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Food Chemistry 102 (2007) 295–301
Food Chemistry
www.elsevier.com/locate/foodchem

Dissociation of natural actomyosin from kuruma prawn muscle induced by pyrophosphate

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Received 16 February 2006; received in revised form 10 March 2006; accepted 15 May 2006

Abstract

Effect of pyrophosphate (PP) on the dissociation and stability of natural actomyosin (NAM) from kuruma prawn muscle was studied in comparison with adenosine 5′-triphosphate (ATP). In the presence of PP up to 5 mM, NAM exhibited lower Mg^{2+}-ATPase activity ($P < 0.05$), while no marked change was observed in NAM treated with ATP at all concentrations tested (0.25–10 mM) ($P > 0.05$). Ca^{2+}-ATPase activity of NAM treated with 5 mM PP decreased markedly when incubated at temperatures greater than 30 °C, suggesting lowered thermal stability of the liberated myosin molecule. Nevertheless, Ca^{2+}-ATPase activity of ATP-treated NAM was similar to the control NAM. In the presence of 5–10 mM $MgCl_2$, NAM treated with 5 mM PP underwent dissociation effectively, as evidenced by a greater decrease in Mg^{2+}-ATPase activity as well as an increased band intensity of actin released. Therefore, addition of PP in combination with $MgCl_2$ was more effective than was ATP in dissociating the actomyosin complex of prawn muscle.

Keywords: Natural actomyosin; Prawn; Pyrophosphate; ATP; Dissociation; ATPase; Muscle

1. Introduction

Phosphates have been used as essential additives in processed meat to improve the texture and to retain the juiciness, flavour and mouthfeel. Increased myofibrillar/cytoskeletal protein extraction by phosphate in the presence of NaCl was associated with increased beef myofibril swelling and increased beef muscle water-holding capacity (Peterson, Parrish, & Stromer, 1988). Phosphate treatment produced a transverse expansion of myofibrils with a simultaneous extraction of myosin from the ends of the A-band in sarcomere (Xiong, 2005). The efficacy of phosphates in increasing the water-binding capacity varied with the type and concentration used (Trout & Schmidt, 1984, 1986). In general, pyrophosphate was the most effective phosphate for increasing water-binding capacity, as well as binding in restructured meat (Trout & Schmidt, 1984). Phosphates also improved the raw and cooked appearance, binding property and acceptability of restructured steaks (Miller, Davis, Seideman, Ramsey, & Rolan, 1986). Phosphates, in combination with sodium chloride, are effective in increasing water-holding capacity by enhancing electrostatic repulsion, which causes the loosening of the myofibrillar structure (Peterson et al., 1988). However, myosin extractability of bovine, chicken and rabbit muscle, by phosphates, depended on the muscle types (Parsons & Knight, 1990; Xiong, Lou, Harmon, Wang, & Moody, 2000).

Polyphosphates, including pyrophosphate, have been used in frozen surimi, together with cryoprotectants. Pyrophosphate neutralized the surimi, in which the protective effect of sorbitol was enhanced (Kumazawa, Oozaki,

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doi:10.1016/j.foodchem.2006.05.021

Iwami, Matsumoto, & Arai, 1990). Addition of pyrophosphate, in combination with 2.5% NaCl, was found to increase the breaking strain of walleye pollack surimi gel set at temperatures above 40 °C (Matsukawa, Sakurada, & Arai, 1992). Pyrophosphate dissociated the actomyosin and enhanced the thermal denaturation of Alaska pollack myosin at high salt concentration (Torigai & Konno, 1996; Konno, 1992).

Prawns are increasingly demanded by consumers, due to their delicacy. Marination of prawns, using various salts, including tripolyphosphate and acids, was found to affect tenderness of the prawn, *Machrobrachium rosenbergii*, differently (Xiong, Xiong, Blanchard, Wang, & Tidwell, 2002). Although pyrophosphate has been widely used in the shrimp-processing industries, no information regarding the effect of pyrophosphate on prawn muscle proteins has been reported. The objective of this investigation was to study the effect of pyrophosphate on the dissociation and thermal stability of natural actomyosin from kuruma prawn.

2. Materials and methods

2.1. 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 and magnesium chloride were obtained from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). Sodium pyrophosphate was procured from Alfa Aesar (Lancs, UK).

