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GELATION OF MYOSIN FILAMENT UNDER HIGH HYDROSTATIC PRESSURE

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Abstract

Gelation of myosin filaments under high hydrostatic pressure was investigated. At room temperature myosin filaments in 0.1 M KCl and 20 mM phosphate, pH 6.0, formed gels at 280 MPa and a protein concentration above 2 mg/ml or at 210 MPa and 3 mg/ml. However, no pressure-induced gelation was observed at 140 MPa. The gel strengths of pressure-induced gels were almost proportional to the protein concentration and they were comparable to those of heat-induced gels. The association of myosin filaments and the structural disturbance seemed to coincide with the formation of the gel. The microstructure of the pressure-induced myosin gel consisted of a fine network and was similar to that of heat-induced myosin filament gels at low ionic strength. Myosin light chains 1 and 3 were easily dissociated from the pressure-induced gels.

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Introduction

Myosin is the major component of skeletal muscle myofibrils and it forms a gel when it is heated at appropriate salt concentrations, pH levels, and protein concentrations. Such heat-induced gelation of myosin contributes to water holding capacity or binding properties of meat (Asghar et al., 1985).

Application of high hydrostatic pressure to various food stuffs including meat is of interest (Macfarlane, 1985). The application of high hydrostatic pressure to some food stuffs improves texture; changes functional properties of proteins, such as gelling properties; and modifies enzyme activities. In addition, some microorganisms are destroyed by this high pressure (Hayashi, 1989).

The purpose of this study was to determine the effects of high hydrostatic pressure on myosin. Heat-induced myosin gels in low ionic strength solutions such as 0.1 M KCl, show higher rigidity as compared to gels formed in high ionic strength (0.6) and optimum pH (6) for thermogelation of myosin (Ishioroshi et al., 1979, 1983). Myosin is in the monomeric state in a high ionic strength solution, but exists as filaments at low ionic strength. The state of myosin affects the microstructure of the heat-induced gel as well as the gel strength. A heat-induced gel formed from filamentous myosin has a fine strand network structure and shows high gel strength, whereas monomeric myosin forms less rigid and aggregated gels (Hermansson et al., 1986; Yamamoto et al., 1988). From these points of view, we have performed the pressurization experiment in 0.1 M KCl at pH 6.0 to determine if pressure is a contributing factor in the gel formation of myosin.

Materials and Methods

Myosin preparation and pressurization

Myosin was prepared from rabbit fast muscle, that is, back and white portion of leg muscles, and purified by the method of Offer et al. (1973). Myosin was dissolved in 0.5 M KCl, then dialyzed against 0.1 M KCl and 20 mM K phosphate, pH 6.0, to prepare the filaments.

The myosin filament suspensions were kept in plastic tubes (14 mm in diameter and 25 mm in depth), sealed with a silicon rubber plug without trapping an air bubble, placed in a pressure vessel filled with water, and pressurized. Three pressure levels (140, 210 and 280 MPa) and duration of 1, 2.5, 5, 10, 15 and 30 min at each pressure level were used. Pressurization was done in a French Press (SLM Aminco, USA) at room temperature.

Gel strength measurement

When a gel was formed by pressurization, a cylindrical plunger with a flat end 5 mm in diameter, was pushed 5 mm into the gel at a speed of 10 mm/min and the peak force in gram was recorded. We assumed this value as the strength of the gel. In case that the gelation did not occur by pressurization alone, the sample was transferred to a cuvette then heated at 65°C and the rigidity of the heat-induced gel was measured as described previously (Yamamoto et al., 1989).

Transmission electron microscopy

Pressurized myosin filament suspensions were diluted with 0.1 M KCl and 20 mM K phosphate, pH 6.0, to the protein concentration of 0.15 mg/ml. A drop of the samples was put on carbon-coated 400-mesh grids, which were rendered hydrophilic by glow discharge, rinsed with several drops of 0.1 M KCl, then negatively stained with several drops of 2% unbuffered uranyl acetate. The specimens were observed in a Hitachi H-800 transmission electron microscope at an accelerating voltage of 100 kV.

Scanning electron microscopy

A pressure-induced myosin filament gel was cut into 1-2 mm cubes and fixed in 2.5% glutaraldehyde in 0.1 M phosphate (pH 7.0) and dehydrated in a graded series of ethanol (50%, 70%, 90%, and 100%). After dehydration, they were transferred into isoamyl acetate and dried by the carbon dioxide critical point method, then coated with gold in a vacuum evaporator. The structure of pressure-induced gel of myosin filaments was observed in a JEOL JSM-T100 scanning electron microscope.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of the supernatant and the precipitate after centrifugation of gels was performed with a minislab gel apparatus previously described (Yamamoto and Moos, 1983). The acrylamide concentration was 7.5%.

