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Changes in conformation and in sulfhydryl groups of actomyosin of tilapia (*Orechromis niloticus*) on hydrostatic pressure treatment

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Abstract

Actomyosin extracted from tilapia muscle was subjected to hydrostatic pressure treatment (50–300 MPa, 10–60 min, 4 °C) to investigate the changes in sulfhydryl groups and on conformation. Transmission electron microscopy showed that the structure of actomyosin was aggregated and disrupted above 100 MPa, and more regular network aggregates were observed as pressure increased. Moreover, pressurisation at 50 MPa for 10 min did not change actomyosin structures. Using SDS–PAGE analysis, molecules larger than the myosin heavy chain were observed when actomyosin was treated at and above 200 MPa. Below 200 MPa actomyosin formed aggregates, mainly with hydrogen bonds. Surface sulfhydryl group content of actomyosin increased with increased pressure, up to 250 MPa. However, total sulfhydryl group content of actomyosin decreased with increased pressure and time. According to this study, 200 MPa would be the critical pressure that induced actomyosin to form regular network structures.

Keywords: Actomyosin; Hydrostatic pressure; Conformation; Sulfhydryl group

1. Introduction

In addition to temperature, pressure also determines the transition phases of natural materials. The application of high hydrostatic pressure as a food-processing tool is gaining popularity. High pressures have been shown to induce gel formation in egg white, egg yolk, rabbit meat, suspension of soy protein, and milkfish actomyosin (Bridgman, 1914; Ko, 1996; Okamoto, Kawamura, & Hayashi, 1990; Yamamoto, Miura, & Yasui, 1990). Recently, the effects of pressurisation on β-lactoglobulin (Pittia, Wilde, Husband, & Clark, 1996), starch gelatinisation (Douzals, Marechal, Coquille, & Gervais, 1996), and inactivation of microorganisms (Aleman et al., 1996; Drake, Harrison, Asplund, Barosa-canovas, & Swanson, 1997; Patterson, Quinn, Simpson, & Gilmour, 1995) have been investigated.

Fish muscle proteins are typically heat-gelled in the manufacture of surimi seafood products. Functional and textural characteristics of fish meat depend mainly on the myofibrillar proteins (Montecchia, Roura, Roldan, Perez-borla, & Crupkin, 1997) due to the collagen content being lower than that in mammalian muscle (Hartshorne, Barns, Parker, & Fuchs, 1972). Actomyosin is the major protein in myofibrils. Protein–protein interactions, in actomyosin, such as association, aggregation and polymerization, are dependent upon temperature, pH and the type of actomyosin used (Deng, Toledo, & Lillard, 1976). Since protein-protein interactions lead to changes in the secondary and tertiary structure of the protein molecule, these changes could affect fat and water-binding affinities of these molecules. Some of the sulfhydryl groups of actomyosin exist deep inside its molecules, and disulfide bonds are formed

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due to the augmented interaction of the interior and exterior protein by extrinsic energy and the change of environment. As structures of actomyosin are exposed more disulfide bonds are formed (Monahan, German, & Kinsella, 1995). In our previous study (Hsu & Ko, 2001; Ko, Jao, & Hsu, 2003), we found that 100 MPa and 150 MPa caused an apparent unfolding of tilapia myosin, by measuring the exposed hydrophobicity, and myosin formed intermolecular disulfide bonds at pressures of 100–200 MPa.

In this study, actomyosin was extracted from tilapia (*Orechromis niloticus*) muscle. Our objectives were to investigate the changes in actomyosin conformation and in the sulfhydryl content of actomyosin, using pressurising treatments ranging from 0.1 to 300 MPa.

2. Materials and methods

2.1. Materials

Live tilapia, *O. niloticus*, weighing about 600 g, were purchased from a store in Taichung. They were kept in ice and dissected immediately, to obtain the ordinary muscle. All chemicals used were of reagent grade.

