Synergistic Solubilization of Porcine Myosin in Physiological Salt Solution by Arginine

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Abstract

Myosin is an important protein resource for food industries and has a bipolar filamentous structure that is composed of subfilaments that occur in vivo. It has been shown that a high ionic strength is required to prevent myosin from forming filamentous structures and to solubilize the protein in aqueous solution. In the presence of 100 to 200 mM NaCl, 50 mM arginine was more effective than other additives tested, including NaCl, in myosin solubilization. Before reaching equilibrium solubility, the myosin solution was initially supersaturated upon the dilution of a stock myosin solution in 1 M NaCl into the test solvents. Arginine slowed the process of equilibration and stabilized the supersaturated solution more effectively than other additives. No structural changes in myosin caused by arginine were observed, which indicated that arginine enhanced the solubility of myosin in a physiological salt solution without affecting the structure.

Keywords: myosin, arginine, supersaturation

1. Introduction

Meat is rich in high-quality proteins and contains all of the essential amino acids for humans [1]. However, meat has not been fully utilized as a protein supplement to the same extent that milk or soybean products have been utilized because of the low solubility of myosin, which constitutes approximately 50 % of myofibrillar proteins. The low solubility of myosin results from the spontaneous formation of filaments that occur in vivo [2–4]. Myosin is practically insoluble in aqueous solution at low ionic strength but is increasingly soluble at high salt concentrations [5–9].

Solubilization of insoluble myosin has been achieved by adding 5 mM histidine in low ionic strength solutions (1-5 mM KCl, pH 7.5) [10–12]. The mechanism by which histidine solubilizes myosin appears to involve structural changes in monomeric myosin and the resulting inhibition of native myosin filament formation [11]. When the salt concentration is increased to a physiological level (0.15 M), 5 mM histidine no longer shows such effects [12]. Considering the observed structural changes caused by histidine, which may limit myosin application, it would be advantageous to achieve high myosin solubility without structural changes even in physiological salt solutions. We have investigated several additives, including histidine and arginine, for their effects on myosin solubility as a function of salt concentration and on the myosin structure.

Arginine is one of the most common solvent additive that suppress protein aggregation without altering or destabilizing the tertiary structure of the proteins [13,14]. Arginine has been used in various applications, including suppression of reductant- or heat-induced aggregation [15–18], enhancement of protein refolding [17–22], crystallization [23], and improved performance of column chromatography [24–27]. The molecular mechanisms by which arginine suppresses protein aggregation have been proposed: (i) the guanidinium group of arginine interacts with aromatic residues by cation-π interactions [28–31], which has

been observed in the enhanced solubility of aromatic compounds [32,33], and (ii) weak preferential exclusion of arginine from the protein surface, which is associated with its weak binding to the proteins and increases the activation energy toward aggregation [34,35]. Thus, we have focused on the solubilization effects of arginine and its influence on myosin structure.

2. Materials and methods

2.1. Materials

Sodium chloride (NaCl) and sodium phosphate were obtained from Nacalai Tesque Inc. (Tokyo, Japan). L-arginine hydrochloride (Arg), glycine (Gly), L-histidine (His), L-lysine hydrochloride (Lys) and guanidine hydrochloride (Gdn) were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). All chemicals used were of reagent grade and were used as received.

2.2. Purification of myosin

Porcine myosin extract was prepared as follows: myosin was extracted from 120 g of lean porcine meat from the inner thigh (semitendinosus) with 450 ml of buffer containing 100 mM pyrophosphate and 5.0 mM MgCl2 (pH 7.0) using a homogenizer at 5 °C for 60 min. The myosin extract was stored at -25 °C. After thawing at room temperature, the myosin extract was dialyzed against 1.0 mM KCl and 2.0 mM Na-phosphate buffer (pH 7.0). The precipitates of the dialyzed solution were washed with 1.0 mM KCl and 2.0 mM Na-phosphate buffer (pH 7.0) and were used as the source of myosin. SDS-PAGE analysis of the prepared myosin was shown in supplemental Figure 1.

2.3. Preparation of myosin solution

The frozen myosin extract (72 mg) was dissolved with 20 mM sodium phosphate buffer containing 1 M NaCl (pH 7.5). After incubation for 1 hour at 25 °C, the myosin solution was centrifuged at 18,800 ×g for 20 min. After centrifugation, the supernatant containing 1 M NaCl was used as the stock myosin solution. As previously reported, myosin was highly soluble and stable in this solvent, which was largely in the monomeric structure [5,6].

