Functional properties and structure changes of soybean protein isolate after subcritical water treatment

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Abstract Subcritical water is an emerging method in food industry. In this study, soybean protein isolate (SPI) was treated by subcritical water (SBW) at various temperatures (0, 120, 160, 200 °C) for 20 min. The changes in the appearances, physicochemical properties and structural changes were investigated. After SBW treatment, the color of SPI solution modified turned to be yellow. The mean particle size and turbidity of SPI had similar behaviors. The mean particle size was decreased from 263.7 nm to 116.8 nm at 120 °C and then reached the maximum at 160 °C (1446.1 nm) due to the aggregation of protein. Then it was decreased to 722.9 nm at 200 °C caused by the protein degradation. SBW treatment could significantly enhance the solubility, emulsifying and foaming properties of SPI. With increasing temperature, the crystalline structure of protein was gradually collapsed. The degradation of the protein advanced structure occurred, especially at 200 °C revealed by ultra-high resolution mass spectrometry. Better functional properties exhibited in hydrolysis products indicating that SBW treatment could be used as a good method to modify the properties of soy proteins isolate for specific purposes under appropriate treatment condition.

Keywords Subcritical water · Soybean protein isolate · Functional properties · Structure

Introduction

Soybean protein isolate is a dominant by-product of producing soybean oil. Soybean proteins have been widely used in many protein-based food formulations because of their excellent nutritional and functional properties, i.e., solubility, emulsifying properties, film-forming and foaming properties. Physical modification (Speroni et al. 2009; Pednekar et al. 2010; Hua et al. 2005; Li et al. 2007), chemical modification (Rocha et al. 2007), and enzymatic modification (Zhao et al. 2011; Tsumura 2009; Amarowicz 2010) have been carried out to improve the functional properties of protein. Subcritical water (SBW) has also been used to produce more functional products from protein. In the past decade, there has been growing interest in using SBW to extract proteins, essential oils, and bioactive components (Hassas-Roudsari et al. 2009; Narita and Inouye 2012; Teo et al. 2010). SBW is cost-effective, readily available almost everywhere in the world; moreover, water is chemically stable, safe, and harmless to the environment. SBW has been applied to process whey protein, bovine serum albumin and soy bean meal to modify the functional properties (Rogalinski et al. 2005; Espinoza et al. 2012; Espinoza and Morawicki 2012; Watchararuij et al. 2008).

There are limited knowledge of functional and structure changes of SPI after SBW treatment (SBW-SPI). In this paper, we evaluated the effects of SBW temperature on the appearance, solubility, emulsifying and foaming properties of SPI. In addition, the structural changes after SBW treatment were investigated. The relationship between structure, appearance and functional properties of SPI after subcritical water treatment was elucidated.

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Materials and Methods

Materials

Soybean protein isolate (SPI) was obtained from Baiaote Company (Shandong, China), soybean oil was purchased from a local supermarket and used directly without further purification. Sodium dodecyl sulphate (SDS) was obtained from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). All other chemicals were of analytical grade.

Subcritical water treatment

SPI was incubated in a high temperature and pressure extractor (GS-1 W, Shandong Longxing Chemical Machinery Group co., LTD, China). SPI was dispersed into deionized water to achieve solid-to-liquid ratios of 1:200. The amount of SPI loaded into reactor was suggested 2/3 of the maximum volume (800 mL) that would fit into the reactor without plugging the vent lines located just 80 % volume of the reactor. The SBW treatment was performed at temperatures ramping from 120 to 225 °C for 15 min. The heat up time need to reach the target temperature ranged from 40 min (100 °C) to 200 min (225 °C) and the cooling time from the target temperature to below 90 °C ranged from 20 min (120 °C) to 180 min (225 °C). The system was cooled to 90 °C to ensure safe handling of the product before opening the reactor to get the solution. Then the insoluble was separated from the liquid phase by air pump filtration. The supernatant was collected, and the solution was lyophilized for further analyses.

