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Conformational changes induced by high-pressure homogenization inhibit myosin filament formation in low ionic strength solutions

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ABSTRACT

Myofibrillar proteins (MPs) of chicken breast are generally insoluble in water. We have developed a new method whereby MPs are solubilized in water by applying high-pressure homogenization (HPH) thus potentially enabling greater utilization of meat in various products. To clarify the mechanism of solubilization of MPs by HPH, we investigated their conformation, solubility and filament forming behavior in low ionic strength solutions induced by 15,000 psi HPH (103 MPa). HPH induces unfolding of MPs which subsequently exposes sulfhydryl and hydrophobic groups to the surface. Our findings, determined by circular dichroism, ATR-FTIR, SDS-PAGE and LC-ESI-MS/MS analysis suggest that HPH leads to unraveling of helical structures and to formation of myosin oligomers through disulfide bond. Due to intermolecular electrostatic repulsion and physical barrier of disulfide bonds in the rod induced by HPH, we suggest that the altered myosin conformation in MPs inhibits filament formation, thus contributing to high solubility of MPs in water.

1. Introduction

Myofibrillar proteins (MPs), comprised of approximately 50% of the total meat proteins, are not readily soluble in low ionic strength solutions or water. The low solubility of MPs is largely attributed to the spontaneous formation of myosin (major protein in MPs) filaments that occurs in vitro at low ionic strength (Chen et al., 2016b). A relatively high concentration of salt (>0.3 M NaCl or KCl) is required for their complete solubilization (Ito, Tatsumi, Wakamatsu, Nishimura, & Hattori, 2003). To increase the utilization of meat, particularly from lower-value cuts, there is a need to determine if MPs can be solubilized at low ionic strength under certain conditions. For example, producing meat products in the form of liquid diet together with low salt for elderly people and dysphagic patients with malnutrition (Nieuwenhuizen, Weenen, Rigby, & Hetherington, 2010; Tokifuji, Matsushima, Hachisuka, & Yoshioka, 2013).

Many studies have investigated the solubilization of MPs in water or low ionic strength media and the mechanism has been determined (Chen et al., 2016b; Hayakawa, Ito, Wakamatsu, Nishimura, & Hattori, 2009; Ito et al., 2003; Katayama, Haga, & Saeki, 2004; Katayama & Saeki, 2007; Takai, Yoshizawa, Ejima, Arakawa, & Shiraki, 2013). More than 80% of MPs from chicken breast were solubilized in a low ionic strength solution containing 5 mM histidine (His) by washing and ultrasonication of muscle tissues (Ito et al., 2003). It was suggested that 5 mM His might affect the secondary structure of myosin (Guo, Peng, Zhang, Liu, & Cui, 2015) and cause elongation of light meromyosin (LMM), resulting in the inhibition of native myosin filament formation (Hayakawa et al., 2009; Hayakawa, Ito, Wakamatsu, Nishimura, & Hattori, 2010). Ultrasonication was used for disruption of the highly-ordered structure of the myofibrils and their solubilization in a low ionic strength solution (1 mM KCl) (Ito et al., 2003). In a physiological salt solution (0.15 M), 50 mM arginine was found to increase the equilibrium solubility and activation energy of self-association of monomeric porcine myosin (Takai et al., 2013). Through the Maillard reaction, water-soluble MPs from fish or shellfish can be prepared by glycosylation with glucose (Katayama, Shima, & Saeki, 2002; Saeki & Inoue, 1997). The increase in negative charge repulsion among myosin molecules and the introduction of the glycosyl units onto the surface of the rod region provide repulsive electrostatic and steric forces which prevent the self-assembly of myosin molecules in low ionic strength medium, thus improving their solubility (Katayama et al., 2004). These results suggest that solubility of MPs depend on the conformational characteristics and the association state of myosin in low ionic strength solutions.

Recently, we established a new method to facilitate solubilization of chicken breast MPs in water without degradation of individual protein polypeptides by applying 15,000 psi (103 MPa) high-pressure

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homogenization (HPH) treatment (Chen, Xu, & Zhou, 2016a). Furthermore, we demonstrated that HPH induced a reduction in particle size and a strengthening of intermolecular electrostatic repulsion of MPs in water. However, the reason that HPH improves the solubility and stability of MPs remains unclear. It has been reported that the cavitation phenomena, high shearing and turbulence by the strong force of HPH can affect the macromolecular conformation of soy and whey proteins (Keerati-u-rai & Corredig, 2009; Lee, Lefèvre, Subirade, & Paquin, 2007; Liu & Kuo, 2016). Therefore, the objective of the present study was to determine the behavior and conformation of MPs solubilized in water induced by treatment with HPH and to elucidate the solubilization mechanism by comparison with the native soluble MPs in high ionic strength solution (0.6 M NaCl, pH 7.0).

2. Materials and methods

2.1. Materials

The frozen chicken breast used during this research was purchased from a local market (Sushi Food Co., Ltd., Nanjing, China).

2.2. Preparation of water soluble MPs by HPH

The frozen chicken breast was thawed for about 12 h at 4 °C and washed chicken breast myofibrils were prepared as previously reported (Chen et al., 2016a). Briefly, the minced meat (100 g) was washed four times with cold (4 °C) deionized, distilled water. In each washing step the mince and water (1:10 *w*/*v*) were allowed to sit for 10 min after an initial homogenization (Ultraturrax T25, IKA, Staufen, Germany) at 8000 rpm for 2 min. The collected sediment after the final step of washing and centrifugation was termed washed myofibrils. Washed myofibrils were then suspended in water or 0.6 M NaCl solution (pH 7.0).

