was then subjected to centrifugation at 3,000 xg for 10 min using a refrigerated centrifuge (Tomy CX250, Tokyo, Japan).

TCA-soluble peptide content in the supernatant was determined using the Lowry method (Lowry et al. 1951) and expressed as μmoles tyrosine/ml homogenate. The supernatant of SDS-solubilized samples was subjected the electrophoretic analysis.

Effect of NaCl on the autolysis of kuruma prawn muscle homogenate

Homogenate (2 ml) was mixed with NaCl solution at different concentrations (2 ml) to obtain the final concentrations of 0, 0.1, 0.3, 0.5, 0.7, 1.0, 1.5 and 2.0 (w/v). The mixtures were incubated at optimal temperature for 60 min. To stop reaction, 1 ml of 12.5% TCA was added and TCA-soluble peptides in the supernatant were determined as previously described.

Effect of metal chelating agents on the autolysis of kuruma prawn muscle homogenate

The solution (2 ml) of different metal chelating agents including 1,10-phenathroline, EDTA, EGTA and Na-pyrophosphate was added into the homogenate (2 ml) to obtain the final concentrations of 1, 5 and 10 mM. The mixtures were incubated at room temperature (25 °C) for 10 min before starting the autolysis assay.

After being incubated at the optimal temperature for 60 min, the autolysis was terminated by addition of 1 ml of 12.5% TCA or 12.5% SDS solution (85 °C). TCA-soluble peptide content and protein patterns in the supernatant were determined after centrifugation at 3,000 xg for 10 min as described above.

Electrophoretic analysis

Protein patterns of homogenate autolyzed under different conditions were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Samples were mixed with the sample buffer at a ratio of 1:1 (v/v) and boiled for 3 min. The samples were then loaded on the PAGEL®-Compact precast gel (10% separating gel) and subjected to electrophoresis at constant voltage of 250 V using a Compact-PAGE apparatus (Atto Corp., Tokyo, Japan).

After electrophoresis, gel was fixed 50% methanol and 10% acetic acid and stained with 0.025% Coomassie brilliant blue in 59% methanol and 10% acetic acid. The gel was then destained with 30% methanol and 10% acetic acid. Molecular weight markers (Fementas Inc., Hanover, MD, USA) were used to estimate the molecular weight of proteins.

Determination of protein content

Protein content was determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin as a standard.

Statistical analysis

The experiment was conducted with two different lots of prawn samples. All analysis was performed in triplicate. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie 1980). Analysis was run using a SPSS package (SPSS 11.0 for window, SPSS Inc, Chicago, IL).

Results and discussion

Effect of different temperatures on ATPase activities of kuruma prawn NAM

Ca^{2+}-, Mg^{2+}-, Mg^{2+},Ca^{2+}- and Mg^{2+}-EGTA-ATPase activities of kuruma prawn NAM assayed at different temperatures are shown in Fig.1. All ATPase exhibited the maximal activities at 35 °C. Among all ATPase, Mg^{2+},Ca^{2+}-ATPase had the highest activity at all temperatures tested. Mg^{2+}- and Mg^{2+},Ca^{2+}- ATPase activities are indicative of the integrity of actomyosin complex in the presence of endogenous and exogenous Ca^{2+} ion, respectively. For Ca^{2+}-ATPase, the similar activity was found in the temperature ranging from 25 to 40 °C.

In the presence of EGTA, Mg^{2+}-ATPase activity was much decreased. Generally, Mg^{2+}-EGTA-ATPase activity can be used to indicate the integrity of troponin-tropomyosin complex (Watabe et al. 1989). The sharp decreases in all ATPase activities were observed when the temperatures were greater than 40 °C, suggesting the thermal denaturation of most proteins in NAM. The decrease in Mg^{2+}-ATPase can be used as the indicator for the selective denaturation of F-actin, which is reported as the activator for myosin Mg-ATPase (Torigai and Konno 1996; Collins and Korn 1980). From the result, the negligible activity was obtained at temperatures above 60 °C.