Characterization of appearances of SPI and SBW-SPI

Color measurement

A Minolta colorimeter (Model CR-10, Konica Minolta Sensing, INC, Japan) was used to measure surface colors of solution samples and the results were expressed in terms of 3-coordinate values (L, a*, and b* color space) also known as CIELAB, in which L indicates lightness of the color (or value) on a numerical scale from 0 (black) to 100 (white). The color coordinates a* and b* represent the positions between red (+a*) and green (−a*), and between yellow (+b*) and blue (−b*). Prior to the color measurement, the instrument was calibrated against a standard white calibration plate. Two readings were taken from randomly selected locations and the mean values of L, a*, and b* were recorded.

Particle size distribution

Particle Size Distributions (PSDs) were determined with a laser scattering Mastersizer S (380/ZLS, NICOMP, Fla., U.S.A.) using the methods of Liu, C. M. and others (Liu

et al. 2011). The Nicomp 380, based on dynamic light scattering (DLS), provides accurate PSD results down to 1 nm. Samples were diluted by approximately 1000 times with deionized water in the sample dispersion unit under stirring (2000 rpm).

Turbidity

The solutions of SPI and SBW-SPI sample were removed and immediately cooled to room temperature in an ice water bath. The turbidity of each solution was estimated according to Hua, Y., et al. (Hua et al. 2005) measuring the absorbance of the solutions at 600 nm in a T6 Vis–UV spectrophotometer. The absorbance was used as an indicator for turbidity.

Characterization of physicochemical properties of SPI and SBW-SPI

Measurement of solubility

Protein solubility (PS) was determined according to the method of Chen et al. (2011) with minor modifications. Protein samples concentration of 5 mg/mL at pH 7.0 was centrifuged at 8000 g for 30 min at 20 °C in a high speed centrifuge (TGL-20B, ShangHai Anting Scientific Instrument Factory, China). The supernatant was collected. The protein content of the supernatant was determined by micro-Kjeldahl method ($N \times 6.25$). The protein solubility was calculated as nitrogen solubility index (NSI, %) = (protein content of supernatant/amount of proteins added) $\times 100$ %.

Emulsifying properties

Emulsifying properties were determined according to the method of VKlompong, S Benjakul, DKantachote (Khuwijitjaru et al. 2011). Briefly, vegetable oil (10 mL) and 30 mL of 1 % protein solution were mixed. The mixture was homogenized using a homogenizer (Ika - Ultra - Tur - rax T25, Germany) at a speed of 10,000 rpm for 2 min. An aliquot of the emulsion (50 μ L) was pipetted from the bottom of the container at 0 and 10 min after homogenization and mixed with 5 mL of 0.1 % sodium dodecyl sulphate (SDS) solution. The absorbance of the diluted solution was measured at 500 nm using a spectrophotometer (T6, Pgeneral, Beijing, China). The absorbance measured immediately (A_0) and 10 min (A_{10}) after emulsion formation were used to calculate the emulsifying activity index (EAI) and emulsion stability index (ESI) as follows:

$$EAI \left(\frac{m^2}{g} \right) = \frac{2 \times 2.303 \times A_{500nm}}{F \times proteinweight(g)}$$

$$ESI(\%) = \frac{A_{10 \times \Delta t}}{\Delta A}$$

where F is the oil volume fraction of 0.25; A_{10} is the absorbance at 10 min after homogenization; $\Delta t=10$ min; and $\Delta A=A_0-A_{10}$.

Foaming properties

Foaming capacity was evaluated according to the method described by Ogunwolu, S.O., et al. (Ogunwolu et al. 2009). The sample (25 mL) was whipped using a homogenizer (Ika-Ultra-Tur-rax T25, Germany) for 3 min. The foam and solution obtained were then transferred to a 100 mL glass cylinder. Volume of the foam portion was recorded at 0 min for foam capacity and up to 30 min for foam stability. Each sample was evaluated at least in duplicate. Foam capacity and foam stability were then calculated:

$$\begin{aligned} \text{Foam capacity (FC)}(\%) \\ = \frac{(\text{Volume after whipping} - \text{Volume before whipping})\text{mL}}{(\text{Volume before whipping})\text{mL}} \end{aligned}$$

$$\begin{aligned} \text{Foam stability (FS)}(\%) \\ = \frac{(\text{Volume after standing} - \text{Volume after whipping})\text{mL}}{(\text{Volume before whipping})\text{mL}} \end{aligned}$$

Characterization of structure of SPI and SBW-SPI

X-ray diffraction analysis

A D8 X-ray diffractometer (Bruker AXS Co., German) was used to analyze crystalline structure according to the method of Zhang et al. (2011) with some modifications. Copper K α was used at 40 kV and 35 mA. The 2θ range was set from 1° to 60° .