2.2. Prawn sample and preparation

Fresh kuruma prawns (*Panaeus japonicus*) (with the size of 50 prawns/kg) were purchased from the market in Tokyo. The prawns, kept in ice at a prawn/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.

2.3. Preparation of natural actomyosin (NAM)

NAM from kuruma prawn muscle was prepared according to the method of Benjakul, Seymour, Morrissey, and An (1997) with a slight modification. Prawn meat (50 g) was homogenised in 10 volumes of chilled 0.6 M KCl, pH 7.0, every 10 s with a 10 s rest interval for (totally) 1.0 min using a blender (National MX-X103, Tokyo, Japan). The homogenate was stirred gently for 10 min in ice to allow solubilisation. The extract was centrifuged at 5000*g* for 30 min at 4 °C using a refrigerated centrifuge (Tomy CX250, Tokyo, Japan). Ten volumes of chilled deionised water were slowly added to the supernatant to precipitate NAM. NAM was then collected by centrifuging at 5000*g* for 20 min at 4 °C. The NAM pellet was then dissolved in 20 mM Tris–HCl containing 0.6 M KCl (pH 7.5). To ensure the complete solubilisation of NAM, the mixture was stirred gently on ice for 15 min. NAM solution was filtered using two layers of cheesecloth to remove the undissolved debris. The filtrate was kept in ice and used immediately.

2.4. Effect of pyrophosphate and ATP on the salt inactivation of actin

NAM (3.5–4 mg/ml), in 20 mM Tris–HCl containing 0.6 M KCl (pH 7.5), was treated with neutralised pyrophosphate or ATP at different concentrations (0, 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, and 10 mM) in the presence of 5 mM $MgCl_2$. The mixture was incubated in ice for 30 min, followed by dialysis against 10 volumes of 20 mM Tris–HCl containing 0.6 M KCl (pH 7.5). The dialysis was performed at 4 °C with 2 changes of dialysis buffer for (totally) 15 h. The dialysed NAM samples were then subjected to Mg^{2+}-ATPase activity measurement. A decrease in Mg^{2+}-ATPase activity indicated the denaturation of actin at high salt concentration.

2.5. Effect of pyrophosphate and ATP on the thermal inactivation of myosin

To NAM (3.5-4 mg/ml), in 20 mM Tris–HCl containing 0.6 KCl (pH 7.5), the neutralised pyrophosphate or ATP was added to obtain final concentrations of 0, 1 and 5 mM in the presence of $MgCl_2$ at the level of 5 mM. The mixture was incubated and dialysed as previously mentioned. After dialysis, NAM with different treatments was incubated at different temperatures (25, 30, 35, 40 and 45 °C) for 5 min, followed by immediate cooling in iced water. The remaining Ca^{2+}-ATPase activity was determined at 25 °C for 10 min. Ca^{2+}-ATPase activity was used to indicate the integrity of myosin.

After dialysis, different NAM samples were also incubated at 30 and 35 °C for different times (0, 1, 3, 5, 7, 10, 15, 20 and 25 min). At the time designated, the samples were cooled rapidly in iced water and assayed for Ca^{2+}-ATPase activity.

2.6. Effect of pyrophosphate and $MgCl_2$ on the dissociation of NAM

NAM (4 mg/ml), in 20 mM Tris–HCl containing 0.6 M KCl (pH 7.5), was mixed with neutralised pyrophosphate (1 and 5 mM) and $MgCl_2$ at levels of 5 and 10 mM. NAM, without any additive, was used as the control. The mixture was incubated in ice for 30 min. All samples were separated into two lots. The first lot was subjected to dialysis against 20 mM Tris–HCl containing 0.6 M KCl (pH 7.5) for 15 h at 4 °C and Mg^{2+}-ATPase activity of dialysate was determined. The second lot was dialysed with 10

volumes of 40 mM Tris–HCl, containing 50 mM KCl (pH 7.5), for 15 h with two changes of dialysis buffer. The mixture was centrifuged at 10,000*g* for 30 min at 4 °C (Iwami & Arai, 1988). The fixed volume of supernatant obtained (15 μl) was subjected to SDS-PAGE analysis using 10% separating gel, according to the method of Laemmli (1970).

