Food Chemistry 232 (2017) 727–732

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Changes in the structure and dissociation of soybean protein isolate induced by ultrasound-assisted acid pretreatment

Liurong Huang [a,b,*], Xiaona Ding [a], Chunhua Dai [a], Haile Ma [a,b]

[a] School of Food and Biological Engineering, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, China
[b] Jiangsu Provincial Key Laboratory for Physical Processing of Agricultural Products, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, China

ARTICLE INFO

Article history:
Received 8 January 2017
Received in revised form 7 April 2017
Accepted 12 April 2017
Available online 13 April 2017

Keywords:
Structure
Dissociation
Ultrasound
Acid
Soybean protein isolate

ABSTRACT

Structure and dissociation properties of soybean protein isolate (SPI) induced by ultrasound and acid were investigated. Results of solubility showed that ultrasound-assisted acid had no effect on the content of soluble aggregates in SPI. Increase of fluorescence intensity and red-shift of maximum emission wavelength indicated that acid induced molecular unfolding of SPI and exposure of hydrophobic groups. Circular dichroism spectra showed that ultrasound-assisted acid pretreatment resulted in increases in the α-helix content by 29.2% and random coils content by 8.3%, while β-sheet decreased by 13.4% ($P < 0.05$), as compared with those of control. Analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and atomic force microscope revealed that the contents of small subunits and particle increased significantly when SPI treated by ultrasound-assisted acid comparing with the SPI treated by single acid and ultrasound treatment. This study illustrated the ultrasound and acid have synergistic effect on the structure unfolding and dissociation of SPI.

1. Introduction

Soybean meal, an abundant by-product of oil production industry, contains about 35–45% (w/w) protein. The proteins from soybean meal have been widely applied in many protein-based food formulations, primarily attributed to their high nutritional value, excellent processing ability, and low cost. 90% of native soy proteins are storage proteins with globular structure consisting mainly of 7S (β-conglycinin) and 11S (glycinin) globulins (Nielsen, 1985). The compact globular structure is stabilized mainly by hydrogen bonds and disulfide bonds, which results in lower molecular flexibility and a rather poor property (Chen, Chen, Ren, & Zhao, 2011). In some food cases, selected superior functional properties such as foaming and dynamic surface properties are needed for the protein. Thus, lots of techniques including physical, chemical and biological modifications have been used to improve the functional properties of soy proteins (Chen et al., 2011; Yuan, Ren, Zhao, Luo, & Gu, 2012).

The dissociation and aggregation of protein subunits play an important role in the microstructure, with consequent changes of functional properties such as rheology, emulsification and gelation (Durand, Gimel, & Nicolai, 2002; Nicorescu et al., 2010; O'Sullivan, Arellano, Pichot, & Norton, 2014). Li, Wang, Hu, and Liao (2014) reported that more intense high pressure CO_2 treatment caused the dissociation of larger aggregates and further aggregation in the smaller aggregates, which resulted in the change of polyphenol oxidase activity of thaumatin-like protein. The effect of heat_induced dissociation and aggregation on the functional properties of soy protein was also reported (Guo et al., 2012; Keerati-u-rai & Corredig, 2009). Both soy 7S and 11S globulin in their isolated form can aggregate on heating, the nature of which depends upon the precise conditions of protein concentration, pH and ionic strength (Li et al., 2007). Therefore, if the protein can be given a full dissociation, and then re-aggregated at the controlled condition, a protein with new properties can be produced.

Recent years, the power ultrasound was widely introduced to improve the functional properties of proteins. They showed that ultrasound broke the peptide bonds, destroyed the noncovalent interactions, and induced the dissociation and aggregation of subunits, leading to the changes of protein functional properties (Hu et al., 2013; Jambrak, Lelas, Mason, Krešić, & Badanjak, 2009; Jin et al., 2015; Tang, Wang, Yang, & Li, 2009). Generally, single modification treatment has relative low efficiency. Therefore, most research focus on the combination of different modification methods to improve the functional properties of protein (Li et al., 2016; Luo et al., 2010; Wei & Ye, 2011). For example, it has been reported that ultrasound treatment combined with transglutaminase could

* Corresponding author at: School of Food and Biological Engineering, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, China.
E-mail address: hlr88888@163.com (L. Huang).

http://dx.doi.org/10.1016/j.foodchem.2017.04.077
0308-8146/

enhance the gelling properties of soy protein (Hu et al., 2015). Zhao, Xin, Zhao, Chen, and Cai (2014) indicated the structural variations of peanut protein isolate under acidic conditions. Due to the intermolecular electrostatic repulsion, the peanut protein isolate unfolded and the subunits dissociated especially at pH values ranging from 2.0 to 3.0. It was also found that glycinin would unfold and disassociate in acid solution (Yuan et al., 2012). In the view of these results, it can be inferred that the combination of acid treatment and ultrasound modification is a good choice to be used for dissociation of SPI.

