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Structural modification by high-pressure homogenization for improved functional properties of freeze-dried myofibrillar proteins powder

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ABSTRACT: To expand utilization of meat in various products, the structural, physicochemical and functional changes of water soluble myofibrillar protein powder (WSMP-P) were investigated as affected by high-pressure homogenization (HPH) intensities (0-20000 psi). HPH modified the structure of WSMP-P by random dissociation (myofibril and myosin polymer dissociation), partial unfolding and rearrangement (actin trimer formation), producing an amorphous protein structure with high thermal stability. α-helix and β-turn conversion to β-sheet structures occurred at pressures above 15000 psi, suggesting an increase in myosin conformation flexibility with minor aggregation. Moreover, HPH was able to improve the water solubility and emulsifying properties of WSMP-P. This might be resulted from its unfolded flexible structure with submicron size and high surface net charge in aqueous suspensions induced by HPH. The findings regarding the improved functionality evidence potential of applying WSMP-P as protein supplements in formulated food or beverage at low ionic conditions.

KEYWORDS: Protein structure; Physicochemical property; Functional property; Water soluble myofibrillar proteins; Freeze-dried powder; High-pressure homogenization.

1. Introduction

Proteins are being increasingly used to facilitate the engineering for fabrication of novel food products, such as sports protein beverages and therapeutic powder foods. The effectiveness of protein utilization in food production depends on their functional characteristics, which can be tailored to satisfy the various demands of food product manufacturers. These properties are influenced by the intrinsic factors (e.g., protein structure and conformation) and extrinsic elements (e.g., pH, ionic strength, temperature and food processing) (Siddique, Maresca, Pataro, & Ferrari, 2016) .

Generally, some native proteins rarely show good functional properties desirable for the food industry. To improve the functional properties for broad applications, protein structural modification is often implemented, and innovative non-thermal processing technologies can serve this purpose. High-pressure homogenization (HPH) is a unit operation where a fluid is forced through a small orifice (valve or nozzle) and several physical factors (shear stress, high hydrostatic pressure, turbulence, cavitation, and impingement) promote dispersion of aggregates, modify the structure of protein and thereby the physicochemical properties of foods (Ye & Harte, 2014). It is, effectively, a continuous process which could be easily scale up. With the development of high pressure technology, HPH has undergone an enormous progress with the change of scale from laboratory devices to the design and assembly of a full pilot-plant scale equipment in the food industry (Chen, Xu, & Zhou, 2016b). Recently, it has been proposed the application of high-pressure homogenization (HPH) to modify the structural and functional properties of dairy (Lee, Lefèvre, Subirade, & Paquin, 2009; Ye & Harte, 2014) and plant proteins (Sun, Dai, Liu, & Gao, 2016), aiming at tailor-making protein substrates having targeted functionality suitable for different formulated foods.

However, the impact of HPH treatments on the physicochemical as well as on the functional properties of meat proteins has been scarcely determined yet.

Meat with high-nutritious proteins provides a broad range of peptides and amino acids for humans. Differently from beans and whole wheat proteins, meat proteins comprise all the essential amino acids, with a particularly high content of lysine, and are all highly digestible (Ito, et al., 2004; Pereira & Vicente, 2013). However for extraction of component, meat has not been exploited as a supplementary protein ingredient to the same extent as milk or soybean, mainly due to the poor functionality of myofibrillar proteins (MP, approximately 50% of the total meat proteins) in water or at low ionic strength (Krishnamurthy, Chang, Hultin, Feng, Srinivasan, & Kelleher, 1996). To enlarge the application scope of meat in various products, we recently proposed the potential application of high-pressure homogenization (HPH) to selectively modify the structure of MP for improved solubility in water without obvious hydrolysis of individual protein polypeptides (Chen, et al., 2016b). However, the physicochemical and functional properties of HPH induced water-soluble myofibrillar proteins (WSMP) still remained unclear. As these properties constitute the major criteria for food product formulation, processing and storage, the physicochemical properties must be elucidated to utilize WSMP as an effective protein supplement.

To enable the application of protein extracts as functional ingredient in food formulation and prolong their stable storage, it is common to convert them into a dry powder form (Aguilera, Chiralt, & Fito, 2003; Huda, Abdullah, & Babji, 2001; Sun, Senecal, Chinachoti, & Faustman, 2002). Freeze-drying has been reported to be an attractive way to extending the shelf life of high-valued materials without quality deterioration and with minimum damage in structure, texture, appearance and flavor (Vega-Mercado, Góngora-Nieto, &

Barbosa-Cánovas, 2001). Therefore, the aim of the present study was to investigate the effects of HPH treatment on the structural, physicochemical and functional properties (solubility and emulsifying properties) of freeze-dried WSMP powder (WSMP-P). So far, there is still limited information available on the structural and functional properties of freeze-dried WSMP-P. Attempts addressed towards physicochemical of WSMP would unlock a promising area of research for development of novel meat products and broaden the application area of HPH technology in food industry.

2. Materials and methods

2.1. Materials

Chicken breast muscle (*Pectoralis major*) was collected at 36 h postmortem from Sushi Food Co., Ltd. (Nanjing, China). Muscle samples were vacuum packaged, stored in a –30 °C freezer, and used within 4 days after slaughter.

2.2. Preparation of freeze-dried WSMP-P

WSMP were prepared according to our previous report (Chen, et al., 2016b). In brief, the minced meat (100 g) was homogenized and washed four times with cold (4 °C) deionized, distilled water (pH 7.0). Then, the washed myofibrils were suspended in water (5 mg/mL protein) and treated by 10000 psi (69 MPa), 15000 psi (103 MPa) or 20000 psi (138 MPa) HPH for two passes. The inlet temperature for the samples should be maintained within 4-6 °C. The HPH was carried out by 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) and implemented with a rapid cooling system for maintaining the outlet temperature blow 20°C. Previous study has shown that no

significant change in pH and ionic strength was detected for the treated suspensions (pH 6.92–7.05 and ionic strength 1.3 mM) (Chen, et al., 2016b). Finally, the HPH treated dispersions were lyophilized for 48 h using a freeze dryer (Alpha 2-4 LSCplus, Martin Christ, Germany) at -80 °C compressor temperature and 0.1 mbar vacuum pressure. The freeze-dried powders were milled and sieved using a screen mesh (0.3 mm in aperture). The freeze-dried myofibril sample without HPH treatment was used as the control. All samples contained about 87% of protein (w/w) and no significant differences in protein, moisture, ash and lipid contents were detected (Supplemental Table 1). Each HPH treatment was conducted for four replicates.

2.3. Determination of the structural and physicochemical properties

2.3.1. Fourier transform infrared (FTIR) measurements

FTIR measurements were performed on flakes of KBr (100 mg) containing sample powder (3 mg). All spectra in the region of 4000-500 cm^{-1} were performed with 64 times scanning on a Nicolet 6700 spectrophotometer (Thermal Fisher Scientific, WI, USA) at ambient temperature (20 °C).