According to our procedure (Chen et al., 2016a), the myofibril dispersions were subjected to 15,000 psi (103 MPa) HPH treatment for two passes by using a high pressure homogenizer (Mini DeBee, Bee International, USA) equipped with a single pressure intensifier and a 75-μm opening Y-type diamond nozzle (Genizer™, Los Angeles, USA) in a modular homogenization cell. The homogenized dispersions were centrifuged at 20,000 g for 20 min and the resulting supernatants were used as HPH induced water-soluble MPs (H-WSMP). For comparison, washed myofibrils suspended in 0.6 M NaCl (pH 7.0) were centrifuged at 20,000 g for 20 min and the resulting supernatants were used as salt-soluble MPs (SSMP). Washed myofibrils suspended in 0.6 M NaCl (pH 7.0) were also treated by HPH with the same procedure of H-WSMP and the resulting supernatants were prepared as HPH treated salt-soluble MPs (H-SSMP). Where preparations were subsequently dialysed with dialysis bags (diameter: 36 mm, MW: 3500 Da) against 1 mM NaCl solution (pH 7.0) they are identified as D-H-WSMP, D-SSMP and D-H-SSMP.

2.3. Protein electrophoresis of water soluble MPs

2.3.1. Reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (reducing SDS-PAGE) and non-reducing SDS-PAGE

Reducing or non-reducing SDS-PAGE was run with a 4% acrylamide stacking gel and a 10% separating gel to observe the original myofibrillar constituents and the cross-linked protein polymers as previously described (Li, Xiong, & Chen, 2012). Protein samples (2 mg/mL) were mixed with an equal volume of sample buffer without or with 5% β-mercaptoethanol (β-ME) then boiled for 4 min. Each well was loaded with 10 μL of samples or markers. The electrophoretic analysis was performed on a Bio-Rad Mini-PROTEAN II System Cell apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA) at a constant voltage of 120 V for 1 h. The stained gel was scanned by using Imager Scanner III (EU-88, Epson, Japan) and the densities of bands were analyzed by Quantity One software (Bio-Rad, Laboratories Inc., Benicia, CA, USA).

2.3.2. Nano liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry (Nano LC-ESI-MS/MS) analysis

For Nano LC-ESI-MS/MS analysis, protein bands in the gels were excised manually and digested with trypsin. The peptides from the digestion were extracted with acetonitrile, and then completely dried in a SpeedVac device (Thermo, CA, USA). The dried samples were then re-dissolved in sample solution (2% acetonitrile, 97.5% water, 0.5% formic acid). Each protein solution was reduced by DTT and all cysteine residues were alkylated by iodo-acetamide and cleaned. The samples were then digested with sequencing-grade modified trypsin (Promega, MI, USA) in digestion buffer (100 mM ammonium bicarbonate, pH 8.5). Following digestion, the peptides were analyzed using an ion trap mass spectrometer (LTQ Linear Ion Trap Mass Spectrometer System (Thermo, CA, USA) coupled with a high pressure liquid chromatography (HPLC) system. All MS data were searched in the non-redundant protein database (NR database, NCBI). The relative abundance of protein in a sample (excised protein band) was determined based on a label-free quantitation method (Griffin et al., 2010).

2.4. Conformational characteristics of water soluble MPs

2.4.1. Reactive sulfhydryl (SH) groups and surface hydrophobicity determination

Determination of reactive SH groups was carried out according to the method as previous described (Chen et al., 2014) with slight modifications. Fifty microliters of 10 mM DTNB solution (20 mM phosphate buffer, pH 8.0) was added to 4 mL of the sample (1 mg/mL) and the reaction was allowed to proceed for 20 min at 25 °C. The absorbance of the mixture was measured at 412 nm with a Microplate Reader (SpectraMax M2, Molecular Devices Limited, USA). The content of sulfhydryl groups was expressed as micromoles of SH per 100 mg protein, using a molar extinction coefficient (EM = 13,600).

The surface hydrophobicity was measured using 8-anilino-1-naphthalene sulphonic acid (ANS) as previously described (Cao, Xia, Zhou, & Xu, 2012) with slight modifications. Ten microlitres of 15 mM ANS solution (in 0.1 M phosphate buffer, pH 7.0) was added to 2 mL of the sample (1 mg/mL). After leaving for 20 min at 25 °C, the fluorescence was determined (SpectraMax M2, Molecular Devices Limited, USA) using an excitation wavelength of 380 nm and an emission wavelength in the range of 410 to 570 nm at a 300-nm/min scanning speed. The surface hydrophobicity was expressed as fluorescence intensity (arbitrary units, a.u.).

2.4.2. Secondary structure analysis by circular dichroism (CD) and Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy

The CD spectrum was measured using a Jasco J-715 spectropolarimeter (Jasco Co. Ltd., Tokyo, Japan). The soluble MPs (0.3 mg/mL) were transferred to a quartz cell with a 0.1 cm light-path. Molecular ellipticity was measured in the range from 200 to 240 nm at a scan rate of 20 nm/min at a regulated temperature. The percentages of α-helix structures were determined using the protein secondary structure estimation program (Yang's method) provided with the Jasco J-715 spectro-polarimeter.

ATR-FTIR spectroscopy was performed on a Thermo-Fisher Nicolet 6700 spectrometer (Thermo Electric Corporation, IL, USA) using a DTGS (KBr beamsplitter) detector with Smart iTX accessory (Li & Xiong, 2015). Each spectrum from 400 to 4000 cm^{-1} was collected with an average of 256 scans at a resolution of 4 cm^{-1}. Buffer background samples to determine the effects of water or 0.6 M NaCl solutions were subtracted before collecting a sample spectra. Fourier self-deconvolution of merged FTIR scans from three independent MP samples (5 mg/mL) were obtained and a quantitative estimation of α-helix content was made on the assumption that any protein can be considered as the linear sum of a few fundamental secondary structural elements and the percentage of each element was only related to