FT-IR spectroscopy

Dried and bulk samples (<5 mg) were ground with KBr powder (spectroscopic grade), pressed into pellets for scanned 32 times with wave numbers from 4000 to 400 cm^{-1} on a Nicolet 5700 FTIR Spectrometer (Thermo Nicolet Corporation, America) (Chen et al. 2012). Data were plotted as transmittance (%) in function of the wave number (cm^{-1}) and

analyzed using OMNIC 7.2 software from Thermo Nicolet Corporation in America.

Fluorescence measurement

Fluorescence measurements of the protein samples were obtained by a fluorophotometer (F4500, Hitachi, Tokyo, Japan). For intrinsic fluorescence measurements were according to Liu, Zhao, Zhao, Ren, and Yang (Adje et al. 2011) with modifications. Protein solutions (0.5 mg/mL) were prepared in 10 mM phosphate buffer (pH 6.8). To minimize the contribution of tyrosine residues to the emission spectra, the protein solutions were excited at 280 nm, and emission spectra were recorded from 300 to 400 nm at a constant slit of 5 nm for both excitation and emission. All the determinations were conducted in triplicate.

Ultraviolet spectrum

Ultraviolet spectrums of samples (1 mg/mL) were measured by UV–VIS 2510PC spectrophotometer (Shimadzu Corporation, Japan). UV absorption spectra were scanned from 240 to 480 nm (Liu et al. 2010).

Molecular weight distribution

FT-ICR-MS identification

We applied Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) to determine the peptides generated from SBW treatment since it offers the highest combination of simultaneous mass measurement accuracy, resolution and sensitivity (Domon and Aebersold 2006). 100 μL of the native SPI and SPI-SBW solution (5.0 mg/mL) was added to Amicon Ultra-0.5 ultrafiltration centrifuge filters (Millipore, MA, USA) with 3 KDa molecular weight cut off. The flow through with lower molecular weight was collected for mass spectrometry analysis.

Mass spectrometry analysis of the protein hydrolyzates was conducted on a 12 T Agilent IonSpec FTICR-MS (Agilent Inc., Santa Clara, CA) equipped with a standard electrospray source. 50 μL samples were first mixed with 50 μL of H_2O : MeOH: HAC (1:1:0.03), then directly infused into the mass spectrometer at 3 $\mu\text{L}/\text{min}$. Since the mass error of this mass spectrometer is around 3 ppm with external calibration, 3 ppm was used as the threshold to identify the peptides by accurate mass.

Statistical Analysis

All measurements were reported as mean $\pm$ standard deviation (SD). The significance of differences among mean values was

determined using one-way analysis of variance (ANOVA), using SPSS version 17.0 (SPSS Institute, Chicago, USA), with a significance level of 5 % ($P<0.05$).

Results and Discussion

Appearances of SPI and SBW-SPI

Colors of SPI and SBW-SPI solutions at different treatment temperatures obtained from Minolta colorimeter are shown in Table 1. The colors of the solutions changed from colorless (natural) to slight yellow (at 120 and 160 °C) and finally to dark brown (at 200 °C). The data showed that the solution varied greatly in color under different temperatures, with L varying from 47.50 to 38.87, a-value from 7.60 to 3.47, and b-value increased from 7.90 to 12.40. These range values indicate that the color of the sample solutions exhibited from light to dark with faint yellow. As the temperature increased, degradation of protein may occur. Khuwijitjaru et al. (2011) reported that hydrolyzate of rice bran and defatted soy meal obtained by SBW treatment at high temperature contains high amount of furfural which is an intermediate product of browning reaction.