2.4. Measurement of myosin solubility

The stock myosin solution was diluted from 20 mM sodium phosphate buffer and 1 M NaCl (pH 7.5) into the test solvents for a final concentration of 50 mM additives (Arg, Lys, NaCl, His, Gly and Gdn) in the presence of 0.05-0.3 M NaCl in the same buffer. The sample solution was incubated for 1 hour at 25 °C and centrifuged at 18,800 ×g for 20 min. The supernatant was diluted 10-fold with the respective buffer to reduce the myosin concentration for the fluorescence measurements. The myosin concentration in the supernatant, which corresponds to the solubility, was measured using a fluorescence spectrofluorometer (FP-6500, Jasco Corp.; Tokyo, Japan) with a 1-cm path-length quartz cuvette. The solution was excited at 280 nm (3 nm slit-width), and the emission spectrum (5 nm slit-width) was collected at 25 °C.

2.5. Circular dichroism (CD) spectra

Far-UV circular dichroism (CD) measurements were performed on a spectropolarimeter (J-720W; Jasco Corp.) using a 1-mm path-length quartz cuvette. The CD spectra of the sample myosin solutions were measured at 25 °C. The CD spectra of the samples were corrected by subtracting the spectra of the respective solvents.

3. Results

3.1. Measurement of myosin concentration by fluorescence intensity

The concentration of myosin in the test solvents was determined by the intrinsic tryptophan fluorescence of myosin as opposed to the absorbance at 280 nm. This approach was used because the formation of filamentous structures causes significant light scattering and makes UV absorbance unreliable for concentration determination. Figure 1 demonstrates the reliability of fluorescence for myosin concentration measurements. Figure 1A shows the fluorescence emission spectra of a serially diluted myosin stock solution of known protein concentration. The spectral shape was independent of dilution with a peak at 334 nm, which indicated no effects of dilution and protein concentration on the tryptophan environments of myosin; note that this fluorescence peak position indicates that the fluorescent tryptophans are at least partially buried inside the tertiary structure of myosin. The fluorescence intensity at 334 nm of these myosin samples were plotted against the concentration of myosin. As shown in Fig. 1B, the fluorescence intensity linearly increased with the sample concentration. The straight line corresponds to a linear regression with a correlation coefficient of 0.999. Thus, the myosin concentration can be reliably determined from the fluorescence intensity at 334 nm. Furthermore, the fluorescence spectra of myosin were not affected by the additives tested and NaCl concentrations (date not shown).

3.2. Effect of arginine on myosin solubility

Figure 2 shows the effects of 50 mM Arg, Lys, NaCl, His, Gly and Gdn on the solubility of myosin in the presence of NaCl concentrations (50, 100, 150, 200, 250 and 300 mM); note that an additional 50 mM NaCl was present over the basal NaCl concentration (a total of 100 mM NaCl for the first column, which corresponds to the 50 mM basal NaCl

concentration). The myosin solubility in the absence of an additional 50 mM NaCl (at the basal salt concentration) is shown as "none" in Fig. 2. As described in the methods section, the myosin solubility was determined in the test solvents by diluting the stock myosin solution in 1 M NaCl, where myosin is largely monomeric, into the test solvents. Thus, myosin filament formation may vary depending on the salt concentration, which suggests that more filaments may be present at lower salt concentrations. The solubility shown in Fig. 2 is the value at 1 hour after dilution and may not be the final equilibrium value, particularly in the presence of 50 mM Arg (for reasons described later).

At the 50 mM basal NaCl concentration (first column in Fig. 2), 50 mM Arg and Lys showed insignificant effects on myosin solubility compared with 50 mM NaCl (total 100 mM NaCl), whereas 50 mM His and Gly slightly reduced myosin solubility compared with the three additives. It is interesting that 50 mM Gdn showed reduced myosin solubility, although only slightly. Thus, the effects of these five additives (i.e., Arg, Lys, His, Gly and Gdn) were marginal at this low basal salt concentration. A small but significant effect of 50 mM Arg was observed at a 100 mM NaCl concentration (second column in Fig. 2). Arg and Lys increased the myosin solubility compared to NaCl alone, and Arg was more effective; 50 mM NaCl was slightly effective when compared with its absence (see "none"). At this NaCl concentration (second column in Fig. 2), 50 mM His, Gly and Gdn were essentially ineffective as myosin solubility in these solvents was nearly identical to the solubility in 100 mM NaCl alone ("none"). At a 150 mM NaCl concentration (third column in Fig. 2), a much stronger effect of Arg was observed. At this NaCl concentration, 50 mM Arg increased the myosin solubility by more than twofold over the level achieved by 50 mM NaCl (200 mM total NaCl) and almost threefold over the value at 150 mM NaCl (see first black bar, "none"). Lys was also effective; however, its effect was greatly reduced when compared to Arg. At 150 mM NaCl, 50 mM His, Gly and Gdn were much weaker than NaCl in solubilizing myosin and nearly identical to the value in the absence of 50 mM salt (but in the presence of 150 mM salt). The lack of