For this reason, the objective of the present work was to investigate effects of ultrasound-assisted acid pretreatment on the structural characteristics of soybean protein isolate. Changes in the solubility, subunits dissociation and particle size, as well as secondary and tertiary structures of soybean protein isolate were investigated. This work would contribute to our further study on the relationship of aggregation control and functional properties of soy protein isolate.

2. Materials and methods

2.1. Materials

Defatted soybean meal (protein content, 39.2%) supplied by Danyang Zhengda Oil Co., Ltd. (Jiangsu, China), was milled and sieved through a 180 μm mesh. Pre-stained protein markers (bands1-8: Mr 15, 20, 25, 35, 50, 70, 100 and 150 kDa) were purchased from Ruichu Biotechnology Co., Ltd (Shanghai, China). Deionized water was used in the experiments, which was purified by Ultra-pure water system (UPN-1V-20L, Ulupure Technology Co. Ltd, Chengdu, China). All the other chemicals used in the present study were of analytical grade.

2.2. Preparation of soybean protein isolate (SPI)

SPI was produced from the defatted soybean meal according to Liu, Zhao, Ren, Zhao, and Yang (2011), with slight modifications. 200 g of soybean meal powder was dispersed in deionized water (1:15, w/w), then the pH was adjusted to 8.0 with 2 M NaOH, and the resultant slurry was mechanically stirred for 2 h at 25 °C. After centrifuging at 4000*g* for 15 min, the protein supernatant was collected and adjusted to pH 4.5 with 2 M HCl, and then centrifuged at 4000*g* for 15 min. The obtained precipitate was dissolved in deionized water, neutralized to pH 7.0 with 2 M NaOH, dialysed against deionized water at 4 °C for 24 h and then freeze-dried to obtain SPI.

2.3. Pretreatments of SPI with ultrasound and acid

SPI were treated by ultrasound, acid and their combination according the methods of Zhao et al. (2014) and Li et al. (2016). Briefly, the suspension containing 1.0 g SPI and 90 ml solvent was stirred at 25 °C for 1 h. For control and ultrasound samples, the solvent was water and pH was maintained at 7.0 by adding 0.1 M NaOH or HCl during the agitation process. For samples acid and ultrasound-assisted acid, the solvent was 0.001 M HCl and pH was maintained at 3.0. Then samples ultrasound and ultrasound-assisted acid were treated by ultrasound. Ultrasonic reactor (GA92-II, Shangjia Biotechnology Co., Wuxi, China) was equipped with a 2.0 cm flat tip probe operating in a pulsed mode (on-time 2 s and off-time 2 s). The suspension was put into a 100 ml beaker, and the beaker was placed in a water bath at initial temperature of 25 ± 2 °C. The probe was submerged to a depth of 2.0 cm in the suspension and sonication was done at 600 W and 20 kHz for 5 min.

In order to compare the effects of different pretreatments, the suspension was diluted to 100 ml with the corresponding solvent. The dilution was centrifuged at 4000*g* for 15 min and the supernatant was collected for further analysis.

2.4. Determination of solubility

After appropriate dilution, the protein content of the supernatant was determined by method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as the standard. The protein solubility was expressed as grams of soluble protein per 100 g of SPI.

2.5. Measurement of intrinsic fluorescence

The fluorescence spectra were recorded using a Cary Eclipse spectrofluorometer (Varian Inc., Palo Alto, USA) at room temperature (25 ± 1 °C). The protein solutions were excited at 282 nm, and emission spectra were recorded from 300 to 500 nm using a 10 × 10 mm quartz cuvette. The maximum emission wavelength was recorded. The constant slit of 5 nm was set for both excitation and emission.