Raw absorbance spectra were cut between 1700 and 1600 cm^{-1} for the analysis of Amide I. Fourier self-deconvolution of Amide I spectra was used to analyze the secondary structure of protein by using OMNIC 7.2 software from Thermo Nicolet Corporation (Thermal Fisher Scientific, WI, USA). The secondary structural contents of α-helix, ß-sheet, turn and random coil were calculated using integrated areas of each peak according to the method of Kong and Yu (2007).

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

Raw myofibril or WSMP-P were mixed with a sample buffer (20% glycerol, 5%

β-mercaptoethanol, 4% SDS, 0.125 M Tris, pH 6.8) to reach a final protein concentration of 2 mg/mL. SDS-PAGE was conducted with a 4% acrylamide stacking gel and a 12% separating gel to observe the myofibrillar profiles following the method previously described (Chen, Xu, Han, Zhou, Chen, & Li, 2016a).

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

Unknown protein bands in the SDS-PAGE gels were excised manually and were pre-treated as previously reported (Chen, et al., 2016a). Treated samples were then 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 for data collection. All the 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 calculated according to a label-free quantitation method (Griffin, et al., 2010) .

2.3.4. Differential scanning calorimetry (DSC) analysis

Thermal characteristics of WSMP-P were determined using a differential scanning calorimeter (DSC 1 STAR^e System, Mettler Toledo, Switzerland). An amount of 18 mg of WSMP-P sample was accurately weighed and sealed in an aluminium pan for heat scanning (from 30 to 120 °C at a heating rate of 10 °C/min). An aluminium pan without sample was used as the reference. The peak (T_d) denaturation temperatures and enthalpy of denaturation (ΔH) were estimated from the thermograms using the associated software (Mettler Toledo, Switzerland).

2.3.5. X-ray diffraction measurement

X-ray diffraction (XRD) measurement was conducted on a D/MAX2500V diffractometer

(Rigaku Corporation, Japan) to analyze the physical structure. Copper Kα was used at a voltage of 40 kV and a current of 40 mA. The XRD scans were set in the range of 2θ from 15° to 45° at an angular speed of 2°/min.

2.3.6. Scanning electron microscopy (SEM) observations

WSMP-P samples were fixed on aluminum stubs by the pieces of double-sided conductive carbon tabs, and sputter-coated (Jeol JFC-1600 Tokyo, Japan) with gold/palladium before the test. The SEM observations were then conducted on a scanning electron microscope (Jeol, JSM 6490LV, Tokyo, Japan) at an accelerating voltage of 20 kV.

2.4. Determination of the functional properties

2.4.1. Solubility

Protein samples were completely dispersed (5 mg/mL) in aqueous solution (10 mM sodium phosphate, pH 7.0). Then, the samples were centrifuged at 8000 g for 20 min (Liu, Geng, Zhao, Chen, & Kong, 2015). The solubility was defined as the ratio of protein concentration in the supernatant relative to that of protein suspension before centrifugation.

2.4.2. Particle properties of protein aqueous suspensions

Protein samples were completely dispersed (0.5 mg/mL) in aqueous solution (10 mM sodium phosphate, pH 7.0). Particle size (*Z*-average diameter) and the zeta potential was determined by dynamic light scattering (DLS) using a Zetasizer Nano-ZS 90 (Malvern Instruments Ltd., Worcestershire, UK) according to the descriptions of Chen, et al. (2016b).

2.4.3. Emulsifying properties

The emulsifying activity index (EAI) and emulsion stability index (ESI) were evaluated to determine the emulsifying properties according to the previous report (Xu & Liu, 2016) with a slight modification. Soy oil (2 mL) and 6 mL of 5 mg/mL protein solution (10 mM sodium

phosphate, pH 7.0) were initially homogenized using a high-speed mechanical shear unit (T25, IKA, Staufen, Germany) at 15,000 rpm for 2 min. Fifty microliters of the emulsion was pipette from the bottom of the emulsion into 5 mL of SDS solution (0.1%, w/v) at 0 and 10 min after homogenization. After shaking the diluted emulsions using a vortex mixer for a few seconds, the absorbance of these diluted emulsions were detected at 500 nm wavelength using a UV-vis spectrophotometer (U-3010, Hitachi, Japan). The absorbance measured at 0 min (A0) and 10 min (A10) was used to calculate the EAI and ESI according to Xu and Liu (2016).

2.5. Statistical analysis

Four independent trials were carried out for each treatment (n = 4). The analyses of data were performed using one-way ANOVA and Duncan's multiple range tests when necessary with the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). A $P < 0.05$ significance level was used to determine the differences between the treatments.

3. Results and discussion

3.1. Effects of HPH on the structural and physicochemical properties of WSMP-P

3.1.1. Protein Conformation

Fig. 1(A) displayed the FTIR spectra of native myofibril powder and WSMP-P modified by various HPH pressures. All samples showed strong and broad bands in the 3200–3600 cm^{-1} region (Amide A), ascribed to intermolecular H-bonded N-H and O-H stretching vibration. The weak peak at 2692 cm^{-1} was attributed to the C-H stretching vibration. Typical protein bands at 1655 and 1546 cm^{-1} are related to amide I (80% C=O stretch, 10% C-N stretch) as well as amide II (60% N-H bend, 30% C-N stretch and 10% C-C stretch)

(Fan, Hu, Zhao, Xiong, Xie, & Huang, 2017; Xu & Liu, 2016; Zhang, et al., 2015).

The entire FTIR profiles of samples were hardly affected by the HPH process, indicating that no major change occurred in the backbone structure. However, slight frequency shifting of Amide A (N-H and O-H stretching vibration) was observed after HPH treatment (Fig. 1A), suggesting the changes of intra/intermolecular interactions in WSMP-P induced by HPH. By 10000 psi treatment, the peak for Amide A was slightly shifted from 3293 cm^{-1} to higher wave number of 3295 cm^{-1}. This might be caused by the dissociation of myofibril under HPH (Chen, et al., 2016b). It is well known that N-H and O-H could participate in the formation of chemical bonds within proteins including the hydrophobic bond and the hydrogen bond (Fig. 5D). The intense mechanical forces (high pressure combining with shearing, turbulence as well as cavitation) occurring during HPH can induce unfolding of MP and affect protein conformation by disrupting the hydrogen bonding and hydrophobic interactions (Chen, et al., 2016a). The weaker intermolecular interactions involving N-H or O-H, the higher the electron density and shorten the N-H or O-H bond. As a result, the energy for displacing the hydrogen atom from the nitrogen or oxygen atom is enhanced and higher photon energy is accordingly required, being responsible for an absorption peak appeared at a higher frequency in the IR spectrum of protein (Harnkarnsujarit, Kawai, & Suzuki, 2015). Interestingly, the position of peak formed by stretching of N-H/O-H shifted back to lower wave numbers (3291 cm^{-1}) with increasing pressure up to 20000 psi (Fig. 1A). The reason might be that the hydrogen bonds or hydrophobic interactions were strengthened resulting from protein aggregation after 20000 psi HPH treatment. It was reported that HPH can dissociate myofibril into subunits and expose protein structure (Chen, et al., 2016a; Chen, et al., 2016b), diminishing intramolecular hydrogen bonding and spreading polypeptide

chains. However, the increase in unfolded extent of protein structure under intense HPH treatment would favor association of neighboring chains, which allowed the formation of strong intermolecular interactions, causing a relatively low amide A wave numbers at 20000 psi HPH. We hypothesized that HPH-induced protein dissociation and unfolding occurred simultaneously with the formation of aggregations. 15000 psi would be the critical pressure that induced dynamical changes between unfolding and aggregation.