solubilization effects of His at 150 mM NaCl is consistent with previous reports [12]. When the basal salt concentration was increased to 200 mM (fourth column in Fig. 2), the solubilization effect of 50 mM NaCl (total 250 mM NaCl) overwhelmed the additive effects, which is seen as the large difference between 200 mM salt (first black bar, "none") and 250 mM salt (fourth dotted bar). The solubility of myosin at 250 mM total NaCl was only slightly less than Arg and Lys. At 200 mM NaCl, 50 mM His was as effective as 50 mM NaCl, whereas Gly and Gdn were significantly less effective. At 250 and 300 mM NaCl (fifth and sixth column in Fig. 2), the high salt concentration essentially determined the myosin solubility, which became independent of the additives tested.

The solubility experiments were performed by diluting a myosin stock solution in 1 M NaCl. Upon dilution into the test solvents, the myosin should spontaneously form filaments depending on the NaCl concentration and presence of additives until reaching a monomer/filament equilibrium, i.e., equilibrium solubility. Immediately after dilution, the myosin solubility may be much higher in the test solvents than the final equilibrium solubility. Namely, the initial myosin solution is supersaturated with myosin monomer. Thus, the time course of myosin solubility was followed immediately after dilution of the stock solution. Figure 3 shows the myosin solubility changes with time in the presence of 50 mM additive and a NaCl concentration of 150 mM. There appeared to be no significant differences in myosin solubility at 1 hour or 45 hours after dilution in the presence of 50 mM Gly and Gdn, which indicates the establishment of solubility equilibrium within 1 hour. Thus, myosin filaments were rapidly formed in these solvents. There appeared to be a slight time dependence of myosin solubility in 50 mM His, which reached equilibrium approximately 5 hours after dilution. The initial solubility of ~35 % reached a plateau value of ~30 % at approximately 5 hours. Although the solubility was greater in 50 mM NaCl (total 200 mM) than in 50 mM His, the time to reach equilibrium was similar, which was determined to be 5 hours by double exponential fitting (initial solubility of 60 % to ~55 % after 5 hours).

Compared to the above test solvents, an increased delay in reaching equilibrium was observed in 50 mM Arg and Lys. The myosin solubility in 50 mM Arg decreased after 9 hours of incubation from 85 % to a plateau value of 78 %. It appeared that the solubility equilibrium was attained after approximately 24 hours, which was determined by curve fitting. Because of large data scattering for 50 mM Lys, there was ambiguity in the data fitting, and approximately 10 hours was estimated for the solution to reach equilibrium.

3.3. Effect of arginine on myosin structure

Previously, His has been shown to increase the myosin solubility in low ionic strength solutions by altering the structure of myosin [11]. Thus, we examined whether such structural changes occur in Arg and Lys, which clearly enhanced the myosin solubility at 150 mM NaCl. We examined the secondary structure of myosin in 50 mM Arg or Lys at 150-300 mM NaCl. Figure 4 shows the CD spectra of myosin solubilized in 50 mM Arg or Lys at 0.15-0.30 M NaCl. As a control, the CD spectrum was collected in 350 mM NaCl. All CD spectra of myosin were identical within experimental errors, which indicate that an identical myosin structure in the presence of 50 mM Arg or Lys with 50-300 mM NaCl or in the presence of 350 mM NaCl was present, and suggests a different solubilization mechanism for Arg and Lys compared to His.