2.6. Circular dichroism (CD) analysis

Circular dichroism technology was used to determine the secondary structure of SPI (Liao, Wang, & Zhao, 2013). The CD spectra were recorded in the far UV range (250–190 nm) with a spectropolarimeter (Jasco J-815, Jasco Corp., Tokyo, Japan) at 20 °C; a quartz cuvette was 10 mm optical path length; an interval of 0.2 nm and scan speed of 100 nm/min were used. Three scanning acquisitions were accumulated and averaged to obtain the final spectrum. The CD data were expressed in terms of mean residue ellipticity, $[\theta]$, in deg.cm^2. dmol^{-1}. The secondary structure contents were calculated using the CD Pro software (Narasimha Sreerama Research Group, Fort Collins, CO, USA) by the CONTINLL method.

2.7. Analysis of subunits composition by SDS-PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis was conducted using the discontinuous Tris-glycine buffer (pH 8.3) system with 12% separating gel and 5% stacking gel according to the method of Laemmli (1970).

2.8. Atomic force microscopy (AFM) characterization

A 10 μl of diluted suspension was dropped on a freshly cleaved mica substrate. The substrate was dried at ambient temperature of 25 °C for 12 h. The mica was placed in an inspection slot. An atomic force microscope (Multimode 8, Bruker Inc. Germany) was used to perform the surface topography and nano-structural properties analysis (Müller, Müller, & Engel, 2011). All the images were scanned in air using standard peak force-mode silicon cantilevers with a force constant of 5 N/m and resonant frequency of 150–200 kHz. Images were analyzed from area of 25 μm^2 of each sample by processing software Nanoscope Analysis 1.4.

2.9. Statistical analysis

Each determination was run in triplicate and the data were expressed as mean ± SD. Analysis of variance (ANOVA) was performed to compare the effects of ultrasound, acid and their combination under the significance level of $P < 0.05$. Graphs and calculations were performed with Origin Pro 8.0 and Microsoft Office Excel 2010, respectively.

3. Results and discussion

3.1. Changes in the solubility of SPI

Solubility is one of the most important functional properties of protein. It can affect other functional properties, including surface active, rheological and hydrodynamic properties. The solubility of SPI exhibited typically U-shape trend at different pH levels, the lowest solubility was reached at around the isoelectric point (pH 4 to 5). Similar results have been reported by Cui, Zhao, Yuan, Zhang, and Ren (2013) and Jaramillo, Roberts, and Coupland (2011). In this research, the pH values of control, ultrasound, acid and ultrasound-acid pretreatments were 7.0, 7.0, 3.0 and 3.0, respectively. The effects of the ultrasound and acid pretreatments on the solubility of the SPI are shown in Fig. 1. No significant changes in solubility were observed between ultrasound pretreatment and control. Solubility of SPI with acid pretreatment was decreased by 8.6% over the control ($P < 0.05$), indicating that acid induced the transformation from soluble aggregates to insoluble aggregates because of charge effect. The decrease of highly charged proteins might be linked to unfolding and exposure of hydrophobic groups from the inner part of proteins (Tang, Wang, & Yang, 2009). The solubility value for ultrasound-assisted acid pretreatment was 64.2%, showing no significant differences compared with that of the control. However, solubility of SPI with ultrasound-assisted acid pretreatment was higher than that of acid pretreatment ($P < 0.05$), indicating that hydrophobicity was influenced by sonication at pH 3.0. In previous studies, we found that ultrasound and coupled ultrasound-acid could increase the solubility of peanut protein isolate, while there were no significant differences in solubility between control and acid pretreatment. The distinction may be attributed to differences in subunit composition and structure of different protein.

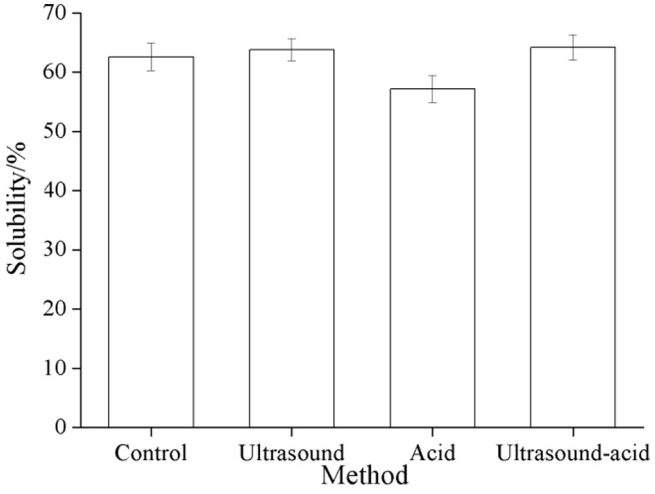

Fig. 1. Protein solubility of SPI with different pretreatments.