The effects of SBW treatment on particle size in SPI solution are shown in Fig. 1A. The average particle size of untreated SPI is 263.7 nm. As the temperature increased, the average particle size was decreased gradually at 120 °C, and then increased to the maximum of 1446.1 nm at 160 °C. After treated with SBW at 200 °C, the particle size increased to 722.9 nm. At first stage (at 120 °C), SPI turned into little granules, and then the granules aggregated at 160 °C. At 200 °C, due to the disaggregation and degradation of protein, the particle size decreased. According to Martínez et al. (2011), temperature and pressure are the major factors that influence the particle size. Particle size would also have effect on the protein functional properties (Jambrak et al. 2009).

Table 1 Color of untreated SPI and SBW-SPI solution at different temperatures (120, 160, 200 °C) for 20 min, expressed in L, a* and b* color space

SBW temperature	Control	120 °C	160 °C	200 °C
L	47.50±0.50 ^d	45.40±0.79 ^c	43.33±0.81 ^b	38.87±0.35 ^a
a*value (–)	7.60±0.01 ^c	8.60±0.26 ^d	4.53±0.25 ^b	3.47±0.45 ^a
b*value (+)	7.90±0.20 ^a	9.43±0.51 ^b	12.40±0.46 ^c	12.40±0.01 ^c

Means ± standard deviations of triplicate analyses are given, superscript letters (a-d) indicate significant ($p<0.05$) difference within the same column

Fig. 1 Mean particle size (A) and turbidity (B) of untreated SPI and SBW-SPI at different temperatures (0, 120, 160, 200 °C) for 20 min. Error bars show mean standard deviation of three determinations. Different letters on the top of the bars denote significant difference ($p<0.05$)

When treated at 160 °C, folding and exposing hydrophobic and SH groups of the globulins may occur.

Figure 1B shows turbidity (absorbance value at 500 nm) of the SPI treated by SBW at various temperatures. The SPI solution exhibited decreased turbidity at 120 °C compared to that of native conditions (0 °C). The turbidity became much higher when the temperatures increased to 160 °C and above. This was most likely because at the relatively low temperature (at 120 °C), the increased solubility was the major factor that caused the decreased turbidity (refer to Fig. 2). When temperature was increased to 160 °C, the SPI started to aggregate as suggested by the increased particle size (Fig. 1A), leading to an increased turbidity. The further increased temperature, however, did not increase turbidity due to the equilibrium between the increasing factor (aggregation) and decreasing factor (disaggregation).

Fig. 2 Solubility of untreated SPI and SBW-SPI at different temperatures (0, 120, 160, 200 °C) for 20 min. Error bars show mean standard deviation of three determinations. Different letters on the top of the bars denote significant difference ($p < 0.05$)

Fig. 3 Emulsifying index (EAI), emulsifying stability index (ESI) (A) and foaming capacity (FC) and foaming stability (FS) (B) of SPI (3.5 mg/mL) at different temperatures (0, 120, 160, 200 °C) for 20 min. Error bars show mean standard deviation of three determinations. Different letters on the top of the bars denote significant difference ($p < 0.05$)

Fig. 4 XRD spectra of untreated SPI and SBW-SPI at different temperatures (0, 120, 160, 200 °C) for 20 min

Characterization of physicochemical properties of SPI and SBW-SPI

Solubility of SPI and SBW-SPI

Solubility of proteins is a critical factor in the acceptability of beverages, additives and fortifier. Fig. 2 shows the effects of SBW treatment (120–200 °C) on the solubility of SPI. The solubility of SPI at 120 °C is significantly higher than that of untreated SPI. The solubility of SBW-SPI at 160 °C became lower than that at 120 °C, while still much higher than untreated SPI. When treated at 200 °C, the solubility climbed to high again. All these solubility changes were consistent with the changes occurred in particle size and turbidity. At 120 °C, the elevated temperature caused the increase of the solubility. When temperature reached 160 °C, the solubility showed decreased value because aggregation started. The further increased temperature induced degradation, with a consequence of increase of solubility.