2.7. Assay of Mg^{2+}-ATPase and Ca^{2+}-ATPase activities

Mg^{2+}-ATPase and Ca^{2+}-ATPase activities were determined as described by Benjakul et al. (1997). To 1 ml of NAM solution (3.5–4 mg protein/ml), 0.6 ml of 0.5 M Tris–maleate, pH 7.0, was added. Magnesium chloride or calcium chloride was added to the system, with the total volume of 9.5 ml, to obtain final concentrations of 2 mM and 10 mM for Mg^{2+}-ATPase and Ca^{2+}-ATPase activity assays, respectively. To each assay solution, 0.5 ml of 20 mM ATP was added to initiate the reaction. The reaction was conducted for 10 min at 25 °C and stopped by addition of 5 ml chilled 15% (w/v) trichloroacetic acid. The reaction mixture was subjected to centrifugation at 6500 rpm, using a high speed refrigerated micro centrifuge (Tomy MRX-152, Tokyo, Japan) for 5 min. The inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). Specific activity was expressed as μmoles inorganic phosphate (Pi) released/mg protein/min. A blank was performed by adding the chilled trichloroacetic acid prior to the addition of ATP.

2.8. Electrophoretic analysis

Protein patterns of NAM with different treatments were analysed 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 (10 μg) were then loaded on to the PAGEL®-Compact precast gel (10% separating gel) and subjected to electrophoresis at a constant voltage of 250 V using a Compact-PAGE apparatus (Atto Corp., Tokyo, Japan). After electrophoresis, the gel was fixed with 50% methanol and 10% acetic acid and stained with 0.025% Coomassie brilliant blue in 50% 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 weights of proteins.

2.9. Determination of protein content

Protein content was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), using bovine serum albumin as the standard.

2.10. Statistical analysis

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

3. Results and discussion

3.1. Effect of pyrophosphate and ATP on the salt inactivation of actin

The effects of pyrophosphate (PP) and ATP, at different concentrations, on Mg^{2+}-ATPase activity of natural actomyosin (NAM) from kuruma prawn muscle are shown in Fig. 1. Mg^{2+}-ATPase activity of NAM decreased sharply

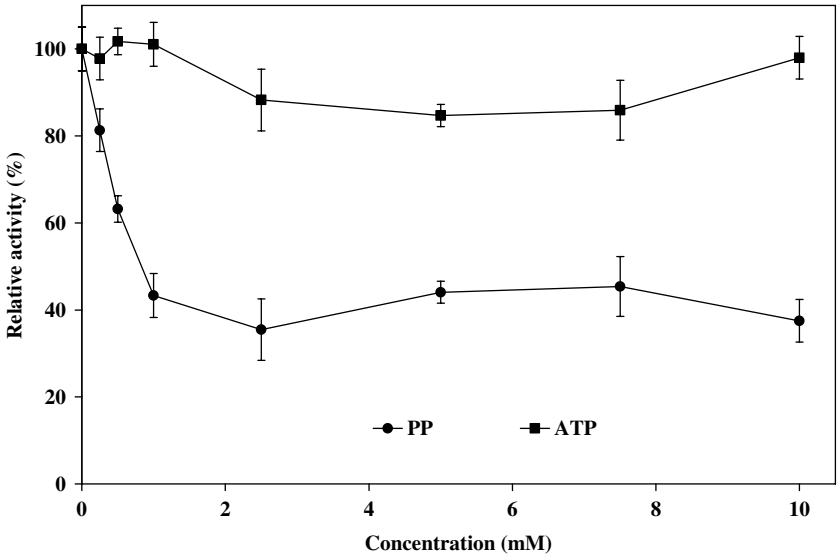

Fig. 1. Changes in Mg^{2+}-ATPase activity of kuruma prawn NAM treated with pyrophosphate or ATP at different levels. Activity was determined after dialysis against 20 mM Tris–HCl containing 0.6 M KCl (pH 7.5). Bars represent standard deviation from four determinations.