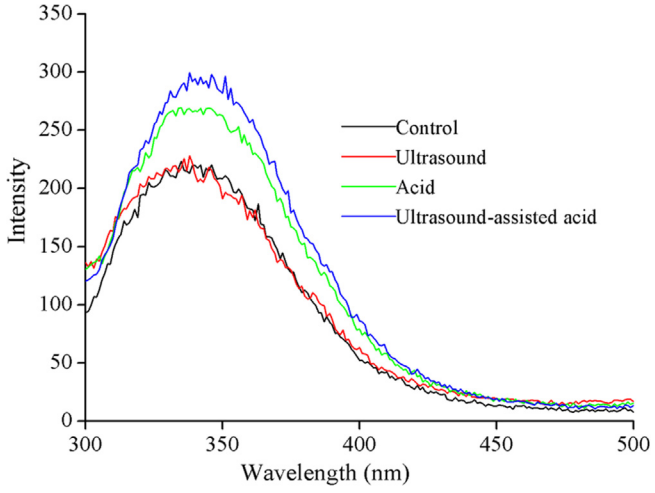

Fig. 2. Effect of ultrasound-assisted acid pretreatment on the fluorescence spectra of SPI.

3.2. Fluorescence spectra analysis

The emission fluorescence spectrum is mainly attributed to the tryptophan (Trp)/tyrosine (Tyr) residues and provides sensitive detection of protein conformational changes during processing (Keerati-u-rai, Miriani, Iametti, Bonomi, & Corredig, 2012). As shown in Fig. 2, the maximum emission wavelength of control was 335.7 nm. Single sonication of SPI showed no significant influences on the fluorescence intensity and maximum emission wavelength over the control. When SPI was treated by acid, maximum emission wavelength shifted by 2.3 nm, coupled with an increase in amounts of the fluorescence intensity ($P < 0.05$). The red shift indicates that more chromophores exposed to solvent due to molecular unfolding under acid condition (Miriani, Iametti, Bonomi, & Corredig, 2012). However, solubility of acid-treated SPI was lower than that of control, suggesting that increases of fluorescence intensity and maximum emission wavelength were from the contribution of the structural changes in soluble aggregates. As far as the effect of ultrasound-assisted acid was concerned, increase in fluorescence intensity was more remarkable than that of single ultrasound or acid pretreatment, indicating that combination of ultrasound and acid could improve the unfolding of the molecular structure, destroying hydrophobic interactions, and thus increasing the fluorescence intensity (Gülseren, Güzey, Bruce, & Weiss, 2007; Jambrak, Mason, Lelas, Herceg, & Herceg, 2008; Jin et al., 2015). This illustrates that the effect of ultrasound is in alliance with acid on the structure unfolding of SPI.

3.3. Effects of ultrasound-assisted acid on secondary structure of SPI

CD spectra of SPI with different pretreatments were scanned and program of CONTINLL was used to calculate secondary structure. The contents of α-helix, β-sheet, β-turn, and random coil are shown in Table 1. It shows that the secondary structure elements of SPI changed mildly by different pretreatments. No significant difference was observed between control and ultrasound pretreatment in the content of α-helical structure. The amplitude of α-helix experienced an increase after acid and ultrasound-assisted acid

Table 1
Secondary structure contents of SPI with different pretreatments.