Because of the presence of extensive hydrogen-bonded structure, water is a highly polar solvent with a high dielectric constant (80 at 25 °C) at room temperature and atmospheric pressure. The high levels of H^+ and OH^- (ion concentrations are perhaps orders of magnitude higher than in ambient water) at subcritical conditions means that many acid- or base-catalyzed reactions were accelerated, such as degradation, biomass hydrolysis and dehydration of carbohydrates (Teo et al. 2010).

Fig. 5 FTIR curves (A) and Fluorescence measurements (B) of untreated SPI and SBW-SPI at different temperatures (120, 160, 200 °C) for 20 min

Emulsifying and foaming properties

SBW treatment increased the emulsifying activity index (EAI) significantly with the increasing temperature (Fig. 3A). The SBW also slightly influenced the emulsifying stability index (ESI). During emulsification, the aggregation state and the hydrophobic interactions are the major factors that alter the emulsifying properties of

proteins (Manoi and Rizvi 2009). The increased EAI values under all the heating conditions suggest that the unfolding of proteins and subsequent exposure of hydrophobic groups generated by SBW treatment might be the dominant factor that enhanced the emulsifying properties. Similar results were reported by Wang et al. (2008). As shown in Fig. 3B, foam properties are also significantly altered. Forming capacity (FC) and forming

Fig. 6 UV absorption spectra of untreated SPI and SBW-SPI at different temperatures (0, 120, 160, 200 °C) for 20 min

stability (FS) exhibited a similar alternation under all the tested temperatures. FC and FS of SPI at 160 and 200 °C were significantly higher than that of control whereas they were lowest at 120 °C. Foam formation is governed by three factors, including transportation, penetration and reorganization of the molecule at air-water interface. SBW treatment increased the surface hydrophobicity and flexibility of the native proteins. At the first stage, unfolding of protein structure and a reduction of the particle size were probably the major factors that may induce the reduction of FC and FS (Yeom 2009). At 160 °C, the aggregation of protein became the major factor causing the increase of FC and FS. At 200 °C, extensive disaggregation and degradation of proteins resulted in formation of some new products, leading to the increase of FC and FS. These are in accordance with the previous studies (Yuan et al. 2012; Ruíz-Henestrosa et al. 2009; Martínez et al. 2009).

Characterization of structure of SPI and SBW-SPI

X-ray diffraction (XRD) analysis

Fig. 4 shows the X-ray patterns of SPI and SBW-SPI. SPI displayed two strong characteristic peaks at 2θ values of around 9° and 19° , similar to the previous reports (Su et al. 2007, 2010). With temperature increasing, the peak intensity of SPI at 9° gradually diminished. Compared to the untreated SPI, the peak of SPI at 19° became gentle with the increased SBW temperature, suggesting that the crystalline structure of SPI had collapsed after SBW treatment. The extent of the structural damage became stronger when higher temperature was applied.

FT-IR spectroscopy

FTIR spectroscopy is one of the most useful methods for investigating the molecular structure (Carbonaro and Nucara 2010). Fig. 5A shows the FTIR spectra of the SPI and SBW-SPI. At room temperature, the bands were in accord with the reported soy protein spectrum with an amide I band (80 % C-N stretch) near 1650 cm^{-1} , amide II band (60 % N-H bend and 40 % C-N stretch) near 1550 cm^{-1} ($1560\text{--}1530\text{ cm}^{-1}$) and amide III band (40 % C-N stretch, 30 % N-H bend) near 1300 cm^{-1} ($1301\text{--}1229\text{ cm}^{-1}$). The absorption near 3294 cm^{-1} referred to the hydrogen-bond association between protein chains and moisture in protein. (Su et al. 2008). Most of the characteristic absorption bands appeared in the Fig. 5A. The absorption of amide I ($1700\text{--}1600\text{ cm}^{-1}$) was around 1652 cm^{-1} , and decreased significantly after SBW treatment at 120, 160 and 200 °C, respectively. With temperature increasing, the peaks of amide II around 1535 cm^{-1} disappeared. The peak of amide III was around 1238 cm^{-1} , while at 200 °C, the peak of 1238 cm^{-1} was diminished. These band shifts suggested that there might be a specific chemical reaction occurring (dissolution, aggregation, disaggregation and degradation) after SBW treatment at different temperatures.