in the presence of PP at concentrations up to 1 mM ($P < 0.05$). Thereafter, no further decreases were noticeable when PP at concentrations above 1 mM were used. For NAM treated with ATP, Mg^{2+}-ATPase activity decreased slightly at all concentrations used (0.25–10 mM). The result indicated that the PP was much more effective than ATP in dissociating the actomyosin complex, as evidenced by the lower Mg^{2+}-ATPase activity remaining in the presence of PP. The decrease in Mg^{2+}-ATPase can be used as an indicator for the selective denaturation of actin, which is reported to be the activator for myosin Mg^{2+}-ATPase (Torigai & Konno, 1996). Depending upon the muscle types, Mg^{2+}-ATPase activity of myosin was activated by F-actin (Collins & Korn, 1980) and other co-factors (Stossel & Hartwig, 1975). Therefore, PP could promote the release of actin and make actin more susceptible to denaturation, especially at high salt concentration (0.6 M KCl). In general, myosin plays a role in protection of actin from salt denaturation (Torigai & Konno, 1996). From the result, a greater efficacy of PP in dissociation of kuruma prawn actomyosin was observed when compared with ATP. This result was in contrast to Torigai and Konno (1996) who reported that ATP was more effective in dissociating the carp myofibrils than was PP. This might be due to the differences in the structural arrangement of muscle proteins between fish and prawn. Apart from myosin, muscle of invertebrates, including prawn, contains paramyosin as another component. Paramyosin was found in both smooth and striated muscle of invertebrates and localised to the A band of the striated muscle (Elfvin, Levine, & Dewey, 1976). Generally, the thick filaments of invertebrate paramyosin have greater diameters than have those of vertebrate striated muscle (Levine, Elfvin, Dewey, & Walcott, 1976). Additionally, Epstein, Aronow, and Harris (1976) found that paramyosin is competitive with F-actin for their effect upon myosin. However, the inhibition of actomyosin by paramyosin appears to have a specific molecular requirement. Therefore, the mechanisms of dissociation of actomyosin of fish and invertebrate muscles by PP and ATP are most likely different.

3.2. Effect of pyrophosphate and ATP on the thermal inactivation of myosin

Remaining Ca^{2+}-ATPase activity of NAM treated with PP or ATP at different concentrations, after subjection to incubation at different temperatures ranging from 25 to 45 °C, was determined (Fig. 2). After incubation at 25 °C for 5 min, Ca^{2+}-ATPase activity of the control NAM and NAM treated with 1 and 5 mM ATP increased ($P < 0.05$), suggesting the activation of Ca^{2+}-ATPase at 25 °C. Lorinczy and Belagyi (2001) reported that nucleotides, including ATP, induced global and local changes of myosin head in muscle fibre. However, no change in activity was found in NAM treated with PP at both concentrations ($P > 0.05$). With prior incubation at 30 °C for 5 min, it was found that the activity increased to a greater extent for NAM and NAM treated with 1 mM ATP ($P < 0.05$) but no changes in activity were observed for those treated with 1 mM PP or 5 mM ATP ($P > 0.05$). Conversely, a marked decrease in Ca^{2+}-ATPase activity was noticeable for NAM treated with 5 mM PP subjected to incubation at this temperature ($P < 0.05$). Generally, Ca^{2+}-ATPase activity of NAM was activated to a greater extent when incubated at 30 °C than at 25 °C. The conformational changes during the incubation at both temperatures might enhance hydrolysis of ATP used as the substrate. The ATPase activity of American lobster myofibrils increased with increasing temperatures and showed a maximal value at 35 °C (Shimada, Ushio, & Yamanaka, 2000). The tight

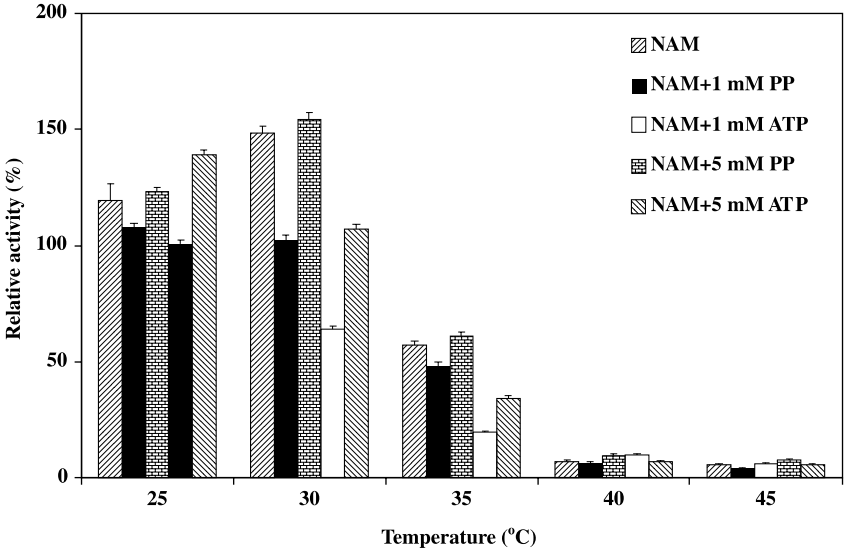

Fig. 2. Changes in Ca^{2+}-ATPase activity of kuruma prawn NAM treated with pyrophosphate or ATP at different levels and incubated at different temperatures. Activity was determined after dialysis against 20 mM Tris–HCl containing 0.6 M KCl (pH 7.5). Bars represent standard deviation from four determinations.

