Stability of Fish Myosins and Their Fragments to High Hydrostatic Pressure

Shoichiro Ishizaki, Munchiko Tanaka, Rikuo Takai, and Takeshi Taguchi

Department of Food Science and Technology, Tokyo University of Fisheries, Minato, Tokyo 108, Japan

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The stability of black marlin and jack mackerel myosins and their fragments to high hydrostatic pressure (100, 200, 300, 400, and 500 MPa) was examined by means of a solubility test, CD measurements, and changes in fluorescence intensity. The solubility of both myosins decreased with the hydrostatic pressure-treatment above 300 MPa. There was a marked decrease in the solubility of S-1, especially in the presence of 0.05 M KCl, though the solubility of rod did not alter. The results of CD measurements showed a slight decrease in the helical content of myosins and S-1s. From the data of binding of ANS (8-anilino-1-naphthalene sulfonate), it was indicated that by pressure treatment the fluorescence intensities of myosins and S-1s increased rapidly. By the changes of tryptophan fluorescence intensity the pressure-treated S-1s in 0.05 M KCl showed rapidly decreasing curves. The pressure-stability of S-1s in 0.05 M KCl was very inferior to that of S-1s in 0.6 M KCl.

Key words: high hydrostatic pressure, myosin, myosin subfragments, denaturation

Protein gelling is an important function which is required for the quality of surimi-based products such as kamaboko. It has been observed that fish muscle protein paste easily forms a gel upon application of high hydrostatic pressure. Denaturation is often considered as a prerequisite to gel formation, as has been suggested by Ferry. It is now widely accepted that conformational changes of protein by pressure take place depending strongly on the volume effect through hydration. Based on the gelation mechanism by pressure, it appears that fish muscle proteins are very sensitive to pressure. Recently, the relationship between a fish living environment and pressure-stability of protein has attracted special interest. By applying the high hydrostatic pressure the study on pressure-stability of fish muscle proteins has been performed. There may be differences in pressure-sensitivity of fish muscle proteins among fish species.

Myosin is the major protein in fish myofibrils, and plays an important role in the kamaboko processing. The objective of this study was to examine pressure-stability of myosin and its fragments.

Materials and Methods

Materials

Black marlin (Makaira mazara, 1–2 kg in frozen block) and jack mackerel (Trachurus japonicus, 150–200 g in body weight) were purchased from a local retail store. They were kept at -30°C until sample preparation. All chemicals were of reagent grade.

Preparation of Proteins

For the preparation of muscle protein, the dorsal muscle of thawed black marlin and jack mackerel was carefully excised and separated into ordinary and dark muscles. Each myosin was prepared by the method described previously. Myosin was extracted from minced muscle (100 g) with a mixture (200 ml) containing 0.7 M KCl, 4.26 mM KH2PO4, and 19.5 mM Na2HPO4 (pH 6.4) plus 15 ml of 0.2 M sodium pyrophosphate (Na4P2O7) and 1.5 ml of 0.2 M MgCl2. The mixture was centrifuged at 10,000×g for 20 min, and the supernatant was diluted with cold water until the ionic strength was decreased to 0.05. The precipitate was collected by centrifuging at 4,000×g for 10 min and dissolved by increasing to 0.5 M with 2 M KCl. For further purification, myosin solution consisting of 0.5 M KCl, 5 mM ATP, and 20 mM Tris-maleate buffer was centrifuged at 100,000×g for 3 h, and myosin in the supernatant was collected by fractional precipitation (40–50% ammonium sulfate saturation). After dialysis against 0.6 M KCl containing 20 mM phosphate buffer (pH 7.0), final preparation of myosin was done by centrifuging at 20,000×g for 1 h.