Results

Fig. 1 shows the gel strengths of pressure-induced gels at 280 MPa as a function of duration of pressurization. After 1 min of pressurization, gelation occurred in the samples having a protein concentration above 2 mg/ml. However, the gel strengths of 1 min pressurized gels were found to be very weak in each sample. The gel strengths increased with the time of pressurization until 10 min. After 10 min, the gel strengths remained at almost constant levels in every sample. The gel strength of pressure-induced gels was nearly proportional to the protein concentration. Also the pressure-induced gel appeared to be more viscous than the heat-induced gel. A myosin filament suspension at a protein concentration of 1 mg/ml did not form a firm gel at 280 MPa. It appeared to be in an intermediate state between a gel and a sol even in samples obtained after 30 min of pressurization.

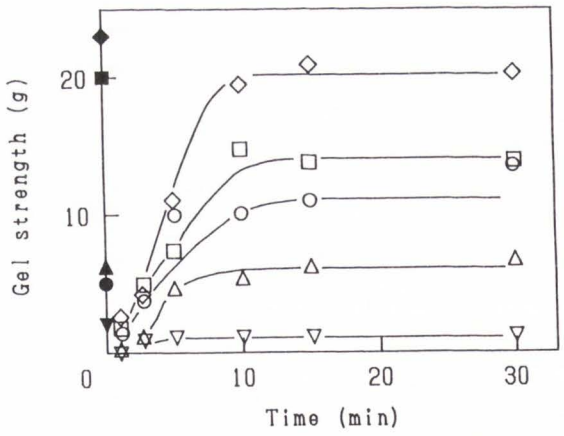

Figure 1. Gel strengths of pressure-induced gels at 280 MPa. Myosin filament suspensions at the protein concentration of 1 (▽), 2 (△), 3 (○), 4 (□), and 5 mg/ml (◇) were pressurized. The gel strength was measured as indicated in the Materials and Methods section. Solid symbols indicate gel strength of heat-induced gels without pressurization at each protein concentration.

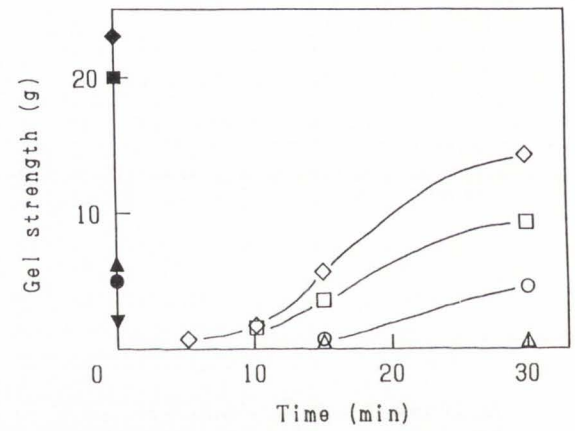

Figure 2. Gel strengths of pressure-induced gels at 210 MPa. Experimental conditions and the symbols, except 1 mg/ml of the sample which did not form a gel, are the same as those in Fig. 1.

The strengths of pressure-induced gels at 210 MPa are shown in Fig. 2. When compared with the pressurization at 280 MPa in Fig. 1, it took a longer time to form a gel at this pressure. Pressure-induced gelations of the

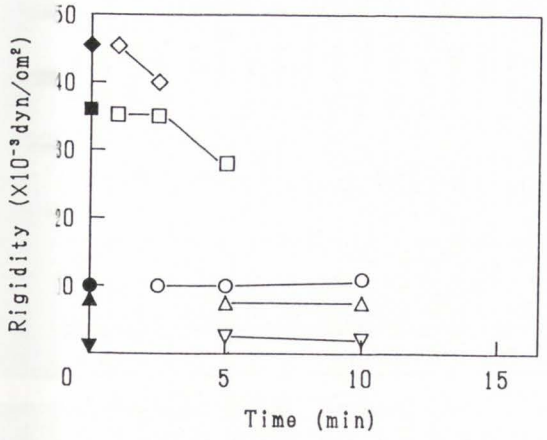

Figure 3. Rigidities of the gels pressurized at 210 MPa then heated at 65°C for 20 min. The symbols are the same as those in Fig. 1.