association of myosin heads with the backbone may contribute to the low level of actin-activated myosin ATPase by restricting the binding of myosin head to actin (Frado & Craig, 1989). Furthermore, ATP was reported to affect the structure of myosin filaments (Frado & Craig, 1989). On the other hand, treatment with 5 mM PP resulted in a lower stability of myosin, suggesting the presence of free myosin, due to the dissociation of actomyosin, caused by PP. When NAM and the treated NAM were incubated at 35 °C or higher temperatures, a sharp decrease in Ca^{2+}-ATPase activity was found, with the different degrees, depending upon the treatments. Among all samples, NAM treated with 5 mM PP had the lowest remaining activity. After incubation at 40 or 45 °C, a negligible activity was retained in all samples. The losses in activity of all NAM samples were caused by thermal inactivation. Thermal denaturation of Ca^{2+}-ATPase resulted from unfolding of protein, the exposure of hydrophobic residues and oligomerisation, especially at temperatures above 30 °C and SH oxidation contributed to these changes (Senisterra et al., 1997). From the results, it can be inferred that PP, at high concentration (5 mM), induced dissociation of the actomyosin complex, releasing myosin, which underwent denaturation easily, particularly at higher temperatures.

The residual Ca^{2+}-ATPase activity of kuruma prawn NAM as a function of incubation time at 30 and 35 °C is depicted in Fig. 3. At 30 °C, the residual activity of all treatments decreased gradually with increasing incubation time ($P < 0.05$) (Fig. 3a). At all incubation times tested, NAM treated with 5 mM PP exhibited the lowest remaining Ca^{2+}-ATPase activity ($P < 0.05$). For other samples, no differences were noticeable, except for NAM treated with 1 mM PP, which had a slightly lower activity. The result suggested that myosin was more released from