2.2. Preparation of actomyosin

Actomyosin was extracted from minced ordinary muscle with 2.5 volumes of Weber–Edsall solution (0.6 M KCl, 0.04 M $NaHCO_3$, 0.01 M Na_2CO_3) for 4 h with slow stirring at 4 °C (Ko, Tanaka, Nagashima, Taguchi, & Amano, 1991). The extract was filtered through gauze and the filtrate was diluted with 2 volumes of cold water. The precipitate collected by centrifuging at 6000*g* for 10 min was dissolved in 0.6 M KCl by addition of 2 M KCl, and then centrifuged at 10,000*g* for 30 min. The precipitation–dissolution step was repeated and the supernatant thus obtained was dialysed overnight against 0.6 M KCl-20 mM Tris–maleate buffer (pH 7.0). Actomyosin thus obtained was adjusted to a protein concentration (determined by the Biuret method) of 5 mg/ml for pressure treatment.

2.3. Pressure treatment

The actomyosin solution (about 6 ml) was filled and sealed in a plastic tube, and then pressurised at 50–300 MPa. A high hydrostatic pressure apparatus (CIP UNIT, Mitsubishi Heavy Industries Ltd., Shinagawa, Japan) was used with an oil-pressure generator and a compressing vessel, in which the internal portion was in the shape of a flat-bottomed cylinder. The vessel temperature during pressure treatment was controlled at 4 °C.

2.4. Transmission electron microscopy

After actomyosin was pressurised, it was diluted with 0.6 M KCl-20 mM Tris–maleate buffer (pH 7.0) to a suitable protein concentration (Sano, Ohno, Otsuka-Fuchino, Matsumoto, & Tsuchiya, 1994). Each sample was put on a carbon-coated 300 mesh grid and then negatively stained with 5% uranyl acetate bihydrate. The specimens were observed in a Japanese JEM-1200EX II transmission electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was done according to the procedure of Weber and Osborn (1969). Separating gels were 10% (v/v) polyacrylamide and stacking gels were 5% (v/v) polyacrylamide. Each sample was centrifuged at 15,000*g* for 5 min to remove insoluble protein. The supernatant was added to the same volume of buffer containing 2% SDS 8 M urea 2% mercaptoethanol 20 mM Tris–HCl (pH 8.0) and 2 drops of tracking dye (0.01 g bromophenol/10 ml glycerol), and incubated for 5 min at 100 °C. Eight microlitres of the treated sample were applied to the stacking gels which were run at 80 V and 140 V. Myosin heavy chain, β-galactosidase, phosphorylase B, bovine albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme were used as protein standards.

2.6. Measurement of surface sulfhydryl (SH) content

The surface SH content was determined by using Ellman's reagent (DTNB) according to the method of Janat-