4. Discussion

Myosin forms filamentous structures at low salt concentrations, which makes it practically insoluble in water or dilute salt solutions [5]. His at 5 mM has been shown to increase myosin solubility in low salt solutions and is accompanied by structural changes [11]. Here, we tested the effects of 50 mM Arg, Lys, NaCl, His, Gly and Gdn on myosin

solubility in the presence of NaCl at different concentrations (50 to 300 mM). The solubility experiments were performed by diluting a stock myosin solution in 1 M NaCl, in which myosin is largely monomeric [5,6], which we confirmed. Assuming that the monomeric myosin is soluble at low and high salt concentrations, it is the formation of filaments that determine the myosin solubility; thus, the protein precipitates are composed wholly of filaments and not the monomers. This finding indicates that the effects of the test solvents on myosin solubility are the results of their effects on the monomer-filament equilibrium. Marginal solubilization effects of all additives tested were observed in the presence of 50 and 100 mM NaCl, unlike the observed solubilization of His in dilute salt solutions, which suggest that the effects of Arg, Lys and other additives are mechanistically different from those of His. Marginal solubilization effects of Arg and Lys at 50-100 mM NaCl may be because of the strong tendency of myosin to self-associate into the filaments at low salt concentrations. When the tendency for self-association becomes weaker at increasing salt concentrations (150-200 mM), the solubilization effects of Arg and Lys are more apparent. Further increases in the salt concentration (250-300 mM) highly favor monomeric myosin, and the effects of the additives are overwhelmed by salt effects on self-association. Thus, the interaction of these additives with myosin (most likely monomeric myosin) that affects myosin solubility may be altered by the presence of salt and may depend on the salt concentration.

Regardless of the solubilization mechanism, two basic amino acids, Arg and Lys, are more effective than His and Gly. As His and Gly are neutral in net charge at the experimental pH of 7.5, a net positive charge (or positively charged side chain) appears to play a role in their interaction with myosin and effects myosin solubility. Electrostatic interactions have been shown to be involved in myosin-myosin interactions in the formation of the filamentous structures [36]. It is expected that high salt concentrations disrupt electrostatic interactions and prevents filament formation, which is observed by the high myosin solubility. A specific interaction of the Arg or Lys cation with myosin may also disrupt the electrostatic interactions

more effectively than NaCl, which increases the myosin solubility over the solubility achieved by salt alone. Arg was always more effective than Lys, which indicates that the positive charge and structure of the side chain play a role. The critical role of the guanidinium group has been implicated in the effectiveness of Arg in the suppression of protein aggregation [17,18]. The guanidinium group alone was insufficient in myosin solubilization, which was shown by the lack of increased solubilization effects because of Gdn. Under the experimental conditions, His displayed no or marginal solubilization effects, which differed from previously reported results [10–12] . Such His effects were attributed to the structural changes of myosin conferred by His [11], which indicates that His has no effect on myosin structure under the present experimental conditions. Further, the effects of Arg and Lys are mediated by their interaction with native myosin, which was indicated by the lack of structural changes.

Interestingly, the equilibrium solubility was attained extremely slowly in the presence of 50 mM Arg with 150 mM NaCl, which took more than 20 hours after dilution of the stock myosin solution in this solvent. Namely, self-association of the myosin monomer to the filaments was slow in this solvent system. The rate of this transition from monomer to filament becomes faster in Lys and further in NaCl. In Gly and Gdn, the transition appeared to occur within one hour. Myosin is in a monomeric structure in 1 M NaCl [5,6]. Upon dilution into 50 mM Gly or Gdn in 150 mM NaCl, the solubility of myosin rapidly reaches equilibrium, which means a low activation energy of the monomer-filament transition. In the presence of 50 mM Arg, the initial solubility is much higher than the equilibrium solubility (~100 % vs. < 80 %). Arg increases the activation energy. Because of this high energy barrier, the supersaturated myosin solution is kinetically stabilized by Arg. The binding of Arg to monomeric myosin may be involved in the stabilization of the monomer as the free energy to dissociate the bound Arg should increase the energy barrier.

In conclusion, Arg increased the equilibrium solubility and activation energy of self-association of monomeric myosin in a physiological salt solution. This stabilization of the

myosin monomer by Arg occurred without altering the structure of myosin. It would be of great interest to test the utility of arginine in processing meat products containing myosin based on its ability to increase the solubility of monomeric myosin.

Acknowledgments

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Figure Captions

Figure 1. (A) The intrinsic emission fluorescence spectra of myosin at different protein concentrations. The stock sample solution was serially diluted (1- to 16-fold) with 20 mM sodium phosphate (pH 7.5). The fluorescence emission spectra were collected at 25 °C with an excitation wavelength of 280 nm. (B) The fluorescence intensity at 334 nm against myosin concentrations. Data are taken from Fig. 1A. The plot was fit by linear regression with a correlation coefficient of 0.999. The measurements were performed three times, and the error bars depict the standard deviation of the mean.