Amide I absorption (1700-1600 cm^{-1}) represents stretching vibration of the C=O bond and has been widely used for determination of protein secondary structures. Fig. 1B showed the curve fitting of the fourier deconvoluted amide bands I for WSMP-P, which allowed to identify six major peaks corresponding to β-sheet (1691, 1626, 1611 cm^{-1}), β-turn (1674 cm^{-1}), α-helix (1657 cm^{-1}), and random coil (1641 cm^{-1}) according to the assignments by Shilpashree, Arora, Chawla, and Tomar (2015a) and Siddique, et al. (2016). The α-helix, β-sheet, β-turn, and random coil contents of WSMP-P were summarized in Table 1. The conformational structure of WSMP-P was sensitive to homogenization pressures. Upon 10000 psi HPH treatment, the content of α-helix and β-turn significantly decreased from 15.27% to 13.23% and from 15.05% to 10.35%, respectively, whereas the random coils increased from 7.05% to 10.80% ($P < 0.05$). In general, α-helix is stabilized by intra-molecular hydrogen bonds between the carbonyl oxygen (-CO) and amino hydrogen (NH-) of the polypeptide chain and is buried in the protein interior site, the β-turn structure is due to the highly ordered protein structure, while the random coil can originate from the unfolding of any higher order structures and related to protein flexibility (Chen, et al., 2016a; Peng, Chen, Luo, Ning, Zhu, & Xiong, 2016; Zhang, Yang, Zhang, Hu, & Zhao, 2017). HPH can disrupt the intact structure of myofibril and may have disturbed intra-molecular

hydrogen bonds, hence accelerating protein molecular motion. The loss of α-helix and β-turn together with increase of random coil revealed that the moderate unfolding played dominated roles in protein structural changes at 10000 psi HPH. With increasing HPH pressures to 15000 psi, an obvious loss in α-helix from 15.27% to 12.21% and β-turn from 15.05% to 9.65% along with an increase in β-sheet from 62.63% to 71.89% were presented ($P < 0.05$) (Table 1), indicating further enhancement of unfolding. Meanwhile, the increase of β-sheet content implied increased protein-protein interactions for the formation of intermolecular β-sheet structures (Harnkarnsujarit, et al., 2015; Lee, et al., 2009). A further increase of the pressures to 20000 psi allowed the formation of β-sheet to a higher extent of 78.40% (Table 1), indicating proteins interactions were enhanced when higher pressure (20000 psi) was used. It was likely that the decrease in the α-helix, β-turn and random coil contributed to an increase in β-sheet conformations at 20000 psi. The protein conformational changes characterized with a structural transition from α-helix and unordered to β-sheet, has been found in aggregated protein in solid state (Harnkarnsujarit, et al., 2015). This is consistent with the results of SDS-PAGE, SEM and XRD which suggested that protein probably went through structural rearrangement under HPH, including early dissociation into sub-units as well as subsequent interactions among reactive side groups facilitated by this dissociation at 20000 psi HPH.

The findings deriving from the FT-IR analysis confirmed the assumption of dissociation and partial unfolding of protein structure induced by HPH treatments (~15000 psi), which could promote the formation of aggregates at 20000 psi.

3.1.2. Protein profiles

Individual protein compositions of native and HPH treated samples were visualized

through SDS-PAGE (Fig. 2). All samples showed a typical protein profile of MP: myosin heavy chain (MHC, 225 kDa), M-protein (160 kDa), α-actinin (103 kDa), actin (43 kDa), tropomyosin (37 kDa) and myosin light chain (MLC1-3, 25 kDa, 17 kDa and 15 kDa), were consistent with previous reports (Eppenberger, Perriard, Rosenberg, & Strehler, 1981; Xiong, 1994). No obvious degradation to the individual proteins after HPH was observed as the SDS-PAGE patterns scarcely show any sign of hydrolysis (Fig. 2). However, there appeared to be some unidentified proteins (A, E, F, G, H, I) that varied with HPH pressures (Fig. 2). For native myofibril powder, polymer band A was presented at the top of the separating gel in the SDS-PAGE pattern. When they were subjected to HPH treatment, polymer band A gradually vanished with increasing HPH pressures from 10000 psi to 15000 psi (Fig. 2). It is likely that HPH can dissociate the polymer protein existed in native myofibril powder. To elucidate this, the corresponding bands including polymer A and E were excised from the gel for Nano LC-ESI-MS/MS analysis and protein identification. The results indicted a relatively high abundance of MHC in those bands: 67.8% and 70.4% for polymer A and E, respectively (Supplemental Table 2), confirming the dissociation of myosin polymers by HPH in WSMP-P. However, 20000 psi HPH could lead to myosin aggregation in WSMP-P (aggregate H in Fig. 2 and Supplemental Table 2). Moreover, the 130 kDa band originally ascribed to myosin-binding protein C in native myofibril powder (Band C in Fig. 2 and Supplemental Table 2) was evolved to be dominant with actin in WSMP-P (Band F, G and I in Fig. 2 and Supplemental Table 2). Since monomer actin possessed a molecular weight of 43 kDa, the actin appeared in 130 kDa band of WSMP-P was suggested to be in their trimer configuration induced by HPH.

Myosin polymers found in native myofibril powder may be caused by minor freeze-dried

denaturation because similar polymer band was also induced in squid myosin profiles by freeze-drying (Deng, Luo, Wang, & Zhao, 2015). Under HPH, the physical forces including high velocity impact, high-frequency vibration, instantaneous pressure drop, intense shear, cavitation and high pressures with a short treatment time (less than 5 s) are induced, which can affect the conformation of proteins (Chen, et al., 2016a). As myosin and actin was the predominant component of MPs, protein dissociation of myosin polymer (10000-15000 psi) and reassembling of actin (10000-20000 psi) were expected. In addition, harsh HPH pressure (20000 psi) could promote muscle protein aggregation (Chen, et al., 2016b), resulting in high molecular weight of myosin aggregate detected in WSMP-P.