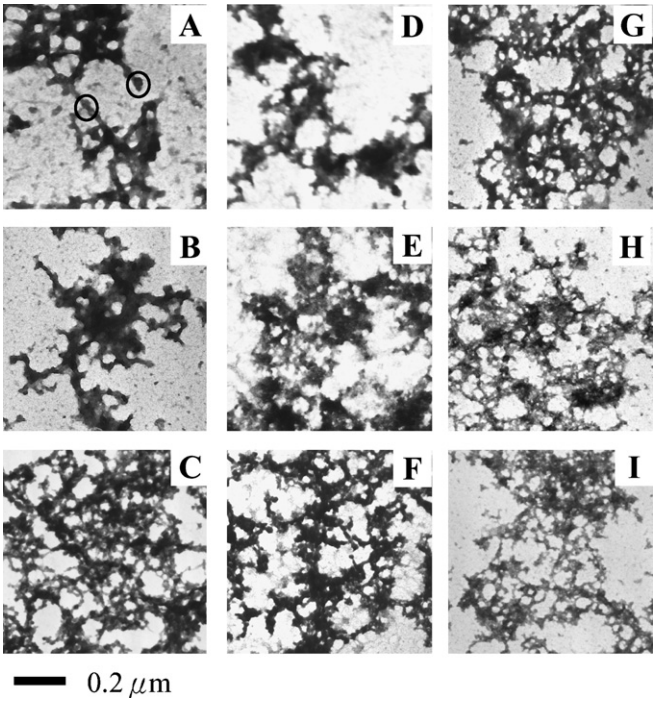

Fig. 1. Transmission electron microscopic observations on the conformation of tilapia actomyosin by pressure-treatment. A: native actomyosin; B: 50 MPa, 10 min; C: 50 MPa 60 min; D: 250 MPa, 10 min; E: 250 MPa, 30 min; F: 250 MPa, 60 min; G: 300 MPa, 10 min; H: 300 MPa, 30 min; I: 300 MPa, 60 min.

ova, Fuller, and Hunter (1968). An aliquot of each sample solution (0.1 ml) was added to 2.9 ml of 6 mM EDTA/0.1 M KH_2PO_4 phosphate buffer (pH 4.0), which was mixed with 0.02 ml of 10 mM DTNB. After reacting for 5 min at room temperature, the absorbance at 412 nm was measured with Hitachi spectrophotometer U-2000 (Ellman, 1959). The SH content was calculated as mM.

2.7. Measurement of total sulfhydryl (SH) content

The total SH content was determined using Ellman's reagent, according to the method of Ellman (1959) and Buttkus (1971). The phosphate buffer contained 8 M urea.

2.8. Statistical analysis

The statistical analysis system (SAS Institute Inc., Cary, NC, USA) was used for analysis of variance (ANOVA) and Duncan's test. The differences among treatments were verified by their least significant difference. Experiments were conducted in triplicate.

3. Results and discussion

3.1. Conformation

The structures of myosin and actomyosin in solution, which show as aggregates, filaments, and soluble status, affect the gelation characteristics of heat-induced gels (Asghar, Samejima, & Yasui, 1984). Transmission electron microscopy is used to obtain information on the changes in the shape of the actomyosin filaments caused by pressure treatment. A characteristic arrowhead structure was found on the filaments from native actomyosin (Fig. 1A). This structure of actomyosin was still observed after pressure treatment at 50 MPa for 10 min, but was disrupted at 50 MPa for 60 min (Fig. 1B, C). At pressures up to 250 MPa, actomyosin formed aggregates and lost the arrowhead structure (Fig. 1). Actomyosin filaments were shortened by pressure treatment, probably due to the dissociation of myosin subunits (Davis, 1981) and depolymerisation of actin (Ko, Tanaka, Nagashima, Mizuno, & Taguchi, 1990; Ikkai & Ooi, 1966). The actomyosin structure of