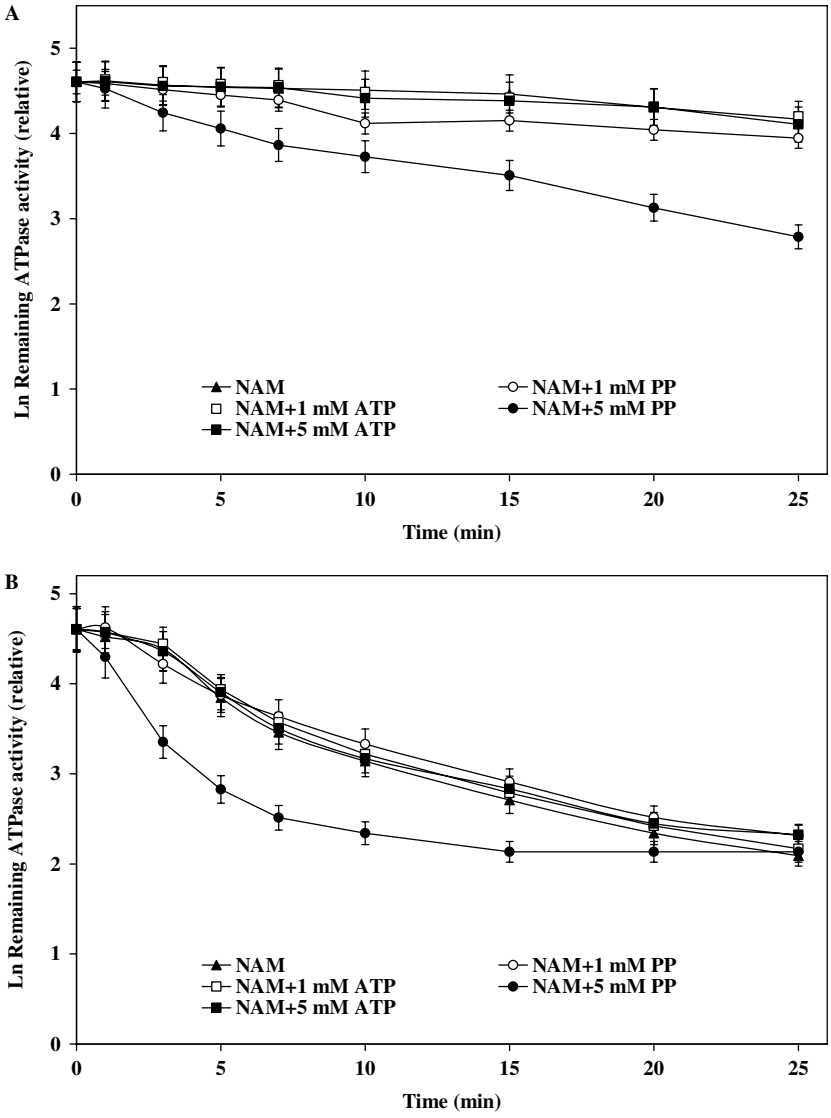

Fig. 3. Changes in Ca^{2+}-ATPase activity of kuruma prawn NAM treated with pyrophosphate or ATP at different levels and incubated at 30 °C (A) and 35°C (B) for different times. Activity was determined after dialysis against 20 mM Tris–HCl containing 0.6 M KCl (pH 7.5). Bars represent standard deviation from four determinations.

NAM when treated with 5 mM PP, compared with NAM alone or other treatments. As a consequence, free myosin could be more susceptible to thermal denaturation, as indicated by the lowered remaining Ca^{2+}-ATPase activity. After incubation at 35 °C for different times (Fig. 3b), it was noted that Ca^{2+}-ATPase activity of all treatments decreased rapidly within 10 min of incubation and the rate of decrease was more intense than that observed for treatment at 30 °C (Fig. 3a). NAM treated with 5 mM PP also exhibited the lowest activity at all incubation times used ($P < 0.05$). No differences were observed among other samples ($P > 0.05$). This indicated that treatment of NAM with PP, at 5 mM, induced the dissociation of actomyosin effectively, leading to release of free myosin to a greater extent than other treatments. Myofibrils containing free myosin generally exhibited a rapid drop phase and a slow decreasing phase of Ca^{2+}-ATPase activity, representing the thermal inactivation for free myosin and remaining actomyosin, respectively (Kawakami, Morita, Takahashi, & Yasui, 1971). From this result, Ca^{2+}-ATPase activity of PP treated NAM had a lower thermal stability, than had ATP- treated NAM. This result was not in accordance with Torigai and Konno (1996) who found that carp myofibrils showed a lower thermal stability when treated with ATP than when treated with PP. The lower thermal stability of Ca^{2+}-ATPase and lower salt stability of Mg^{2+}-ATPase of kuruma prawn NAM treated with a higher amount of PP confirmed that PP could dissociate the actomyosin complex of prawn muscle more effectively than could ATP.

3.3. Effect of pyrophosphate and $MgCl_2$ on the release of actin from NAM

Protein patterns of supernatant obtained after precipitation of NAM treated with PP at different concentrations with $MgCl_2$, at various low salt concentrations are shown in Fig. 4. NAM consisted of myosin heavy chain and actin as the major constituents (lane 1). Protein with the apparent molecular weight of approximately 120–140 kDa was also found. It is reported that paramyosin is a protein commonly found in invertebrate muscle (Elfvin et al., 1976; Levine et al., 1976). From the result, the actin band intensity of 1 mM PP- treated NAM supernatant (lane 3) was not different from that of the control NAM (lane 2). A marked increase in band intensity was found in the presence of 5 mM $MgCl_2$ (lane 4). However, the actin band intensity was reduced when 10 mM $MgCl_2$ was combined. For 5 mM PP-treated NAM supernatant, actin band intensity was more pronounced when $MgCl_2$, at levels of 5 and 10 mM, was used (lanes 7 and 8), compared with that without $MgCl_2$ (lane 6). In the presence of 1 mM PP, MHC band intensity was greater than that of other treatments, indicating the loss of MHC in the supernatant. From the result, tropomyosin band intensity in the supernatant was inversely proportional with the band intensity of actin released in the supernatant. It is postulated that the tropomyosin strand in the groove of F-actin might be released during the dissociation of actomyosin. As a consequence, the free tropomyosin was probably denatured and precipitated at high salt concentration. Therefore, a lower band intensity was observed when a greater band intensity of actin released in the supernatant was noticeable. Apparently, PP and $MgCl_2$ at the appropriate concentrations could enhance the dissociation of actomyosin complex effectively, as evidenced by the increased band intensity of soluble denatured actin in the supernatant.