Myosin subfragment-1 (S-1) was prepared by the method of Weeds and Pope with some modifications. The digestion was performed at 10°C for 20 min with chymotrypsin in an amount of 1/130 (w/w) relative to myosin (10 mg/ml) in 0.05 M KCl, 20 mM Tris-maleate (pH 7.5), 1 mM EDTA, and 1 mM dithiothreitol (DTT). After stopping digestion by the addition of 0.5 mM phenylmethylsulfonyl fluoride (final concentration), the digest was ultracentrifuged at 80,000×g for 60 min. The S-1 in the supernatant was further purified on a Sephacryl S-300 column (2.2×88 cm) equilibrated with 20 mM Tris-maleate (pH 7.5) containing 0.05 M KCl and 0.1 mM DTT. Finally, each S-1 in 20 mM phosphate buffer (pH 7.0) containing 0.05 M and 0.6 M KCl was used.

From the ultracentrifugal pellet of the S-1 preparation, myosin rod was prepared by using the ethanol fractionation procedure reported by Samejima et al. The insoluble residue was dissolved in 20 mM phosphate buffer (pH 7.0)
containing 0.6 M KCl. The ethanol treatment (3 vol 95% ethanol) was conducted at 25°C for 1 h. The fibrous residue was collected by centrifugation and dialyzed at 4°C for 2 days against 20 mM phosphate buffer (pH 7.5) containing 0.6 M KCl. Centrifugal supernatant at 10,000 × g for 30 min was washed three more times with 0.1 M KCl by the same procedure. Finally, the rods dissolved in 20 mM phosphate buffer (pH 7.0) containing 0.6 M KCl were ultracentrifuged at 80,000 × g for 60 min and the supernatants were used as “myosin rod preparation.” Typical SDS-polyacrylamide gel electrophoretograms (7.5% gel) of myosins, S-1s and rods are illustrated in Fig. 1.

Hydrostatic Pressure Treatment
A hydrostatic pressure apparatus with oil-pressure generator and flat bottomed cylindrical compressing vessel (Hikari Koatsu Kiki Co. Ltd.) was employed. The vessel temperature during compressing was kept at 0°C in ice. The protein solution (0.1–8.0 mg/ml) was sealed in a plastic tube (diam 1.2 cm × ht 4.0 cm) without any head space, and compressed at up to 500 MPa for 10 min with hydrostatic pressure. After pressure release, pressure-treated samples of myosin, S-1, and rod were obtained.

Solubility Test
The solubility of protein in 20 mM phosphate buffer (pH 7.0) containing 0.6 M KCl before and after hydrostatic pressure treatment was expressed as a percentage = ((the protein concentration of supernatant after centrifugation) / (the protein concentration before centrifugation)) × 100. Protein concentration was determined by the biuret method.7)
changes in rabbit myosin induced by hydrostatic pressure. They showed that irreversible aggregation of myosin molecules at pH 7.0, expressed by turbidity, was observed at the range of 300-500 MPa. Our results obtained in Fig. 2 are in agreement with theirs. Therefore, the decreasing solubility suggests that fish myosin molecules irreversibly associated to form aggregates after exposure to and release of high hydrostatic pressure. Yamamoto et al. also showed, using transmission electron microscopy, that the pressure-induced oligomers were only formed through head-to-head interaction, neither head-to-tail nor tail-to-tail interaction being observed. To clarify this suggestion, we examined which fish myosin fragment was related to the decrease in solubility of myosin molecule. The solubility of rods did not decrease all over the pressurization up to 500 MPa, whereas in the presence of 0.05 M KCl, both the S-1s exhibited a marked decrease of solubility upon application of high pressurization (Fig. 2). From the results in Fig. 2, it was suggested that the changes in solubility of fish myosin molecules depend on those of S-1 portion. However, the degree of decrease in the solubility of S-1s in 0.6 M KCl was lower than that of 0.05 M KCl-S-1s. In general, it has been considered that the volume change induced by application of high pressure leads to a conformational change of protein. It is not clear from the results of this experiment but it seems that there may be appreciable differences in hydration properties between S-1 in 0.6 M KCl and that in 0.05 M KCl. In case of S-1 in 0.6 M KCl, the pressure-stability of black marlin S-1 was higher than that of jack mackerel S-1. Further study regarding pressure-stability of fish S-1s will be necessary.