Method	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
Control	6.5 ± 0.3	36.7 ± 0.2	23.0 ± 0.2	33.8 ± 0.3
Ultrasound	6.6 ± 0.1	33.0 ± 0.2	23.6 ± 0	36.7 ± 0.1
Acid	7.0 ± 0.2	36.7 ± 0.1	22.7 ± 0.2	33.6 ± 0.2
Ultrasound-acid	8.4 ± 0.2	31.8 ± 0.3	23.0 ± 0.1	36.6 ± 0.2

pretreatments, indicating a formation of α-helical structure induced by acid. Zhao, Yuan, Luo, and Zhao (2011) reported that acid could increase the content of α-helix, which coincides with the results of the present study. Meanwhile, ultrasound and ultrasound-assisted acid caused conformational changes of the protein with the loss of β-sheet and formation of random coli structure ($P < 0.05$). From Table 1, the contents of secondary structure processed by ultrasound-assisted acid had changed remarkably compared with single ultrasound and acid processing, suggesting that acid and ultrasound have synergistic effects. When SPI was pretreated by ultrasound-assisted acid, contents of α-helix increased by 29.2%, β-sheet decreased by 13.4%, and random coil increased by 8.3%, as compared with those of control ($P < 0.05$). Stathopulos et al. (2004) reported that increase of β-structure and decrease of α-helix gave signs to the aggregation of protein. On the contrary, the result of ultrasound-assisted acid in this study might give clues to the dissociation of SPI, which was attributed to charge repulsion induced by acid and mechanical destruction of ultrasound. This explanation was supported by the SDS-PAGE results (Fig. 3).

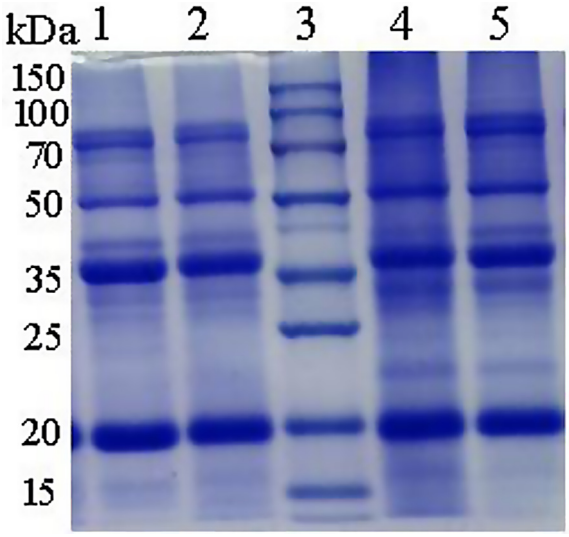

Fig. 3. SDS-PAGE of SPI with different pretreatments. Lanes 1–5 were control, ultrasound, standard protein, ultrasound-assisted acid and acid, respectively.

3.4. SDS-PAGE analysis

SDS-PAGE was used to examine the subunit compositions of SPI obtained by different pretreatments (Fig. 3). It was found that the control and ultrasonic-treated SPI displayed similar profiles (Fig. 3, lanes 1 and 2), which comprised of similar subunits with molecular weight (MW) of 20, 38, 50, 80 and 85 kDa. As shown in Fig. 3, the subunits at MW of about 33 and 22 kDa could be obviously observed (Fig. 3, lanes 3 and 4) when SPI was treated by acid and ultrasound-assisted acid. The differences suggested that some larger subunits might be dissociated into smaller subunits by acid. Compared with the SPI treated by acid alone, the content of smaller subunits increased when SPI treated by ultrasound-assisted acid, indicating that ultrasound could improve the dissociation induced by acid. The synergistic effect is in consistent to the results determined by CD.