Fluorescence measurement

The fluorescence spectra of SBW-SPI at different temperatures were observed and the results are shown in Fig. 5B. It can be noted that the SBW treatment caused an increase in relative fluorescence intensity at 120 and 160 °C, but a decline of relative fluorescence intensity appeared at the 200 °C. As temperature increased, the wavelength of fluorescence emission peak was red-shifted from 348 nm of the untreated to 366 nm of SPI under 200 °C. It was red-shifted to 350 nm at 120 °C and 358 nm at 160 °C. It is probable that the SBW modification of the SPI conformation in the vicinity of the Trp and Tyr residues contributed to the changes in fluorescence spectra, the relative fluorescence intensity and the red shift. It is well known that the fluorescence intensity of Trp and Tyr is quenched by polar solvent. It was possible that the Trp and Tyr residues in hydrophobic environment were more or less exposed to the less hydrophobic environment due to the unfolded and aggregation at 120 and 160 °C. At 200 °C, the significant decline of relative fluorescence intensity was due to the quenching of fluorescence. This indicated that Trp and Tyr contact with more and more polar solvent at 200 °C. The fluorescence spectra analysis indicated that changes of tertiary and quaternary structure of SPI occurred after SBW treatment.

UV absorption spectra

The UV absorption spectra of untreated SPI and SBW-SPI are shown in Fig. 6. The maximum absorption peak was at

Fig. 7 FT-ICR positive ion mass spectrum of native SPI (A), SPI following SBW at 120 °C for 20 min (B), SPI following SBW at 160 °C for 20 min (C), and SPI following SBW at 200 °C for 20 min

(D). The inset is an expansion of the region around 500 m/z where the mass resolving power was approximately 5,000

280 nm, and its intensity showed a significant change after SBW treatment. The absorption intensity of untreated SPI at 280 nm was 0.195. SPI treated by 120 °C showed a big increase and its absorption intensity was 0.287. The absorption intensity exhibited an increasing trend with the increasing temperature. The intensities were 0.290 and 0.365 at 160 and 200 °C, respectively. The increase in absorption intensity was probably because of the gradual exposure of hydrophobic groups when SPI was treated by SBW. The red shift of the absorption peak indicated that treatment passes had a prominent effect on the conformational changes of SPI molecule.

Molecular weight distribution

To confirm the degradation induced by SBW, FTICR-MS was used to identify the peptides generated from the SPI after SBW. The lower molecular weight samples, cut off by 3KDa ultrafiltration centrifuge filters,

were then analyzed by the FTICR-MS. Due to the extreme high mass accuracy of FTICR-MS, most of the peptides generated by SBW treatment can be identified by accurate mass. Fig. 7 compared the FT-ICR mass spectrum of native SPI with SBW treated SPI. Before SBW treatment, only a few peaks of peptides were detected. When treated at 120 °C for 20 min, the number of peaks was increased slightly between 500 and 800 Da (Fig. 7B). When the temperature was increased to 160 °C, there was an apparent increase of the number of peptides (Fig. 7C). The higher temperature (200 °C) further increased the number of the fragmented peptides (Fig. 7D). Similar observations were reported by Espinoza et al. (2012) when using subcritical water to hydrolyze whey protein isolate, with majority of the peptides showing the molecular weight lower than 1.4 KDa. Owing to the high mass accuracy of FTICR-MS, we were able to identify many main peptides from SPI-SBW directly using accurate mass result from the degradation of SPI.

Conclusions

SBW treatment can significantly enhance the solubility, emulsifying and foaming properties of soybean protein isolate. The structure of SPI was significantly changed measured by X-ray, FT-IR, UV spectra, fluorescence spectroscopy and high resolution mass spectrometry. The results indicated SBW treatment can degrade the protein advanced structure. Better functional properties exhibited in hydrolysis products indicates that SBW treatment could be used as an alternative method to modify the properties of soy proteins isolate for specific purposes under appropriate conditions.

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