3.1.3. Thermal properties

The effect of HPH treatment on the thermal behavior of myofibril powder and WSMP-P was presented in Fig. 3A and Table 1. All samples were found to have a major endothermic peak ranging from 51 to 59 °C without any remarkable changes in the peak shape. For meat proteins containing high amount of water, the thermal transition temperatures ranged from 38 to 67 °C was typically ascribed to myosin (Chen, Li, Nishiumi, Takumi, Suzuki, & Chen, 2014). and protein becomes stable toward heat when its water content becomes extremely low (Ghribi, Gafsi, Blecker, Danthine, Attia, & Besbes, 2015), thus it was suggested that the transition peak of myofibril dried powder (Control) at high temperature of 51 °C was the result of myosin denaturation. However, the denaturation temperatures (T_d) shifted to higher values and the enthalpy of denaturation (ΔH) decreased to lower values with increasing HPH pressures (~20000 psi) (Fig. 3A and Table 1). Likewise, studies conducted on the soy protein isolate (Keerati-u-rai & Corredig, 2009) and zein nanoparticles (Sun, et al., 2016) have also observed an increase of their thermal transition peak temperature after they were treated by

HPH. The protein might be more denatured upon HPH treatment prior to heat treatment, thus partially denatured proteins required less energy for further denaturation as the thermograms showed a weakening of the peaks and reduction of ΔH with increasing HPH pressures (Fig. 3A and Table 1). Certain method of processing that induces a well defined structure influences the thermodynamic property of proteins (Ghribi, et al., 2015; Sun, et al., 2016). These significant changes of T_d and ΔH after HPH may be caused by structure rearrangements in MP during HPH.

The thermal stability of a protein is reflected by T_d (the temperature point that cause protein denaturation) and ΔH (the amount of input heat energy required to denature protein) (Kaushik, Dowling, McKnight, Barrow, Wang, & Adhikari, 2016). The increase of T_d and reduction of ΔH as shown in Fig. 3A and Table 1 indicated that HPH enhanced the heating stability of WSMP-P. We have previously discovered that HPH can disrupt the integrated structure of myofibril and modify protein structure by unfolding and inducing soluble myosin oligomers via disulfide bonds in an aqueous condition (Chen, et al., 2016a; Chen, et al., 2016b). The disulfide linkages between the trimers in glycinin (Keerati-u-rai & Corredig, 2009; Staswick, Hermodson, & Nielsen, 1984) and inter-/intra-chain disulfide bonds in napin (Schwenke, Drescher, Zirwer, & Raab, 1988) have been reported to assist with stabilizing its conformation. It was possible to speculate that the compact protein structure having minor disulfide bonds induced by HPH led to significant improvement of thermal stability of WSMP-P. On the other hand, It was shown that the ΔH reflected the conformation status of food proteins (Ghribi, et al., 2015). The reduced value observed for WSMP-P might be attributed to the loss of helical structure of the protein as discussed from the results of FTIR. Moreover, disruption of hydrophobic interactions is the exothermic

reaction which lower the observed enthalpy (Boye, Alli, & Ismail, 1996; Siddique, et al., 2016). During the HPH treatment, the intra/inter molecular hydrophobic bonds were disrupted and exposed to the surface in WSMP (Chen, et al., 2016a), thus the energy required for the complete thermal unfolding decreased in WSMP-P, which was consistent with the observed reduction of enthalpy. Overall, DSC results suggested that HPH could denature native protein and produce compact internal structure to enhance the thermal stability of WSMP-P.

3.1.4. XRD properties

Food materials in solid states are classified to be crystalline, semi-crystalline or amorphous. The XRD patterns in Fig. 3B showed that all samples displayed a predominant amorphous halo, a wide band with a peak at 2θ= 20°, confirming the amorphous structure of WSMP-P. A dominant amorphous halo peaked at 2θ= 20° was also observed for soy protein isolate (Zhang, Song, Wang, & Wang, 2012), flax seed protein isolate (Kaushik, et al., 2016) and chickpea protein concentrates (Ghribi, et al., 2015). With HPH pressures increasing to 15000 psi, the peak intensity at 20° gradually diminished, suggesting that the intact structure of myofibril had collapsed after HPH treatment and more amorphous structure was presented in WSMP-P as HPH proceeded (Zhang, et al., 2015). This was confirmed by our previous finding which showed that, by 15000 psi HPH, the filament structure of myofibril completely split and randomly disassociated into sub protein species such as thick/thin filament, oligomers or monomers in WSMP (Chen, et al., 2016b). However, higher pressure at 20000 psi can lead to an “over-processing” effect and enhance the formation of protein aggregation (Chen, et al., 2016b). Due to the myosin aggregation (Fig. 2 and Supplemental Table 2), the intensity of the diffraction peak in WSMP-P rebounded back to higher value

when harsher pressure (20000 psi) of HPH was applied (Fig. 3B). It could be concluded that HPH could break the intact structures and produce amorphous structure in WSMP-P. HPH at 15000 psi was sufficient for optimal structure modification. The amorphous protein powders always have the superiorities of demonstrating higher solubility, water retention and compressibility (Bhandari & Roos, 2012).

3.1.5. Microstructure

Native myofibril powder and WSMP-P were monitored through SEM to study the appearance changes after various pressures treatment. As shown in Fig. 4A, native myofibril freeze-dried powder exhibited smooth lamellar or flake-like structures with large particle size and irregular geometry shapes. It was structurally similar to the freeze-dried egg white protein hydrolysates (Chen, Chi, & Xu, 2012), lentil protein isolates (Joshi, Adhikari, Aldred, Panozzo, & Kasapis, 2011) and milk protein concentrate (Shilpashree, et al., 2015a). The larger particles and flake-like structure may be ascribed to lack of forces for breaking up the frozen liquid into droplets or for substantially altering their surface topology during the freezing evaporation process (Chen, et al., 2012). By HPH, the morphology of WSMP-P was obviously disrupted, deviating from the original flake-like to random geometric entities with small particle size. And it was mainly depended on the specific pressures. When the pressure was set at 10000 psi, large portion of protein seemed to be broken down into small particles. Although some large particle size species were still retained, the appearance of protein became rough with twisted shape (Fig. 4B). After HPH treatment at 15000 psi, the shapes of WSMP-P became more irregular and extensively damaged which eventually disintegrated into dispersive particles with reduced particle size (Fig. 4C). This was in accordance with our atomic force microscope results in WSMP which showed that the intact structure of

myofibril substantially degraded into monomers, oligomers or filament fragments after 15000 psi HPH (Chen, et al., 2016b). It was also reported that soy protein isolate and zein particles were broken into small particles after exposure to super-high shear force induced by HPH (Song, Zhou, Fu, Chen, & Wu, 2013; Sun, et al., 2016). These results suggested that modification by the strong physical force of HPH contributed to the severe disruption of myofibril integrity and sufficient dispersion of particles in WSMP-P. When the pressure was beyond 20000 psi, some large irregular aggregates were observed in WSMP-P (Fig. 4D), evidencing the aggregation of proteins under harsh pressure of HPH (Fig. 2), which was in a good agreement with the XRD results (Fig. 3B). A possible explanation was that HPH destructed intermolecular hydrophobic interactions and hydrogen bonding, and the reassembling of molecular structure arose when the pressure reached to 20000 psi, leading to formation of protein aggregates through intra/inter particle interactions. Our observations suggested that different HPH pressure could give rise to various microstructures for WSMP-P.