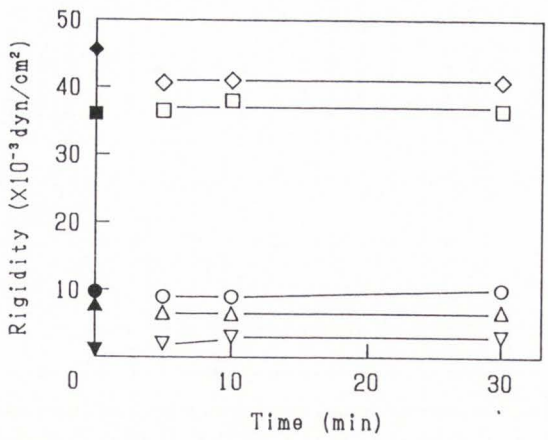

Figure 4. Rigidities of the gels pressurized at 140 MPa then heated at 65°C for 20 min. The symbols are the same as those in Fig. 1.

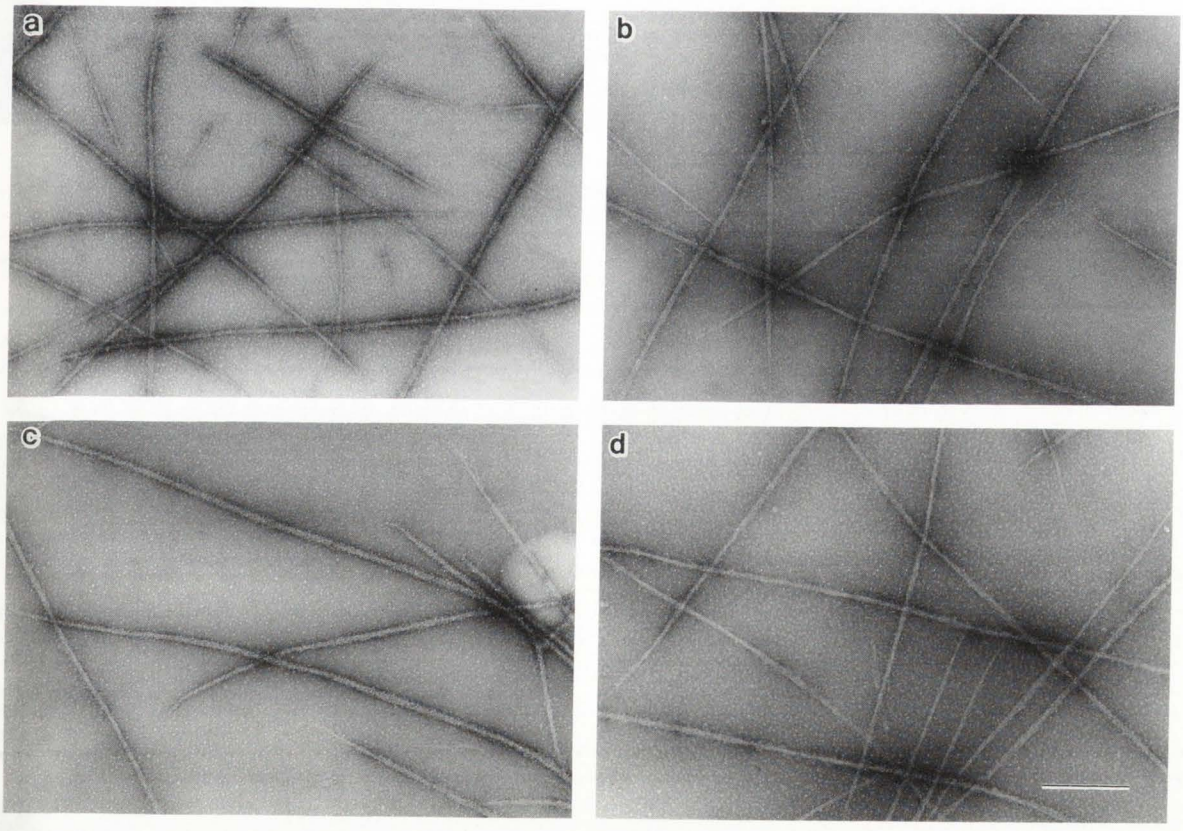

Figure 5. Transmission electron micrographs of myosin filaments pressurized at 140 MPa. a, unpressurized control; b, c, and d are pressurized for 5, 10, and 30 min, respectively. Bar indicates 0.5 μm.