The American lobster Mg^{2+}-ATPase showed the maximal activity at 35°C in the presence and absence of Ca^{2+} (Shimada et al. 2000). The higher optimal temperature of Mg^{2+}-ATPase was found for Japanese spiny lobster, compared with that of American lobster (Shimada et al. 2000). Therefore, the ATPase activities of kuruma prawn NAM were affected by temperature and might be associated with its habitat temperature. Since paramyosin was found in both smooth and striated muscle of invertebrates including prawn (Elfvin et al. 1976), it might affect the properties of ATPase activities of kuruma prawn NAM. Paramyosin is competitive with F-actin for their effect upon myosin (Epstein et al. 1976).

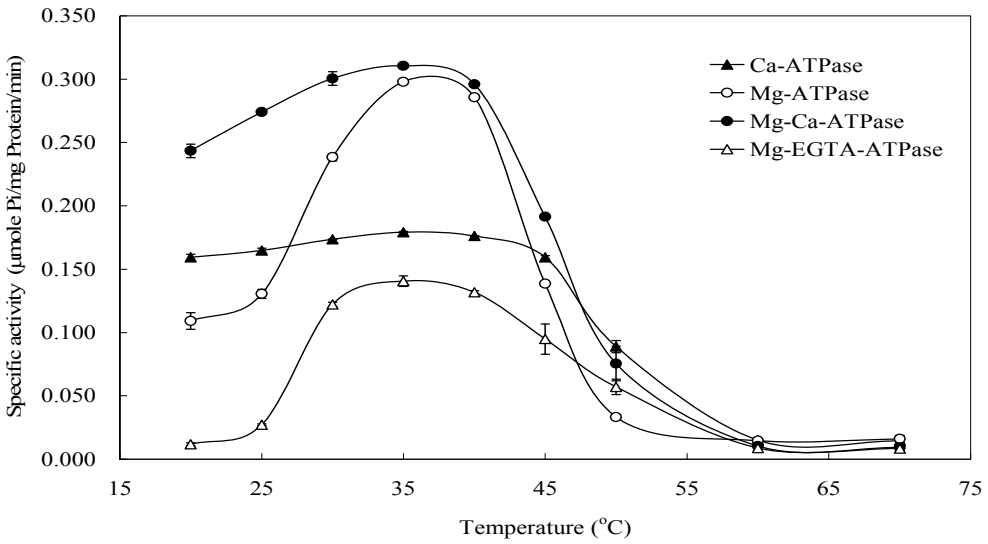

Fig. 1. Temperature profile of ATPase activities of kuruma prawn natural actomyosin. Bars represent standard deviation (n=4).

Effect of different temperatures on Ca^{2+}-sensitivity of kuruma prawn NAM

Ca^{2+}-sensitivity of kuruma prawn NAM at different temperatures is depicted in Fig. 2. A slight decrease in Ca^{2+}-sensitivity of NAM was observed when incubated at 20 and 25 °C. The reduction of Ca^{2+}-sensitivity by 50% was

found when incubated at temperatures ranging from 30 to 45 °C. Thereafter, the marked decrease in Ca^{2+}-sensitivity was noticeable as the temperature was above 50°C. Ca^{2+}-sensitivity of American lobster myofibrils decreased rapidly between 30 and 35 °C, whereas that of Japanese spiny lobster sharply decreased at temperature above 35 °C. Ca^{2+}-sensitivity can be a good indicator of Ca^{2+} regulation of myofibrillar proteins and was dependent on the affinity of troponin molecule for Ca^{2+} ion (Ebashi et al. 1968).

The decrease in Ca^{2+}-sensitivity of NAM at high temperature suggested that troponin might be denatured and the Ca^{2+}-regulation could not function. Temperature causing the loss in Ca^{2+}-sensitivity depended on the species (Shimada et al. 2000). Regulatory proteins in American lobster were less thermally stable than those from Japanese spiny lobster (Shimada et al. 2000). Therefore, heat treatment severely resulted in the denaturation of myofibrillar protein of kuruma prawn as indicated by the decreases in ATPase activities as well as Ca^{2+}-sensitivity. Thermal denaturation of Ca^{2+}-ATPase resulted from unfolding of protein, exposure of hydrophobic residues and oligomerization, especially at temperature higher than 30 °C and SH oxidation was found to contribute to those changes (Senisterra et al. 1997).