3.2. Effects of HPH on the functional properties of WSMP-P

3.2.1. Solubility in water

The solubility of native myofibril and WSMP-P as affected by HPH pressures was shown in Fig. 5A. Without HPH treatment, it was reasonable that the solubility of native myofibril powder in water was extremely low. It was agreed with the previous report, suggesting the insolubility of MP in water without specific processing procedures (Ito, et al., 2004). However, it was of interest to discover that HPH (10,000-20,000 psi) treatment could markedly enhance ($P < 0.05$) the solubility of WSMP-P up to 45% (Fig. 5A). With pressure increased from 0 psi to 15,000 psi, the solubility showed a progressive increase ($P < 0.05$) up

to 60%. When pressure reached 20000 psi, the solubilization effect of HPH was weakened comparing to that of 15000 psi because of protein aggregation as discussed above. These findings suggested that WSMP continued to have high water solubility in its powder state. HPH was able to enhance the solubility of WSMP-P in water and 15,000 psi was the optimal HPH condition.

The protein structure, molecular size and exposed ionizable amino and carboxyl groups influence the sensitive balance between repulsive and attractive intermolecular forces which regulated the solubility of proteins (Chen, et al., 2012; Shilpashree, et al., 2015a). The enhancement of solubility was consistent with the previous observations of the effects of HPH treatments on proteins structure. Due to the modification of HPH, native myofibril were randomly disrupted and unfolded as observed in SEM and FTIR, exposing charged residues of the amino acid to the protein surface. The newly exposed groups can interact with water molecules via stronger hydrogen bonds and thereby being more soluble in water (Chen, et al., 2012; Siddique, et al., 2016). In addition, the rise of solubility was also in a good agreement with the results of particle size and zeta potential (Fig. 5B). Muscle myofibril structure is primarily composed of thin and thick filaments, the backbone of the thick filament is made up of myosin while the large component of the thin filaments consists of protein actin (Pearce, Rosenvold, Andersen, & Hopkins, 2011). Thus a large particle size for native myofibril (> 3000 nm) was exhibited in water (Fig. 5B). After an intense HPH ($\geqslant$ 15000 psi) treatment, the complex macromolecular structure could be dissociated and fragmented to small particles with submicron range (about 500 nm) size in water (Fig. 5B). The nano-/submicron size particles can undergo strong Brownian motion, which would markedly reduce centrifuged aggregation (Chen, et al., 2016b). Small particle size of

WSMP-P in aqueous solution can increase the molecular surface area which increased the ability of water-protein interactions and resulted in electrical repulsion between identical charges (Chen, et al., 2016b; Shilpashree, Arora, Chawla, Vakkalagadda, & Sharma, 2015b). The increased net negative charge on protein molecular surface (high absolute zeta potential value as observed in Fig. 5B) and the enhanced electrostatic repulsion between them can retard the formation of protein aggregates and thereby improve the solubility of the proteins in water (Fig. 5A). Minor protein aggregation through disulfide cross-linking or hydrophobic interactions might contribute to the loss in protein solubility treated at 20000 psi (Chen, et al., 2016a; Liu, et al., 2015). Overall, the results indicated myofibril has undergone thorough disruption and dissociation after HPH treatment. HPH is capable of inducing WSMP-P aqueous suspensions with small particle size and high surface charge, promoting the solubilization of WSMP-P in water.

3.2.2. Emulsifying activity and emulsion stability

As depicted in Fig. 5C, HPH obviously improved the emulsifying activity and stability of WSMP-P, indicating that WSMP-P were more favorable of forming an interfacial membrane, thus facilitating the dispersion of oil droplets and stabilizing emulsion droplets with respect to untreated native proteins. Among the HPH pressures, 15000 psi HPH treatment rendered proteins with the greatest surface activity (Fig. 5C).

Emulsifying properties of proteins are strictly affected by the surface charge, hydrophilic-lipophilic balance and conformational flexibility (Chen, et al., 2012; Xu & Liu, 2016). HPH had been reported to partially unfold and expose their hydrophobic groups of whey protein, and this structural state may facilitate interactions with the lipid phase (Lee, et al., 2009). The higher value of EAI in WSMP-P can be attributed to the moderate

dissociation and unfolding of native protein structure caused by HPH treatment as described earlier. Protein unfolding can result in the exposure of hydrophobic amino acid residues and the increase of conformational flexibility, consequently enhancing the surface activity and protein adsorption at the oil-water interface (Ghribi, et al., 2015; Xu & Liu, 2016; Zhang, et al., 2015). The effect of HPH on the EAI and ESI of WSMP-P were also found to be consistent with the variation trends of solubility, particle size and surface charge. WSMP-P with high solubility (Fig. 5A) and small particle size (Fig. 5B) could move and diffuse rapidly to the interface for adsorption (Shilpashree, et al., 2015a). Additionally, proteins generally can form a coating over oil droplets to prevent agglomeration for emulsions stabilization. The electrostatic repulsion between protein coated oil droplets relies on electrical potential of the ionized layer they possess (Kaushik, et al., 2016). The high net charge in WSMP (Fig. 5B) can generate strong inter-particle repulsion and lead to a more stable cohesive interface, therefore preventing emulsion coalescence (Chen, et al., 2012; Shilpashree, et al., 2015b). Notably, the EAI of 20000 psi treated WSMP-P was lower than that of 15000 psi (Fig. 5C). This case might be linked to unfolded-structure of WSMP-P with slight decrease in the surface charge (Fig. 5B) and partly weakened conformational flexibility due to minor protein aggregation.

4. Conclusion

To sum up as proposed in Fig. 5D, the protein conformation, protein profile, thermal property, XRD property, and microstructure analysis led to the general conclusion that HPH induced myofibril and myosin polymer dissociation (10000-15000 psi), protein unfolding and rearrangement of myosin and actin (20000 psi). The structural changes were found to

mainly involve tertiary and secondary structure, in which loss of α-helix and β-turn with formation of β-sheet was presented. Also an "amorphous" protein structure with relatively high thermal stability was produced. Whereas water solubility and emulsifying properties were significantly improved after HPH treatment at 15000 psi, HPH produced unfolded flexible structures with smaller particle size and higher surface charge in aqueous solution that were clearly more diffused, more surface-active and more capable of stabilizing emulsion droplets than control. It would be of interest to test the effects HPH on structural, physicochemical and functional properties of meat proteins that can be used beneficially as food additives in formulated food or beverage at low ionic strength for pediatric and adult nutrition.

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■ Figure captions

Fig. 1 (A) FTIR spectra and (B) Fourier deconvoluted amide I (1700-1600 cm^{-1}) of (a) native myofibril powder and WSMP-P treated by (b) 10000 psi, (c) 15000 psi, (d) 20000 psi HPH, respectively.

Fig. 2 Representative SDS-PAGE patterns of (1) native myofibril powder and WSMP-P treated by (2) 10000 psi, (3) 15000 psi, (4) 20000 psi HPH, respectively. M: marker, MHC: myosin heavy chain, MLC: myosin light chain. The gel sections from the preparative lanes (A-I) were sliced for LC-ESI-MS/MS analysis.

Fig. 3 (A) DSC thermograms of and (B) X-ray patterns of native myofibril powder and WSMP-P treated by (B) 10000 psi, (C) 15000 psi, (D) 20000 psi HPH, respectively.