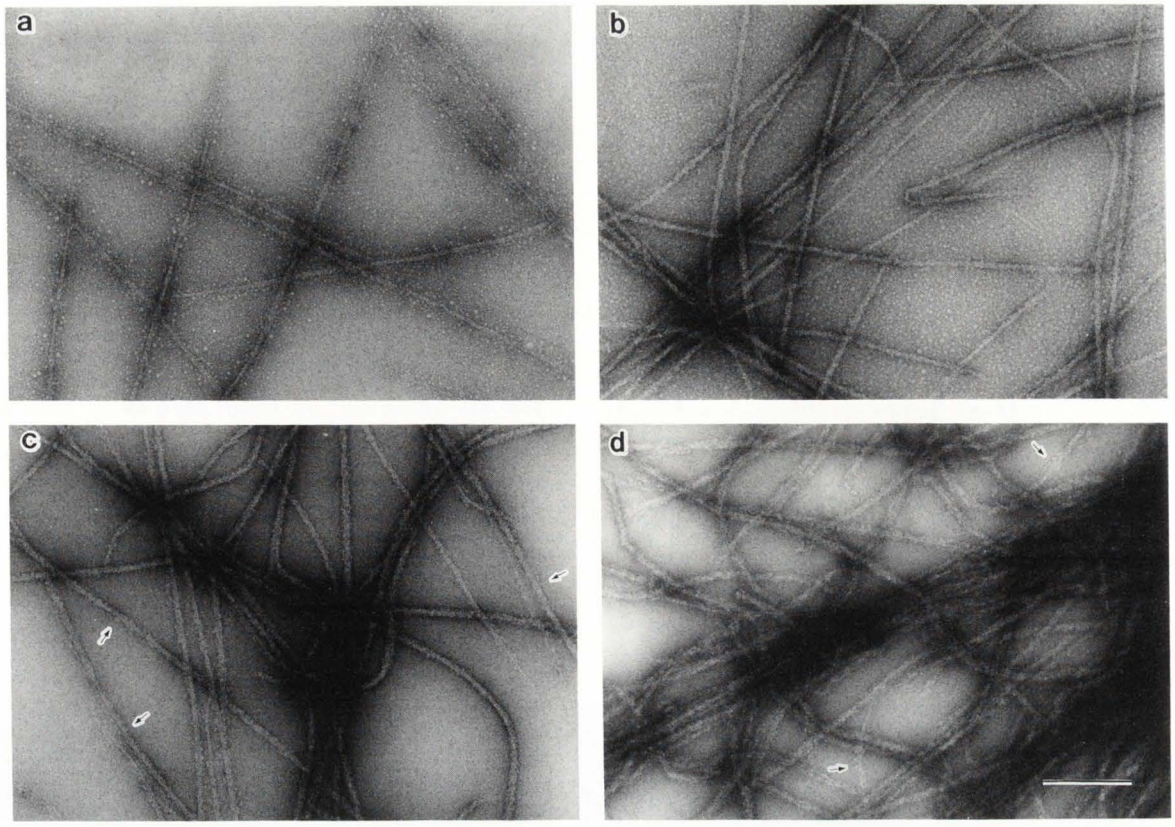

Figure 6. Transmission electron micrographs of myosin filaments pressurized at 210 MPa for 2.5 (a), 5 (b), 10 (c), and 30 min (d). Arrows indicate particles on the surface of the filaments. Unpressurized control is the same as that in Fig. 5a. Bar indicates 0.5 μm.

samples at protein concentrations of 5 mg/ml, 4 mg/ml, and 3 mg/ml were observed at 5, 10, and 15 min, respectively. The gel strengths of all the samples at 210 MPa were lower than those at 280 MPa and never reached a constant value within 30 min.

The gelation did not occur at 210 MPa when the duration of pressurization was short; namely, 2.5, 5, and 10 min for 5, 4, and 3 mg/ml of the samples, respectively. These samples were then heated at 65°C for 20 min to form gels, and the rigidities of heat-induced gels were measured. As shown in Fig. 3, the rigidities of heat-induced gels from pressurized myosin filaments were almost the same as those in heat-induced gels without pressurization (solid symbols). But the rigidities in the samples containing high protein concentrations such as 5 and 4 mg/ml were decreased with the time of pressurization.

When myosin filaments were pressurized at 140 MPa, no gelation was observed at any protein concentration within 30 min. These pressurized samples were also heated and the rigidities were measured (Fig. 4). No differences were observed between the pressurized and the pressurized/heated samples, except the 5 mg/ml sample which showed lower rigidities than those of heat-induced gels, just as the 5 mg/ml sample at 210 MPa shown in Fig. 3.

Transmission electron microscopy was carried out to investigate changes in the myosin filaments induced by pressurization. Fig. 5 shows the pressurized myosin filaments at 4 mg/ml and at 140 MPa for 0 (a), 5 (b), 10 (c), and 30 min (d). No gelation occurred at this pressure (Fig. 4). There were no apparent differences between the control myosin filaments and the pressurized filaments. The filaments dispersed in the field and no interfilamentous association was observed. Furthermore, no significant changes were observed in the individual filaments even in the 30 min pressurized sample.