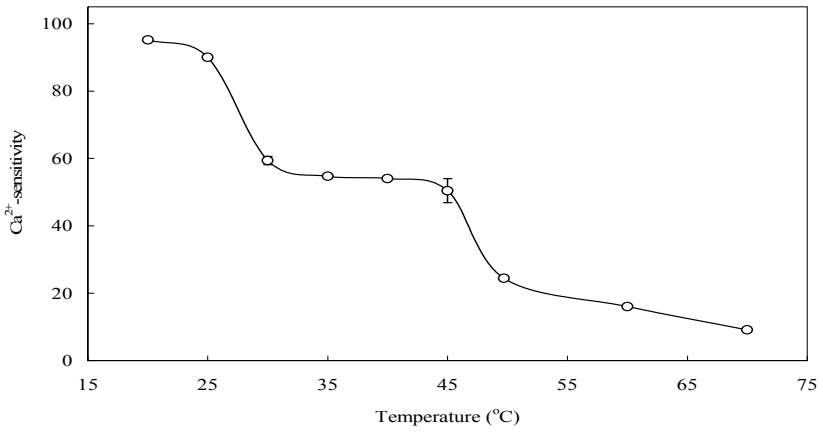

Fig. 2. Ca^{2+}-sensitivity of kuruma prawn natural actomyosin at different temperatures. Bars represent standard deviation (n=4).

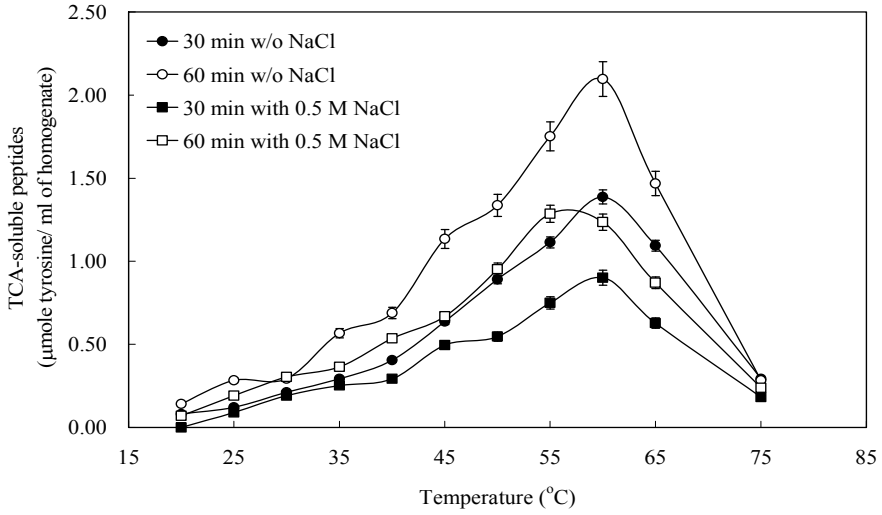

Fig. 3. Temperature profile of autolytic activity of kuruma prawn muscle homogenate in the absence and the presence of 0.5 M NaCl. The autolysis was conducted at different temperatures for 30 and 60 min. Bars represent standard deviation (n=4).

Effect of different temperatures and NaCl on the autolysis of kuruma prawn homogenate

Autolytic activity of kuruma prawn homogenate increased as the incubation temperature increased and reached the optimal at 60 °C (Fig. 3). The longer incubation time (60 min) resulted in the greater hydrolysis, compared with the shorter time (30 min), as evidenced by the higher content of TCA-soluble peptides produced. Nevertheless, the lowered autolytic activity was noticeable when the incubation temperatures were higher than 60 °C. This was probably due to the heat denaturation of endogenous proteases localized in the prawn muscle. From the result, the autolysis was partially suppressed in the presence of 0.5 M NaCl. This suggested that NaCl possibly inhibited or retarded the proteolytic activity to some extent. NaCl might induce the conformational changes of proteases, in which the losses of activity occurred. In the presence of 0.5 M NaCl, the myofibrillar proteins were more likely solubilized and the looser configuration could facilitate the hydrolysis by proteases. Nevertheless, the autolysis was suppressed in the presence of NaCl. This suggested that the inhibitory effect toward proteases was more pronounced than the accessibility of protein induced by NaCl. As a consequence, the decrease in autolysis was found. The electrophoretic study also revealed that the highest autolysis was observed at 60 °C as indicated by the lowest MHC band intensity. However, MHC band was more retained with increasing temperature, suggestion the thermal inactivation of proteinases in kuruma prawn muscle (data not shown). Thus, it can be inferred that the optimal temperature for kuruma prawn autolysis was 60 °C.