Fig. 4 SEM images of (A) native myofibril powder and WSMP-P treated by (B) 10000 psi, (C) 15000 psi, (D) 20000 psi HPH, respectively. Arrows in D indicated protein aggregates.

Fig. 5 (A) Water solubility, (B) particle size and zeta potential, (C) emulsifying activity index (EAI), and emulsion stability index (ESI) of native myofibril powder and WSMP-P treated by 10000 psi, 15000 psi, 20000 psi HPH, respectively. Values are means ± SD (n = 4), a-d or a′-d′ indicates that the different letters are significantly different ($P < 0.05$). (D) Proposed mechanism of protein structural changes modified by HPH for improved

functional properties in WSMP-P. See texts for details.

■ **Figure graphics**

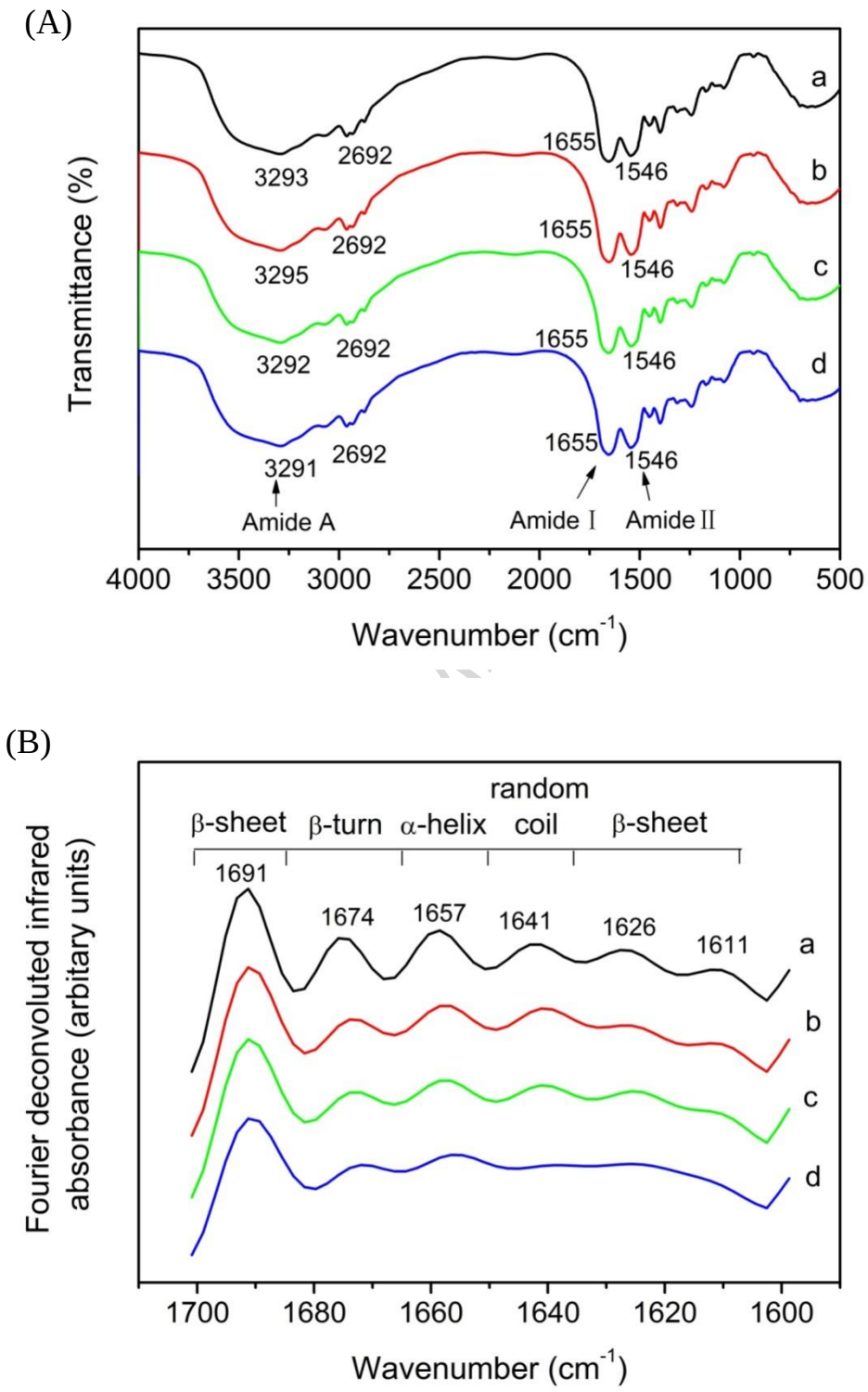
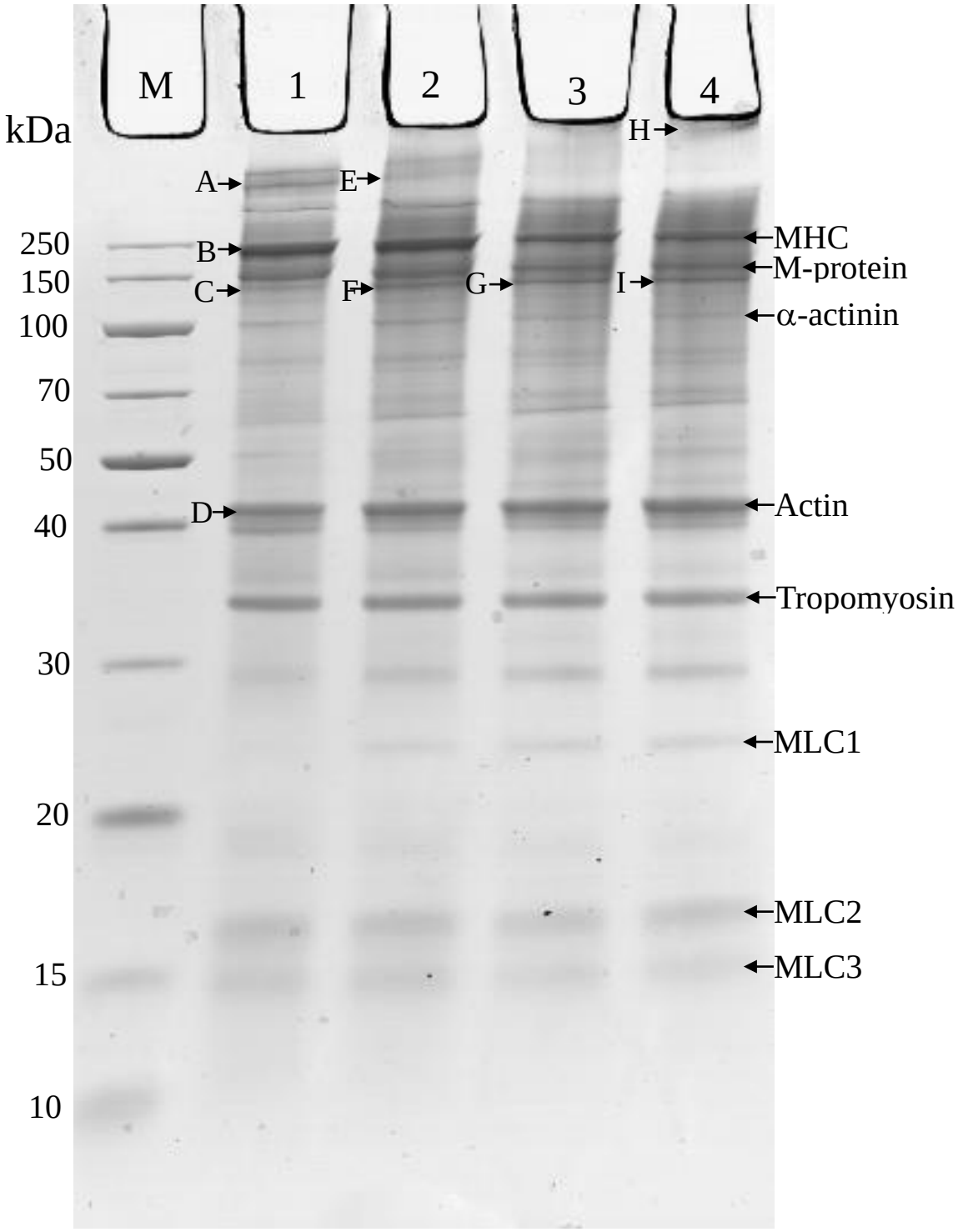