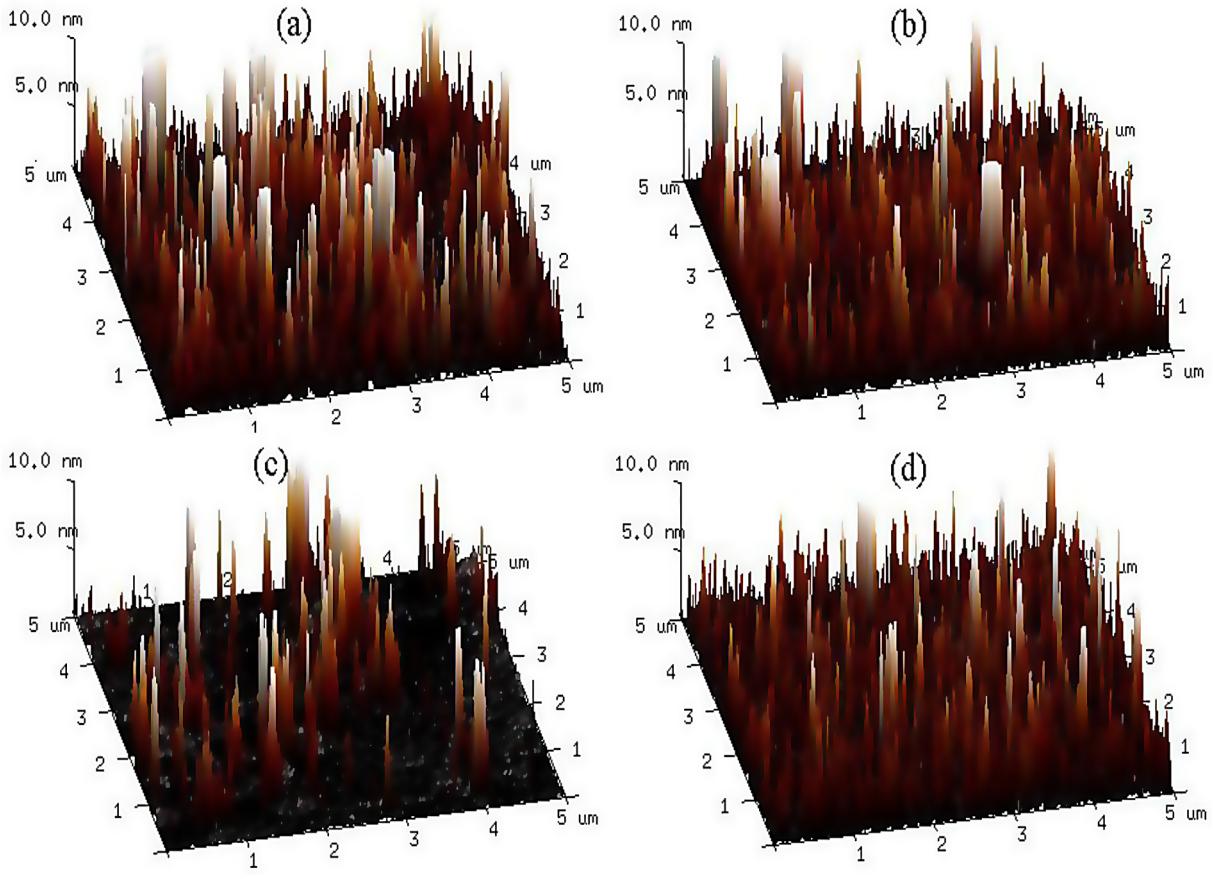

Fig. 4. The effects of ultrasound and acid pretreatments on surface morphology of the samples. (a) Control; (b) Ultrasound; (c) Acid; (d) Ultrasound-assisted acid.