The effect of NaCl on the autolytic activity of kuruma prawn homogenate is shown in Fig. 4. The autolytic activity was gradually decreased as the NaCl concentration increased ($P < 0.05$). In the presence of 1 M NaCl, the autolytic activity decreased by approximately 50%. The remarkable decreases in autolytic activities were obtained in the presence of NaCl at levels of 1.5 and 2.0 M.

With the high salt environment, the electrostatic interaction stabilizing the structure of proteases might be destroyed, causing the losses in their activity. Therefore, the addition of NaCl might inhibit the degradation of prawn muscle proteins, particularly in gelly products, which the salt addition is required to solubilize the protein prior to thermal gelation process.

Effect of chelating agents on the autolysis of kuruma prawn homogenate

Inhibitory effects of various chelating agents at different concentrations toward the autolysis of kuruma prawn homogenate are shown in Table 1. All chelating agents could inhibit the autolysis of kuruma prawn homogenate in a concentration dependent manner. However, the inhibitory activity varied with the chelating agents. At the same levels used, the 1,10-phenathroline showed the highest inhibitory activity, followed by EDTA, EGTA and pyrophosphate, respectively.

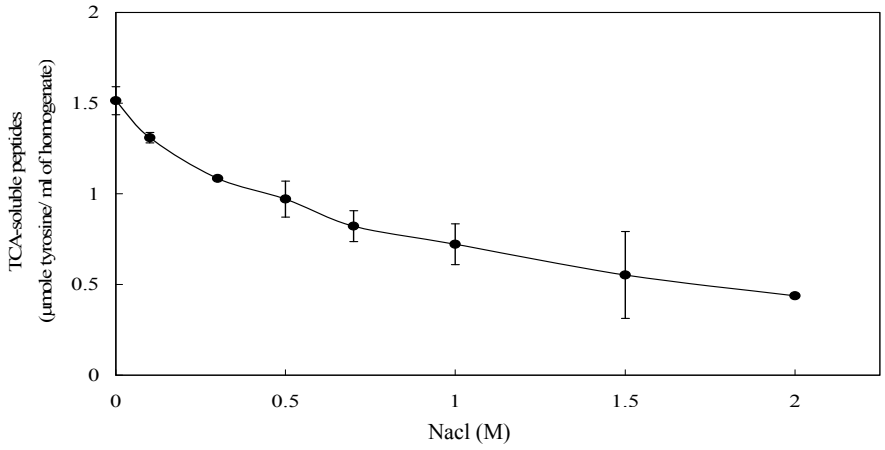

Fig. 4. Effect of NaCl concentrations on autolysis of kuruma prawn homogenate. Bars represent standard deviation (n=4).

Metalloproteinase can be inhibited by 1,10-phenanthroline. EDTA is metalloproteinase inhibitor, which chelates the bivalent metal ion and EGTA is the specific chelator for Ca^{2+} ion. PP also scavenges the metal ion. Since the autolytic activity was inhibited by all metal chelators, the metalloproteinases could play an important role in heat-activated autolysis of kuruma prawn. Metalloproteinase inhibitors including EDTA and 1,10-phenanthroline

significantly suppressed postmortem tenderization of Japanese flounder muscle (Kubota et al. 2001). Due to the inhibitory effect of EGTA against autolysis, Ca^{2+} ion was most likely required for proteinases in the kuruma prawn muscle. Ca^{2+}-dependent proteinase found in crab claw muscle was able to degrade the myofibrillar proteins including myosin heavy chain, paramyosin, actin and other muscle proteins (Mykles and Skinner 1983).