Fig. 1 Xing Chen et al.



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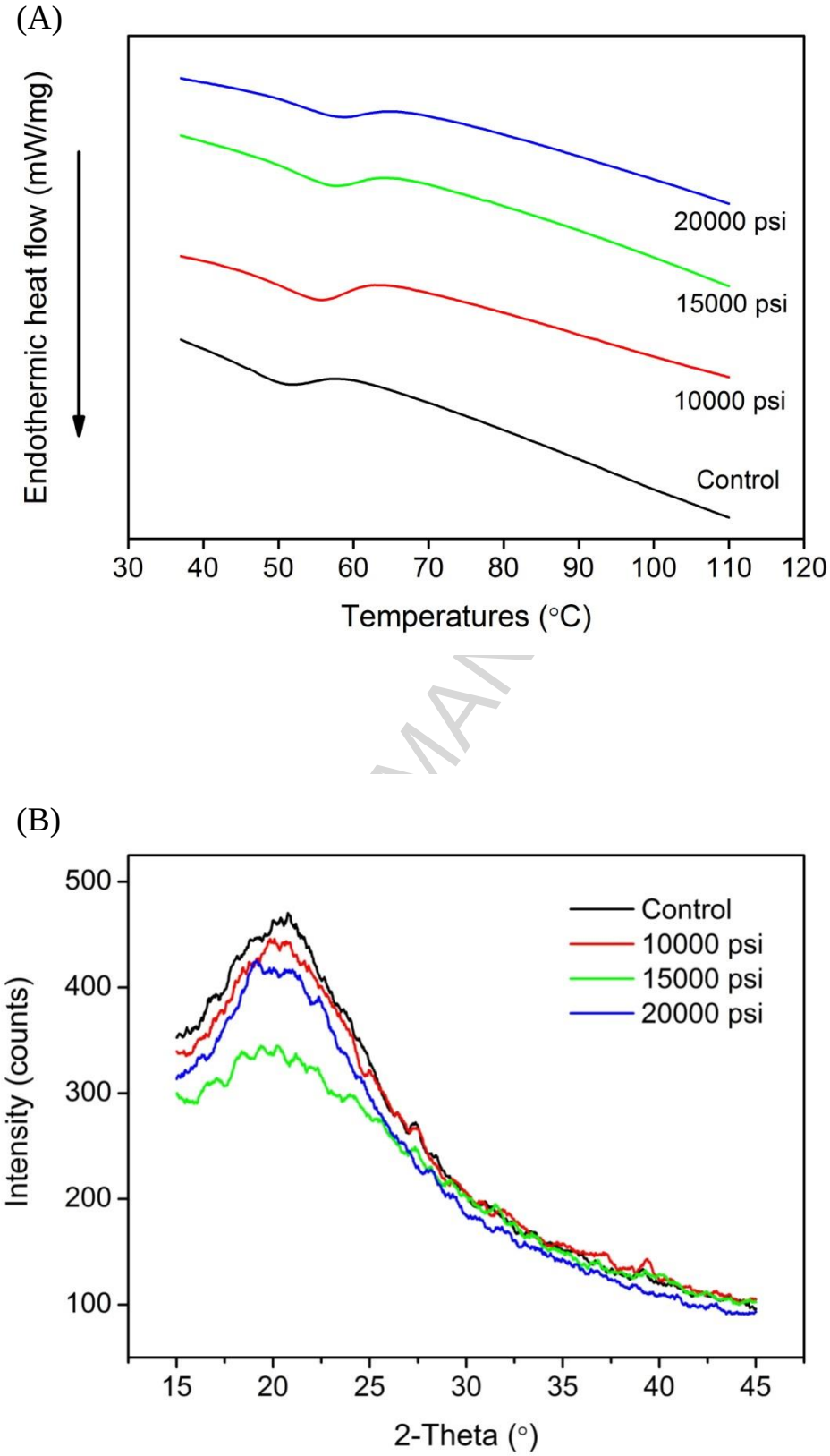

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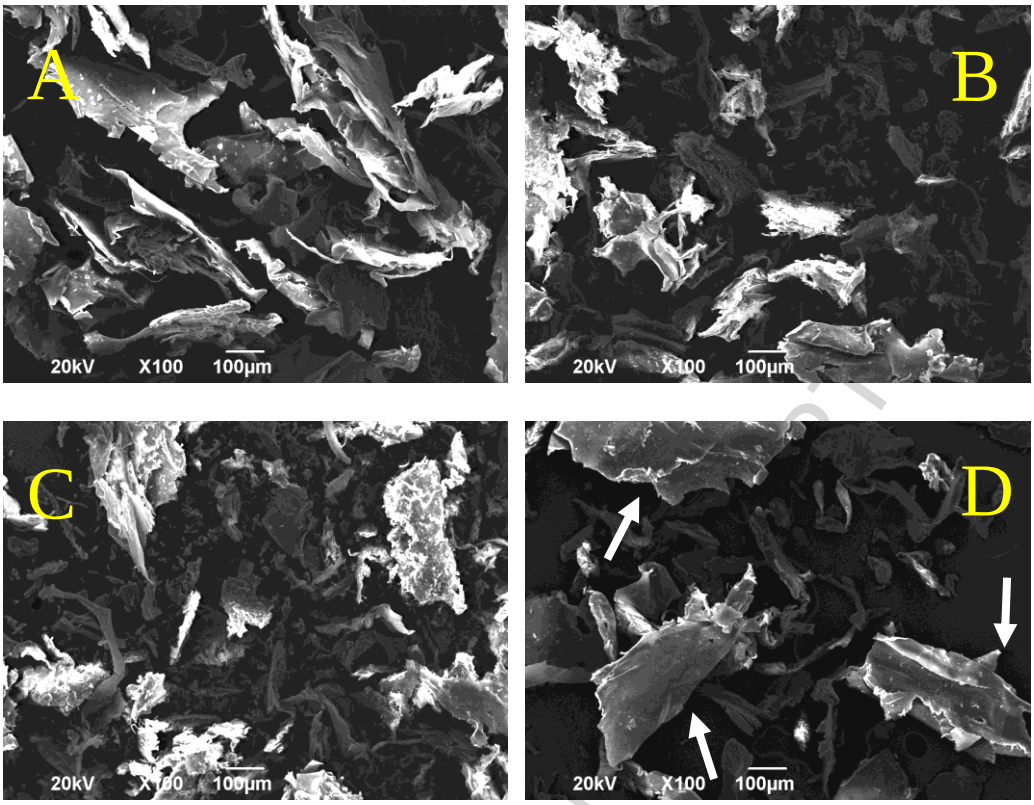