Fig. 6 shows the filaments pressurized at a protein concentration of 4 mg/ml and 210 MPa for 2.5 (a), 5 (b), 10 (c), and 30 (d) min. The nonpressurized control is the same as that in Fig. 5a. The gelation occurred at 10 min of the pressurization at 210 MPa and 4 mg/ml as indicated in Fig. 2. Myosin filaments began to associate upon pressurization, and the association of filaments became more apparent with extended duration of pressurization. In

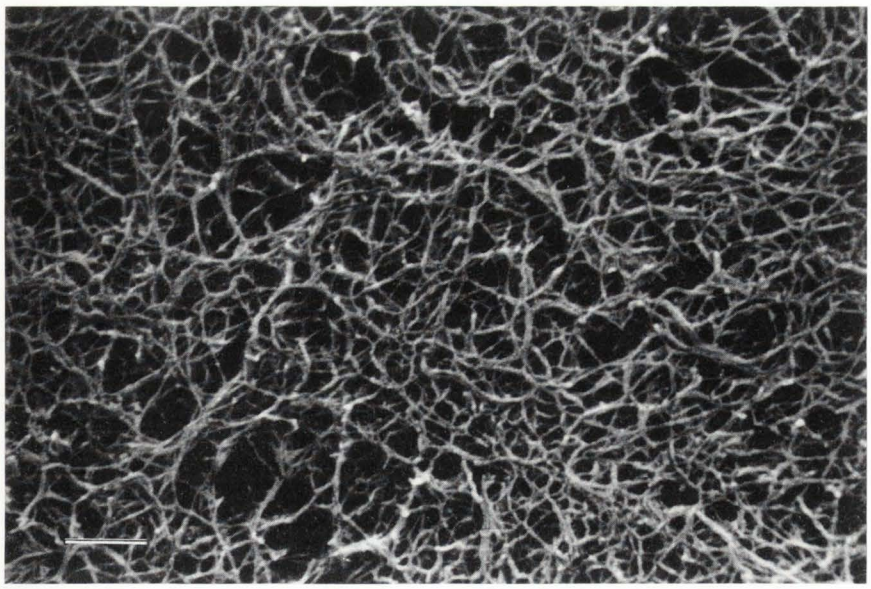

Figure 7. Scanning electron micrographs of pressure-induced gel at 280 MPa for 30 min. Bar indicates 1 μm.

addition, the shaft of the filament seemed to become irregular and distorted with pressure (Fig. 6d). Particles were also observed on the surface of the filaments when the pressurization time was extended (Fig. 6 c and d), suggesting conformation changes in the tail region of myosin molecules as well as aggregation of myosin heads projected from the surface of the filaments.

The microstructure of the pressure-induced gel which was formed at 280 MPa for 30 min and a protein concentration of 5 mg/ml is shown in Fig. 7. The gel structure consisted of a network of fine strands. Such structures are quite similar to those in the heat-induced gels of myosin filaments at low ionic strengths (Hermansson et al., 1986; Yamamoto et al., 1988).

A pressure-induced gel as well as a heat-induced gel were centrifuged and each of the supernatants and precipitates analyzed on SDS-PAGE to investigate the types of subunits of myosin molecule which were involved in the formation of gels (Fig. 8). The gel was formed at 210 MPa with a protein concentration of 5

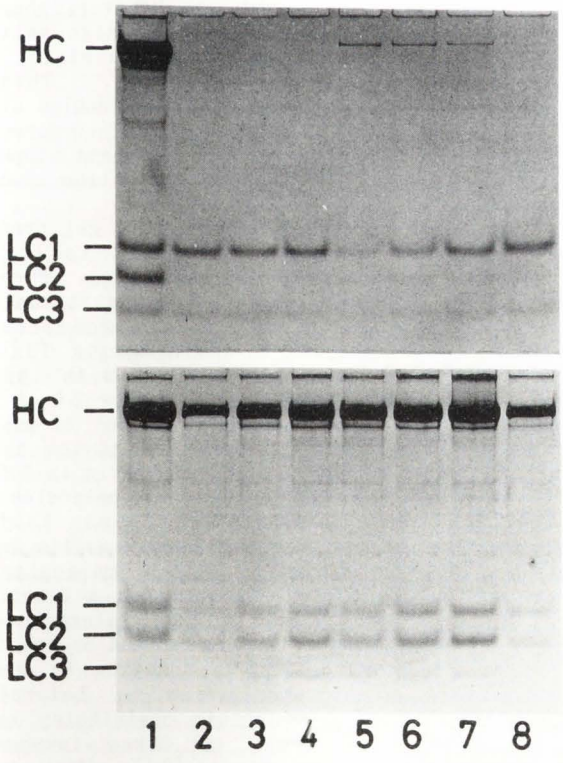

Figure 8. SDS-PAGE of the supernatant (upper gel, except row 1) and the precipitate (lower gel, except row 1) after centrifugation (15,000 rpm, 30 min) of gels. Row 1 (both of upper and lower gels), control myosin filament; row 2, heat-induced gel without pressurization; rows 3 and 4, pressurized for 1 and 2.5 min then heated, respectively; rows 5 to 8, pressurized for 5, 10, 15, and 30 min, respectively. HC and LC denote myosin heavy chain and light chain, respectively.