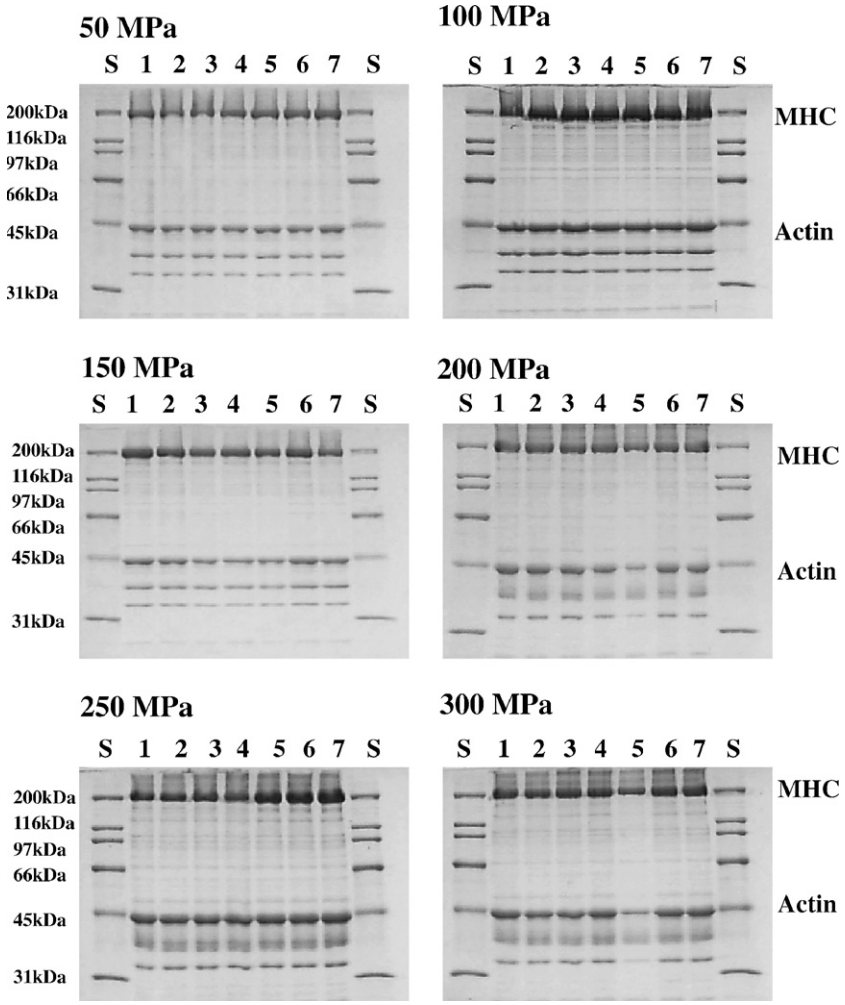

Fig. 2. Analysis by SDS–PAGE of the effect of pressure on tilapia actomyosin. S: standards, Lane 1: native actomyosin, Lane 2: treated for 10 min, Lane 3: treated for 20 min, Lane 4: treated for 30 min, Lane 5: treated for 40 min, Lane 6: treated for 50 min, Lane 7: treated for 60 min. MHC: myosin heavy chain.

rabbit skeletal muscle was disrupted at 150 MPa for 5 min (Ikeuchi, Tanji, Kim, & Suzuki, 1992).

Gelation is the result of protein denaturation, which leads to intermolecular covalent and non-covalent interactions, including disulfide bonds and hydrophobic interactions (Lee & Lanier, 1995). After centrifuging at 15,000*g* for 5 min, actomyosin precipitates were not discovered in all treatments. However, molecules over 200 kDa, appeared with pressurisation at 200 MPa and above (Fig. 2). The large molecules could not pass through the pores of the polyacrylamide gel, and stayed in the stacking gel. Shoji, Saeki, Wakameda, Nakamura, and Nonaka (1990) also reported that myosin heavy chains of a salted paste of Alaska pollock treated by hydrostatic pressure above 200 MPa polymerised into large molecules. Large molecular aggregates were formed from each single actomyosin molecule by covalent bonds. Gilleland, Lanier, and Hamann (1997) demonstrated that pressure-induced fish protein gels were constructed with covalent bonds, such as disulfide bonds, and the result was proved in our previous study (Ko et al., 2003). In our previous study, we revealed that myosin and subfragment-1 (S-1) in tilapia apparently aggregated at 150 and 200 MPa, respectively (Ko, Hwang, Jao, & Hsu, 2004; Ko et al., 2003).

3.2. Sulfhydryl groups

Many hydrophobic residues and SH amino groups buried in the interior of the native muscle proteins would be exposed under pressure (Ishioroshi, Samejima, Arie, & Yasui, 1980; Kato & Nakai, 1980). Fig. 3 showed the changes in surface SH contents of actomyosin under various pressure levels. A marked increase in the surface SH content of actomyosin was not observed at 50 MPa, after 10 min. However, a marked change in structure was shown at the same pressure for 60 min (Fig. 1). This might result from actomyosin aggregates formed by intra-molecular interactions, such as hydrogen bonds and disulfide bonds (Hsu & Ko, 2001). At 100 MPa and above, the surface SH content of actomyosin increased greatly with increasing pressure, and reached the highest value of 0.62 mM above 200 MPa. The surface SH content of pressurised actomyosin at 200 MPa for 10 min was 1.3 times higher than that of native actomyosin ($P < 0.05$). Pressurisation at 200 MPa increased the surface SH content of rabbit actomyosin by 40%, compared with native actomyosin (Ikeuchi et al., 1992).