Figure 2. Myosin solubility in 50 mM additives or "None" indicated in the figure as a function of basal NaCl concentration. A stock myosin solution in 1 M NaCl was diluted into the test solvents and incubated for 1 h. The concentration of myosin in 50 mM additives or "None" in the presence of 50-300 mM NaCl was determined by measuring the intrinsic fluorescence intensity at 334 nm. The myosin solubility in test solvents was normalized to the value in 50 mM Arg and 300 mM NaCl, which was set as 100 %. The measurements were performed three times, and the error bars depict the standard deviation of the mean.

Figure 3. Time course of concentration changes of myosin. The concentration of myosin in 50 mM additives in the presence of 150 mM NaCl was followed with incubation for 1 hour after dilution into the test solvents. The continuous line though the data points is a fit with a double exponential with offset.

Figure 4. Far-UV CD spectra of myosin in the presence of Arg or Lys as a function of NaCl concentration. The far-UV CD spectra were measured in 50 mM additives (Arg, Lys and

NaCl) in the presence of 150-300 mM NaCl. Each CD spectrum was shifted downward by 2 mdeg for visual comparison. a: 50 mM Arg/150 mM NaCl; b: 50 mM Arg/200 mM NaCl; c: 50 mM Lys/200 mM NaCl; d: 50 mM Arg/300 mM NaCl; e: 50 mM Lys/300 mM NaCl; f: 350 mM NaCl.

Fig.1

Synergistic Solubilization of Porcine Myosin in Physiological Salt Solution by Arginine