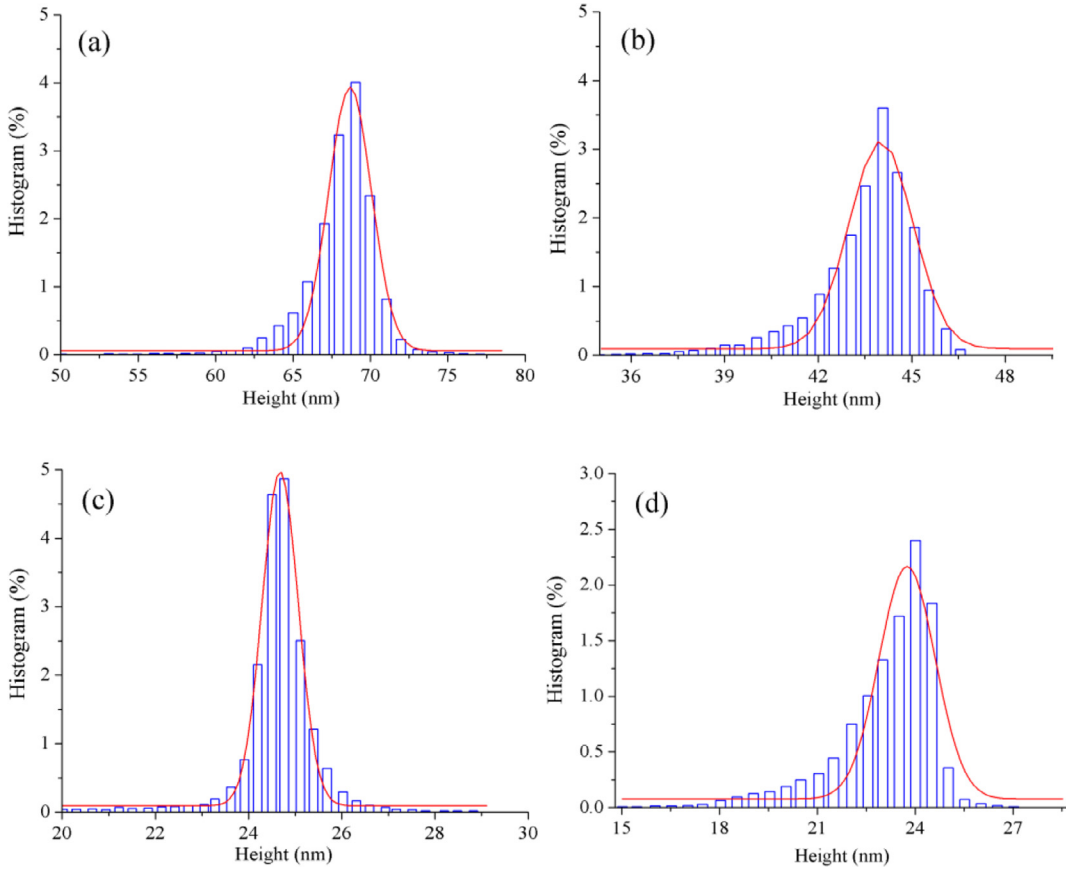

Fig. 5. The effects of ultrasound and acid pretreatments on height of SPI. (a) Control; (b) Ultrasound; (c) Acid; (d) Ultrasound-assisted acid.

3.5. Effect of ultrasound-assisted acid on the surface morphology of SPI

Atomic force microscope (AFM) is a powerful tool to measure the surface morphology, particle size and distribution, which are always related to functional properties of protein (Jin et al., 2016; Lin et al., 2014; Zhang et al., 2016). The AFM images of SPI treated by ultrasound and acid are shown in Fig. 4. The particle analysis was carried out by using the Nanoscope Analysis software to create size histograms of height distribution then fitted to a Gaussian curve based on the data of Fig. 4, and is shown in Fig. 5.

It can be observed that the structure of untreated SPI (control) exhibited a larger aggregate particle with 69 nm height (Fig. 4a and Fig. 5a). Fig. 4b and Fig. 5b shows that the larger aggregated protein collapsed to form smaller particles after ultrasound pretreatment due to the cavitation and mechanical effects (Chandrapala, Oliver, Kentish, & Ashokkumar, 2012). When the protein was treated by acid, the diameter of the particle increased and the height decreased compared to that of the control (Fig. 4c and Fig. 5c). Yuan et al. (2012) reported that the increase of particle diameter was due to the loose structure of SPI induced by acid, which was in agreement with the present result. When the loose subunits were further completely dissociated, height of the particle will decreased. Having undergone the ultrasound-assisted acid pretreatment, height of SPI decreased by 45 nm over the control (Fig. 5d). Moreover, Fig. 4d showed that the small particle increased in number, indicating that ultrasound-assisted acid induced dissociation of SPI aggregates, which was in agreement with the result obtained by SDS-PAGE.

4. Conclusion

In this study, the effects of ultrasound-assisted acid pretreatment on the structure and dissociation of SPI were investigated. Pretreatment of ultrasound-assisted acid had no significant effect on the solubility of SPI. Significant changes were observed in fluorescence spectra of SPI with different treatments. The maximum emission wavelength was red shifted and fluorescence intensity was increased under the influence of acid, indicating the unfolding of the molecular structure. When SPI was treated by ultrasound-assisted acid, it had the highest content of α-helix, and also the lowest β-sheet compared with other methods. Meanwhile, the changes of subunits composition and particle size further confirmed that larger aggregated proteins collapsed and dissociated to the greatest extent under the combination of ultrasound and acid. In conclusion, the ultrasound-assisted acid pretreatment is an effective method to promote the unfolding and dissociation of SPI. In view of the demand for superior functional foods, the dissociated subunits were easy and flexible to recombine to form new functional protein.

Acknowledgements

This work was supported by China Postdoctoral Science Foundation (2015M571697), Jiangsu University Research Fund for Senior Professional Technical Talent (14JDG064) and Agricultural Science and Technology support Program of Zhenjiang in China (No. NY2014012).

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