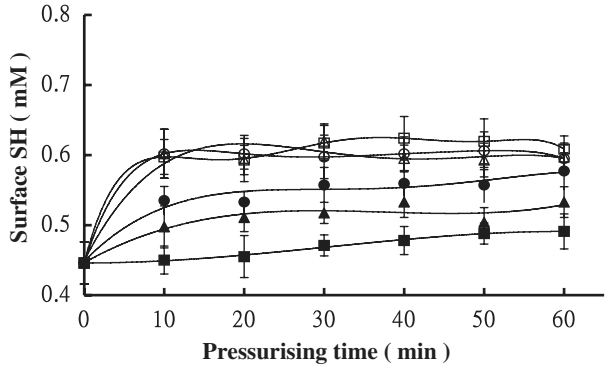

Fig. 3. Changes in the surface sulfhydryl groups of tilapia actomyosin due to pressure treatment. Protein concentration of actomyosin: 5 mg/ml. Treated pressure: 50 MPa (■), 100 MPa (▲), 150 MPa (●), 250 MPa (□), 200 MPa (△), 300 MPa (○).

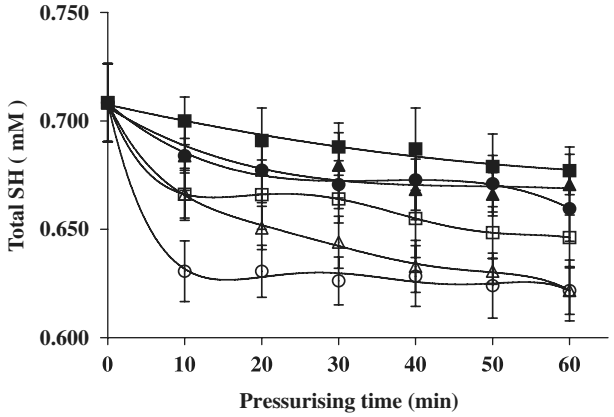

Fig. 4. Changes in the total sulfhydryl groups of tilapia actomyosin due to pressure treatment. Treated pressure: 50 MPa (■); 100 MPa (▲); 150 MPa (●); 200 MPa (□); 250 MPa (△); 300 MPa (○).

The total SH content of actomyosin decreased with increasing pressurisation time at 50 MPa ($P < 0.05$) (Fig. 4), which showed that intra- or extra-protein disulfide bonds were formed from sulfhydryl groups. Pressures above 50 MPa induced actomyosin unfold and exposed sulfhydryl groups to form aggregates by disulfide bonds (Berg, Lebedeva, Markina, & Ivanov, 1965; Gilleland et al., 1997; Ko et al., 2003). Above 200 MPa, the total SH content decreased substantially with increasing pressure (Fig. 4), although the surface SH content was maintained (Fig. 3), and larger molecules were discovered with SDS–PAGE (Fig. 2). The result showed that sulfhydryl groups reacted to form disulfide bonds at pressurisation above 200 MPa. According to our previous study, S-1 and myosin also formed disulfides bonds by pressurization at 200 and 150 MPa (Ko et al., 2003; Ko et al., 2004).

4. Conclusions

Pressure promoted the denaturation of protein. Below 150 MPa, the pressure treatment caused the actomyosin structure to be disrupted and to form intramolecular disulfide bonds. Above 200 MPa, the structure of actomyosin was still fragmented, and aggregates were formed due to disulfide bonds. According to our previous studies, 200 MPa would be the critical pressure that induced actomyosin to form aggregates caused by myosin and S-1.

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