mg/ml. When the pressurization time was reduced to 1 or 2.5 min, no gelation occurred by pressure alone, but gels were formed upon heating. It was clear that neither myosin heavy chains were detected in the supernatant from a heat-induced gel (row 2) nor in heat-induced gels from pressurized myosin filaments (rows 3 and 4). On the other hand, myosin heavy chains were detected in the pressurized sample (rows 5 to 8). The density of the bands of myosin heavy chains decreased with the time of pressurization. Also, light chains 1 and 3 were detected in the supernatants of both heat- and pressure-induced gels. Although the precipitates from the pressure-induced gels as well as the heat-induced gels contained light chains 1 and 3, those light chains decreased as compared to the unheated control. These results indicate that a part of light chains 1 and 3 are dissociated from myosin molecules when they are heated or pressurized.

Discussion

The present results clearly demonstrates that myosin filaments in 0.1 M KCl and 20 mM phosphate, pH 6.0, form a gel under high hydrostatic pressure. As shown in Fig. 1, gel strengths linearly increased with the time of pressurization up to 10 min, suggesting that gel formation proceeds continuously until 10 min and the reactions involved in the formation of a gel is complete around 10 min of pressurization at 280 MPa.

Suzuki and Macfarlane (1984) reported that pressurization (up to 150 MPa and 30 min) of myosin prior to heating modified the characteristics of heat-induced gel; however, they were unable to find gelation of myosin by pressurization alone even at high protein concentrations, such as 30 mg/ml, in 0.5 or 0.25 M NaCl at pH 6. Their results do not contradict the present study (Fig. 4), which reveals no gelation at 140 MPa. As indicated in Figs. 1 and 2, over 200 MPa of pressure was required for the gel formation of myosin filaments in 0.1 M KCl at pH 6.

Heat treatment of pressurized myosin filaments, which did not form gels by pressurization alone, was performed to investigate whether pressurization prior to heating affects the mechanical properties of heat-induced gels. The rigidities of heat-induced gels from pressurized myosin filaments were almost the same as those induced by heat alone, except for the samples of 5 and 4 mg/ml at 210 MPa (Fig. 3) and 5 mg/ml at 140 MPa (Fig. 4). The reason for the decrease in the rigidities of heat-induced gels of high protein concentrations of myosin filaments is unclear, although pressure-induced partial denaturation of myosin filaments may affect the subsequent heat-induced gelation.

No apparent morphological changes were observed in the filaments pressurized at 140 MPa (Fig. 5), and no gelation took place at this pressure (Fig. 4). On the other hand, when gelation was induced just by pressure at 210 MPa and above, the shaft of myosin filaments looked to be irregular and distorted (Fig. 6d). This suggests that the tails of myosin molecules are subjected to pressure-induced denaturation and such denaturation causes disturbances in regular packing of the tails in the filaments. Furthermore, the projection from the filament surface seemed to be aggregated to form particles upon pressurization (Fig. 6 c and d), suggesting that myosin heads projected from the surface of the filaments are also subjected to pressure-induced denaturation. These morphological changes in myosin filaments induced by high hydrostatic pressure are similar to those observed in heat treated filaments (Yamamoto et al., 1988).

Tumminia et al. (1989) showed that myosin filaments at pH 7.0 shortened with pressure (up to 100 MPa and 60 sec) due to partial depolymerization of filaments and the dissociated myosin molecules reaggregated after release of pressure to form another population of filaments, which were shorter than the original filaments. Moreover, only a slight decrease (6%) was observed in myosin ATPase activity after the pressure treatment at 100 or 140 MPa for 1 min. However, no apparent reduction in the lengths of the filaments as well as the appearance of newly formed populations of the filaments upon pressurization were observed in the present study (Figs. 5 and 6). The discrepancy in the morphology of the filaments between the present results and the results of Tumminia et al. (1989) can be attributed to the higher pressures with extended duration of pressurization and also the lower pH used in this study. Myosin molecules are in the monomeric state in a high ionic strength and neutral pH solution; however, they associate to form filaments even in high salt solution if the pH is acidic (Morita et al., 1987; Hermansson and Langton, 1988). This indicates high affinity among myosin molecules at acidic pHs rather than neutral pHs. Therefore, it is probable that the myosin filaments at pH 6.0 are more resistant to depolymerization upon pressure than those at pH 7.0.