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Fig.1

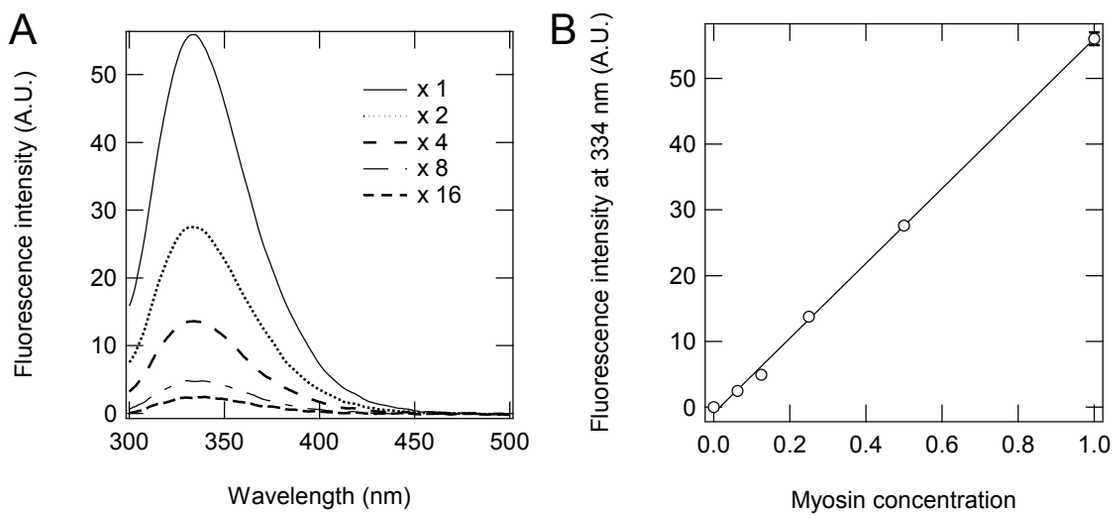

Fig.2

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Fig.2

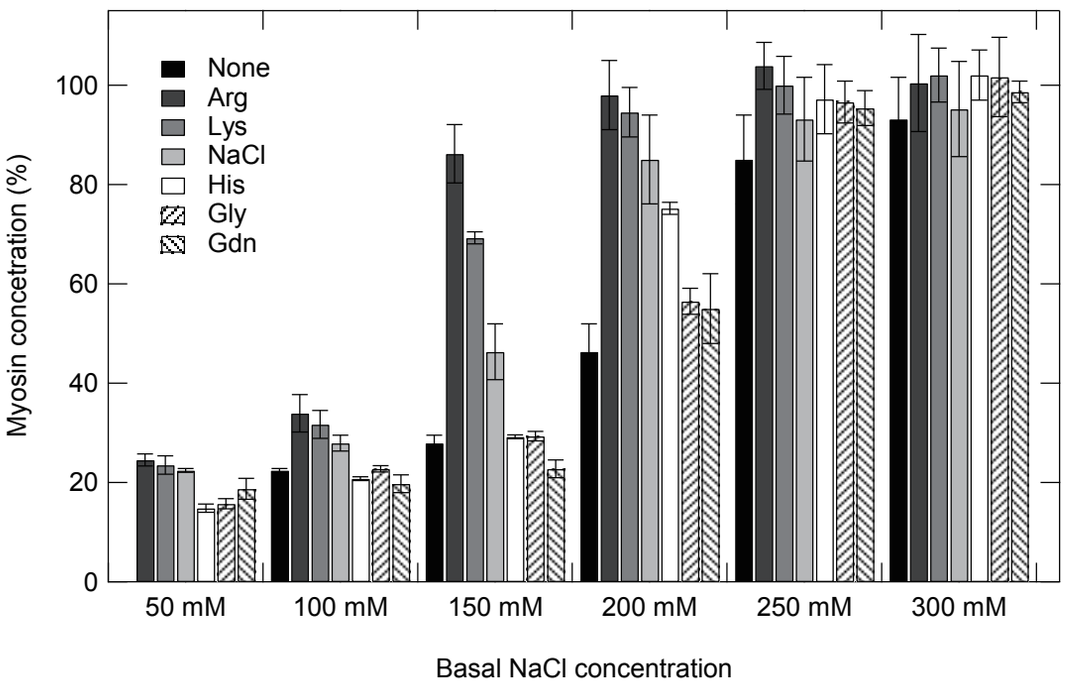

Fig.3

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Fig.3

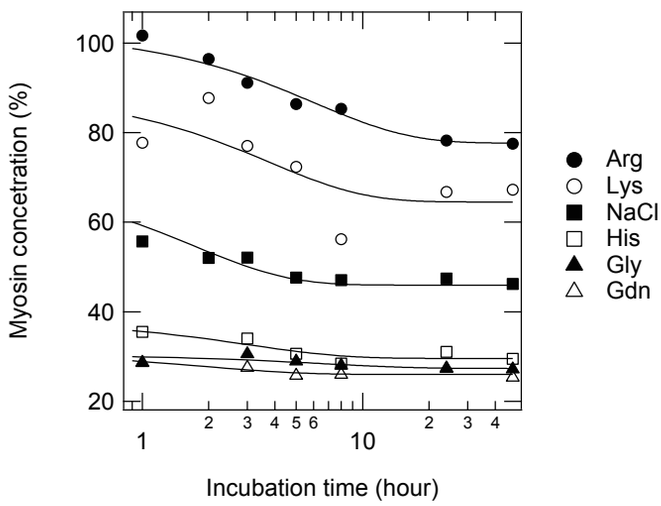

Fig.4

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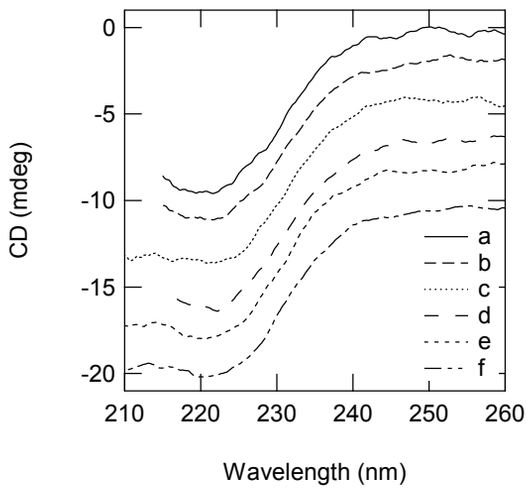
Fig.4



Supporting Information for Synergistic Solubilization of Porcine Myosin in Physiological Salt Solution by Arginine

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SDS–PAGE

The prepared myosin sample was resolved in loading buffer containing 2% (w/v) SDS, 10% (w/v) glycerol, 0.04 M DTT, 0.01% (w/v) bromophenol blue, and 62.5 mM Tris–HCl (pH 6.8). The samples were boiled for 5 min; then the samples and the standard ladder marker were loaded on 15% polyacrylamide gel. The standard marker for SDS–PAGE was obtained from Apro Life Science Institute Inc. (Tokushima, Japan).

Supplemental Fig. 1. SDS-PAGE analysis of the prepared myosin. Lane 1: The standard ladder. Lane 2: The myosin dissolved with 50 mM NaCl and 20 mM sodium phosphate buffer (pH 7.5). The filament formed and monomeric myosin is indicated by the red and black arrows, respectively.