Table 1. Effect of various metal chelating agents on autolysis of kuruma prawn homogenate

Chelators	Concentration (mM)	% Inhibition	
Pyrophosphate	1	0.6	± 0.4
	5	26.6	± 2.7
	10	41.6	± 1.8
1,10-Phenanthroline	1	24.1	± 8.0
	5	53.4	± 0.9
	10	79.4	± 5.7
EGTA	1	50.9	± 2.7
	5	59.4	± 1.3
	10	59.7	± 0.0
EDTA	1	44.1	± 0.9
	5	60.3	± 2.7
	10	65.6	± 3.1

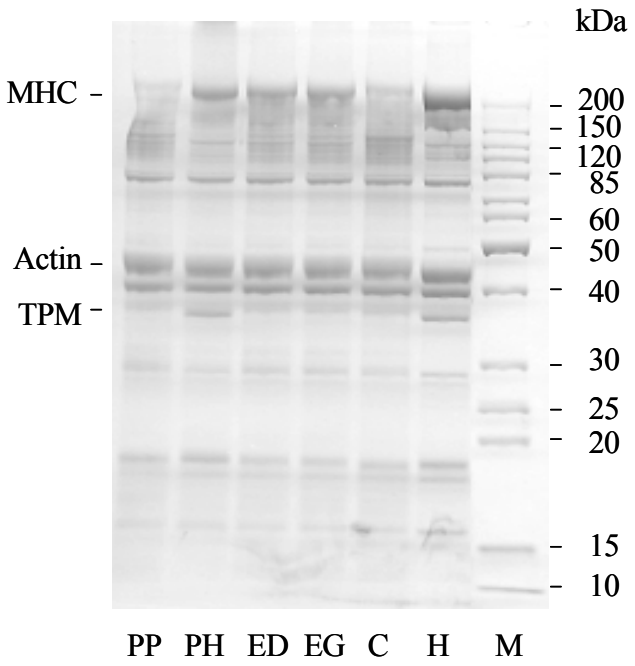

Fig. 5. Protein patterns of kuruma prawn homogenate in the absence and the presence of various metal chelators. The homogenate was incubated without and with metal chalators at a level of 10 mM for 60 min. PP: pyrophosphate; PH: 1,1-phenanthroline; ED: EDTA; EG: EGTA; C: control; H: homogenate; MHC: myosin heavy chain; TPM: tropomyosin; M: molecular weight markers.

Protein patterns of kuruma prawn homogenate incubated at 60 °C in the absence and the presence of various metal chelators at a level of 10 mM are shown in Fig. 5. Without the metalloproteinase inhibitors, MHC almost disappeared and the proteins with molecular weight of 37 kD and 130-140 kDa were completely degraded.

However, actin was resistant to hydrolysis. Actin from Pacific whiting muscle was more tolerant to hydrolysis than MHC (Benjakul et al. 1997). Protein with the molecular weight of 80 kDa and tropomyosin were also resistant to When all metal chelators were added, MHC band was prevented from autolysis at different degrees, depending on the types of chelators and concentrations used.

Among all metal chelators, 1,10 phenathroline showed the highest inhibitory effect toward autolysis as evidenced by the greater MHC band retained. PP exhibited the lowest efficacy in inhibiting the autolysis of kuruma prawn autolysis. Heat-activated protease was reported to degrade the MHC of crucian carp (Ohkubo et al. 2004). Therefore, the degradation at elevated temperature of kuruma prawn homogenate was mostly caused by metalloproteinases. However, other proteinases might also contribute to the autolysis of kuruma prawn.

Conclusions

Properties of natural actomyosin from kuruma prawn were affected by temperatures. At temperatures above 40 °C, myofibrillar proteins underwent denaturation as indicated by the losses in ATPase activities and Ca^{2+}-sensitivity. Autolysis occurred at high temperature and was inhibited by metalloproteinase inhibitors, suggesting the role of heat-activated metalloproteinases in the kuruma prawn muscle at elevated temperatures.

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