It seems that the formation of a gel upon pressurization is closely related to the structural changes in the filaments. When gelation did not occur upon pressurization, e.g., at 140 MPa (Fig. 4), no changes were observed in the appearance of the filaments (Fig. 5), while gel formation coincided with the association and also the disturbance of the filaments (Figs. 1, 2, and 6). Ivanov et al. (1960) reported that high pressure causes an increase in the molecular weight of myosin in 0.6 M KCl, indicating aggregation of the molecules. O'Shea et al. (1976) also found that pressurization of myosin in 0.6 M KCl results in the formation of aggregates with a molecular weight approximately that expected for a dimer. It is likely that the head to head interaction of myosin is involved in the lateral and/or cross interfilamentous association in addition to the intrafilamentous head aggregation. Lateral association of myosin filaments contributes to the formation of fine strands, and cross linkages of strands form network structures. With an

increase in the duration of pressure application, myosin was irreversibly denatured and a gel formed.

Heat denaturation of myosin is known to result in selective dissociation of light chains 1 and 3 (Dreizen and Richards, 1973), and it is also known that light chains are not essential subunits for the formation of heat-induced gels since heavy chains of myosin molecules form a thermogel, which is comparable to that of myosin (Siegel and Schmidt, 1979; Samejima et al., 1984). As shown in Fig. 8 (row 2), light chains 1 and 3 were detected in the supernatant, while those in the precipitate were decreased as compared to the unheated control after centrifugation of heat-induced gel, whereas light chain 2 was not released from the gel. This supports the finding that light chains 1 and 3 are not essential subunits in the formation of heat-induced gels. As with heat induced gels, a pressure-induced gel also releases light chains 1 and 3 with centrifugation. Furthermore, a trace of heavy chain was detected in the supernatant after the centrifugation of a pressure-induced gel, especially in the 5 or 10 min pressurized samples which produced very weak gels. However, the density of the band of the heavy chain decreased with increased time of pressurization. This suggests that a small amount of free myosin molecules remain without being incorporated in the gel at the early stage of pressurization and that they are trapped in a network structure. These molecules are squeezed out from a gel by centrifugal force.

The present results suggest that high hydrostatic pressure is one of potential energy sources in the gel formation of myosin.

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Discussion with Reviewers

J.J. Macfarlane: As indicated by the authors, pressure treatment is known to disaggregate myosin filaments and to affect their subsequent gelation by heat. Therefore the apparent absence of such effects in the present study is unexpected. Pressure-induced disaggregation is favoured by lowering the temperature and as the authors carried out their experiments at ambient temperature, do they think treatment temperature might be part of the reason for a lack of effect.
Authors: We performed the pressurization experiment at pH 6 instead of neutral pH because

a heat-induced gel at pH 6 is known to be stronger than that at pH 7 and we expected an advantage of pH 6 over other pHs in not only heat-induced gelation but also pressured-induced gelation of myosin filaments. As pointed out by the reviewer, temperature may be one of the reasons for a lack of disaggregation of myosin filaments in our experiment. However, we think that acidic pH (6.0) is a more important factor for disaggregation of the filaments. We would like to point out that myosin molecules form filaments in the acidic pH regions even at high ionic strengths such as 0.5 M KCl and no filaments are formed in 0.5 M KCl at neutral pHs (Morita et al., 1987). This suggests strong affinity among myosin molecules to form filaments in acidic pH regions.

R.G. Cassens: What is the maximum size of sample which can be pressure treated? Could the technique be extended to a commercial condition?
Authors: The maximum size of a sample in our apparatus is 2.5 cm in diameter and 7 cm in length. Many types of pressure chambers are now available in the market. A pressure chamber having an inner diameter of 2 m and depth of 3 m (maximum pressure; 200 MPa) for commercial use is manufactured in Japan.

G.R. Schmidt: Was the temperature of the sample measured before, during and after pressurization? Could the sample have heated during pressurization?
Authors: We did our experiments at room temperature and did not measured the temperature of the sample during or after pressurization. No temperature control apparatus was attached to our device. A rise in temperature of water during pressure increase is calculated to be about 2°C per 100 MPa in an adiabatic condition, and a temperature drop will also occur during pressure release. We do not think that the sample temperature rose above 30°C during pressurization even at 280 MPa.