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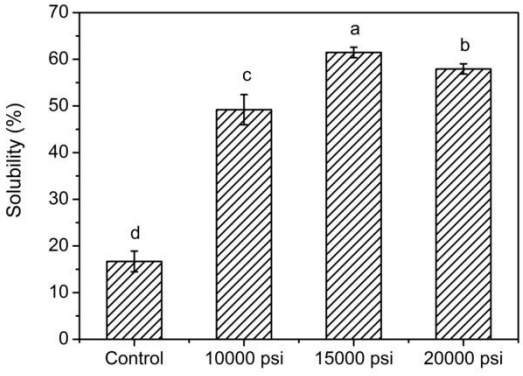
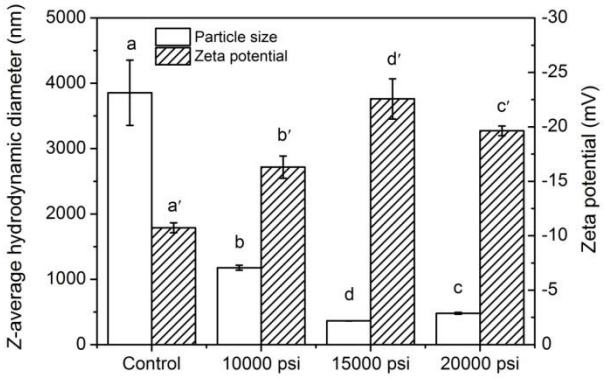


(A)



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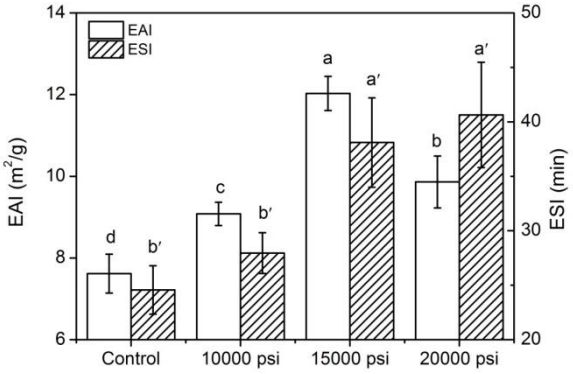
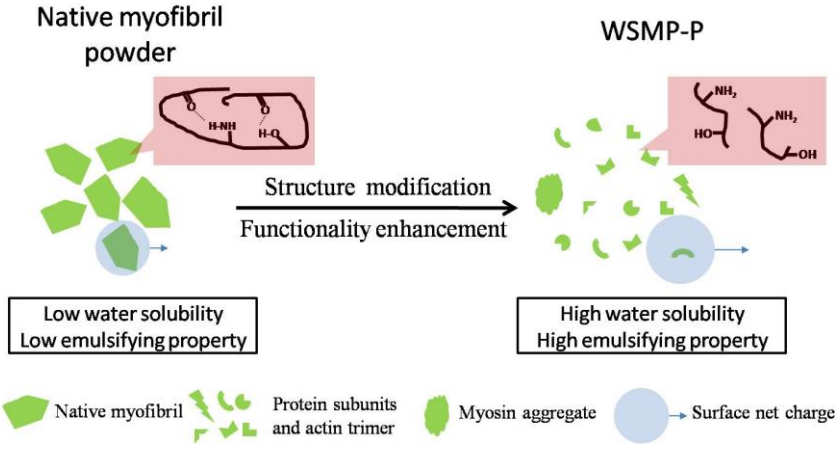




(D)

Fig. 5 Xing Chen et al.

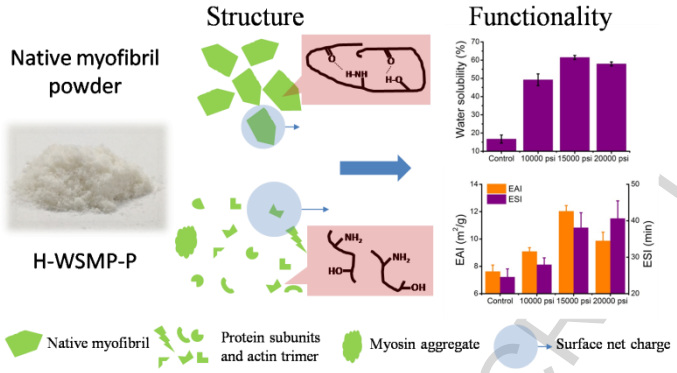
■ Tables

Table 1 Effect of HPH on the endothermic transition peak (T_d), enthalphy (ΔH), and the secondary structure content estimated from deconvoluted FTIR spectra of WSMP-P

Treatment	T_d (°C)	ΔH(J/g)	α-helix (%)	β-sheet (%)	β-turn (%)	Random coil (%)
Control	51.49 ± 0.77d	0.27 ± 0.01a	15.27 ± 0.47a	62.63 ± 1.64c	15.05 ± 1.73a	7.05 ± 1.02b
10000 psi	55.89 ± 0.26c	0.22 ± 0.04b	13.23 ± 0.81b	65.62 ± 2.14c	10.35 ± 0.93b	10.80 ± 1.73a
15000 psi	57.81 ± 0.34b	0.17 ± 0.01c	12.21 ± 0.62b	71.89 ± 0.19b	9.65 ± 0.68b	6.25 ± 1.01b
20000 psi	58.89 ± 0.26a	0.13 ± 0.02c	10.79 ± 0.57c	78.40 ± 1.49a	8.47 ± 0.44b	2.33 ± 1.34c

. Note: Means (n = 4) within the same column sharing no common letters (a−d) differ significantly ($P < 0.05$). Control means non-HPH treated sample.

■ Graphical abstract



■ Highlights

- High-pressure homogenization (HPH) modified myofibrillar protein powder (MP-P).
- HPH induced protein dissociation, unfolding and rearrangement in MP-P.
- HPH produced amorphous protein structure with high thermal stability in MP-P.
- 15000 psi HPH increased protein flexibility for enhanced functionalities in water.
- MP-P can be used as protein supplements in formulated food at low ionic strength.