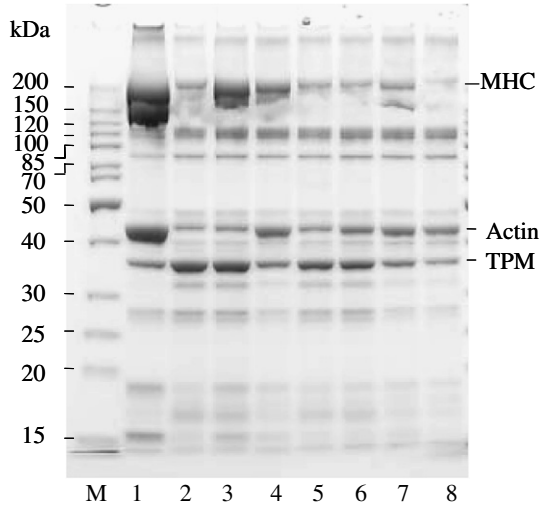

Fig. 4. Protein patterns of supernatant of kuruma prawn NAM treated with pyrophosphate and $MgCl_2$ at different levels after dialysis against 40 mM Tris–HCl containing 50 mM KCl (pH 7.5), followed by centrifugation at 10,000*g* for 30 min. The supernatant was subjected to SDS-PAGE analysis. MHC: myosin heavy chain; TPM: tropomyosin; M: molecular weight standards. lane 1: NAM (without treatment); lane 2: the control NAM; lanes 3, 4 and 5: NAM treated with 1 mM PP in the presence of 0, 5 and 10 mm $MgCl_2$, respectively; lanes 6, 7 and 8: NAM treated with 5 mM PP in the presence of 0, 5 and 10 mm $MgCl_2$, respectively.

For the supernatant of NAM treated with ATP in the presence of $MgCl_2$ at different levels, the actin band intensity in the supernatant of all treatments was similar to that of the control NAM (data not shown). This suggested that ATP had a low ability to dissociate the actomyosin complex in prawn muscle.

Mg^{2+}-ATPase activity of NAM treated with PP or ATP at different levels in the presence of $MgCl_2$, at 5 and 10 mM, is shown in Table 1. Mg^{2+}-ATPase activity of

Table 1
Effect of pyrophosphate and ATP, in the presence of various concentrations of $MgCl_2$, on Mg^{2+}-ATPase activity of kuruma prawn NAM (% of original activity)

$MgCl_2$ (mM)	PP		ATP	
	1 mM	5 mM	1 mM	5 mM
No addition	60.7 ± 4.9^{bc}	43.3 ± 1.3^{a}	94.1 ± 5.2^{d}	95.7 ± 3.5^{d}
(5)	52.7 ± 5.1^{b}	41.5 ± 3.6^{a}	100 ± 6.0^{e}	96.1 ± 5.4^{de}
(10)	65.9 ± 4.0^{c}	55.1 ± 3.5^{b}	108 ± 8.0^{e}	92.4 ± 3.7^{d}

Values are means ± SD from four determinations.
Different superscripts indicate significant differences ($P < 0.05$).

NAM decreased when treated with 5 mM PP ($P < 0.05$). Lower activity was found in NAM treated with PP in the presence of $MgCl_2$ ($P < 0.05$). The result was in accordance with the greater release of actin into supernatant when NAM was treated with 5 mM PP in combination with $MgCl_2$ (Fig. 4).

From the results, a slight decrease in Mg^{2+}-ATPase activity was noticeable when NAM was treated with 1 mM PP, either with or without $MgCl_2$. For NAM treated with ATP at all levels, only a slight decrease in Ca^{2+}-ATPase was observed. However, a lower activity was found when $MgCl_2$ was incorporated. This result was coincidental with no marked changes in actin band intensity found in the supernatant of NAM treated with ATP after dialysis at low salt concentration and centrifugation. This result reconfirmed the low dissociation efficacy of actomyosin in prawn muscle by ATP.

4. Conclusion

PP was superior to ATP in dissociating the actomyosin complex in kuruma prawn muscle. The enhanced efficiency of PP was observed in the presence of appropriate levels of $MgCl_2$.

Acknowledgement

Authors would like to thank the Japanese Society for the Promotion of Science for the financial support.

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