G.R. Schmidt: Did the act of transferring the sample from the plastic tube to the cuvette alter some preliminary aggregation caused by pressurization? Was the cuvette the same size as the plastic tube? How long were the samples heated? Was the actual temperature of the sample measured during heating?
Authors: Heating the samples was done only for those gels not formed by pressure alone, and no obvious morphological changes were observed in those filaments before heating. Therefore, we believe the act of transferring the sample from the plastic tube to the cuvette does not affect the subsequent heat-induced gelation. The cuvette, which was used for heat-induced gelation after pressurization, was an ordinary spectrophotometric one (1x1x4 cm). We measured the actual temperature of the samples during heating in some cases, but not for all of the samples; it was measured to be 65°C. The samples were heated for 15 min.

G.R. Schmidt: How were protein concentrations determined and adjusted?
Authors: The protein concentration of myosin in 0.5 M KCl was measured by UV absorption at 280 nm. The concentration was adjusted to 5 mg/ml with 0.5 M KCl and then the myosin solution was dialyzed against 0.1 M KCl and 20 mM phosphate (pH 6.0). The volume change before and after dialysis was negligible. Dialysate was used for further adjustment of protein concentration such as 4, 3, 2, and 1 mg/ml. In some cases, we used the biuret method, which gave a consistent result with UV absorption.

G.R. Schmidt: For transmission electron microscopy, why was the protein concentration adjusted to 0.15 mg/ml? Does this manipulation upset the structure of the myosin filaments? Also, Fig. 6 mentions different protein concentrations. Had these previously been adjusted to 0.15 mg/ml?
Authors: The protein concentrations of all the samples were adjusted to 0.15 mg/ml for transmission electron microscopy. This is ordinary technique for negative staining. If the protein concentration is too high, a specimen grid is crowded with filaments and this makes observation impossible. We believe that the adjustment of protein concentration does not upset the structure of the filaments.

A.M. Hermansson: As stated by the authors, the gel strength of the pressure induced gels at 280 MPa was almost proportional to the protein concentration. This is not the case for the heat-induced gels, where there is a pronounced difference between the concentrations 2-3 mg/ml and 3-5 mg/ml. Have the authors any comments to this difference in concentration dependency?
Authors: At present, we cannot explain the difference in the gel strengths between the pressure-induced and heat-induced gels. However, we would like to point out that a pressure-induced gel seems to be more visco-elastic compared to a heat-induced gel. More studies, such as microstructural morphology of gels as well as textural measurements are needed to elucidate the difference between the pressure- and heat-induced gels.

A.M. Hermansson: The background of Fig. 5a-d and Fig. 6a and b contains numerous globular particles. Is this an artifact or part of the structure?
Authors: We think globular particles in the background in some micrographs are artifacts probably due to hydrophobicity of the supporting carbon film.

E.A. Elgasim: Because of possibility of synergistic effects of pressure and heat, you don't have heat-induced gel, rather you have pressure-heat-induced gel!
Authors: We agree, the results shown in Figs. 3 and 4 are from pressure-heat-induced gels.

E.A. Elgasim: Myosin filaments were equilibrated to pH 6.0, yet samples for the SEM were fixed at pH 7.0. Why did you fix it at the pH of 6.0 to

minimize possible artifacts?
Authors: We have employed the method of Yasui et al. (J. Food Sci., 44, 1201, 1979) for fixation. We suppose that the gel began to be fixed as soon as it was transferred into a fixative solution.

E.A. Elgasim: Since 280 MPa was more effective in inducing myosin gel formation, why did you not use it for your SDS-PAGE studies?
Authors: At 210 MPa, the gel strength continued to increase until 30 min; on the other hand, it remained at a constant level from 10 to 30 min of pressurization at 280 MPa. Therefore, we expected corresponding changes in the protein constitution of the gels during pressurization at 210 MPa.

E.A. Elgasim: What do you mean by "the appearance of new population of the filaments"?
Authors: According to Tumminia et al. (1989), when myosin filament is exposed to increased hydrostatic pressure at 0.1 M KCl and pH 7.0, followed by quick return to atmospheric pressure, the original filaments shorten linearly with increasing pressure; in addition, a second population of filaments is seen, presumably the result of reaggregation of myosin after release of pressure. In the present study, no new population of filaments was observed. This indicates that the myosin filaments did not depolymerize during pressurization at 0.1 M KCl, pH 6.0, and under the pressure from 140 MPa to 280 MPa.

E.A. Elgasim: Would the authors expect the pH of myosin filament to change with pressurization?
Authors: We have no way to measure the actual pH of the solution in our apparatus during pressurization. A description that the pH of the solution remains relatively unaffected, with a temporary shift toward the acid of no more than about 0.2 pH units at 14,000 psi (which is about 100 MPa) and 4°C appeared in the paper by Tumminia et al. (1989).