In Fig. 3, the changes in α-helix content of black marlin and jack mackerel myosins, S-1s, and rods due to pressurization are given. The α-helix content of pressurized myosins and S-1s gradually decreased up to 300 MPa. However, the α-helix content of rods estimated to be 95% was not affected by pressurization up to 500 MPa. It appeared that though the values for α-helix content of myosin and S-1s were low, there were considerable changes in α-helix content due to pressurization. These suggest that unfolding of the polypeptide chain occurs mostly in the S-1 portion of myosin molecule and not in the rod portion. These are also identical to the results of Yamamoto et al. They also reported that pressure-induced morphological changes in rabbit myosin molecules are similar to that induced on heating. It is, however, considered to cause the entanglement between the tail portions due to a helix-coil transition in the case of heating. As in Fig. 3, a helix structure in the rod was remaining even after pressurization up to 500 MPa. This indicates that pressure denaturation is not always the same as thermal denaturation. From the fact that the pressure-stability of protein is given by enthalpy through volume effect, it is suggested that S-1s, especially in 0.05 M KCl were readily susceptible to change the differential volume by pressurization. Since both hydrophilic and hydrophobic groups of protein surface act like an active substance, it can be considered that hydrophobic hydration also plays an important role in the pressure-stability.

In Fig. 4, the changes in the surface hydrophobicity of black marlin and jack mackerel myosins, S-1s, and rods after pressurization as evidenced by an increase in fluorescence intensity by ANS are shown. The fluorescence intensity of black marlin and jack mackerel myosins after pressurization from 100 MPa to 500 MPa caused a two- to threefold increase. It appeared that an unfolding of protein with more hydrophobic alterations occurred upon application of high pressure. Though there was no appreciable change in the fluorescence intensity of rods due to pressurization, the fluorescence intensity of myosins and S-1s exhibited a marked increase. This indicates that the changes in the solubility of fish myosins as described in Fig. 2 are caused by the increase in the surface hydrophobicity of the S-1 portion, at least. However, the degree of the increase in fluorescence intensity of S-1s in the presence of 0.6 M KCl was slightly lower than that of S-1s in 0.05 M KCl. The variation in fluorescence intensity of S-1s was quite similar to that of solubility obtained from Fig. 2. The pressure-effect for hydrophobicity as shown by the protein-ANS fluorescence may be due mainly to an unfolding induced by a differential volume between hydration compressibility and solvent one. The changes in the tryptophan fluorescence intensity of black marlin and jack mackerel myosins, S-1s, and rods after pressurization are shown in Fig. 5. The tryptophan fluorescence intensity of both myosins was not influenced
by pressurization up to 500 MPa. For rods a slight decrease was observed, though it was reported that the presence of urea gave a rapidly decreasing effect for tryptophan fluorescence intensity. However, the fluorescence intensity of S-1s markedly decreased. The extent of the decrease exhibited higher values in 0.05 M KCl than those in 0.6 M KCl, suggesting that there may be appreciable difference in hydration between both S-1s. Unexpectedly, both the myosins did not show noticeable changes in tryptophan fluorescence intensity, although both the S-1s showed a marked decrease. Exact solution to the cause could not be obtained. Further investigation on surface hydrophobic regions containing tryptophan sites will be necessary.

In conclusion, though direct measurements under pressure were not performed, it would be reasonable to conclude that pressure-sensitive myosin fragment is S-1. The pressure-conformational changes of proteins accompanying the decrease in solubility, the unfolding state, and the variables of hydrophobic surface will result from the volume change through hydration. In addition, it appeared that the pressure-stability of S-1s in 0.05 M KCl was very inferior to that in 0.6 M KCl. Since it was pointed out that there are certain relationships between fish living environment and pressure-stability of myofibrillar proteins, it will be necessary to obtain information about the difference in pressure-stability of S-1 among species.

References