



Characterization of complexes of soy protein and chitosan heated at low pH

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ABSTRACT

The objective of this research is to investigate the complex of soy protein isolate (SPI) and chitosan (CS) heated (121 °C, 15 min) at low pH. The formation and functional properties of heated SPI–CS complex were investigated by electrophoresis, Fourier transform infrared spectroscopy (FTIR), solubility, ζ -potential and emulsifying property. The results indicated that the heated SPI–CS complex is probably more like a soluble SPI–CS aggregate which is driven by electrostatic interaction. Heated SPI–CS complex exhibited improved solubility than SPI (less than 10%) and unheated SPI–CS mixture (about 50%) at pH 4.0 because of the increased electrostatic repulsion (ζ -potential) after heat treatment. The effects of mixing ratios, molecular weights (MW) and charge densities (CD) of chitosan on the stability of heated SPI–CS complex showed the influence was mainly dependent on mixing ratios and CD of chitosan. Concomitantly, the emulsifying activity index of SPI at pH 4.0 was significantly improved by heated complexation and the emulsion stability index was not affected. Additionally, the heated SPI and/or SPI–CS complex exhibited slightly decreased pepsin and trypsin digestibility. The results suggested that the physicochemical and functional properties of SPI could be modulated by heated complexing with chitosan, using appropriate mixing ratio, MW and CD of chitosan.

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1. Introduction

Soy proteins are the most important representative of legume proteins due to their high protein level and well-balanced amino-acid composition. Soy proteins have been widely used to formulate foods with the goal of improving their nutritional and/or functional qualities. β -Conglycinin (7S globulin) and glycinin (11S globulin) are the two main fractions of soy protein isolate (SPI). β -Conglycinin is a trimeric glycoprotein consisting of at least six combinations of three subunits: α , α' and β (Thanh & Shibasaki, 1977). Glycinin is composed of an acidic (38 kDa) and a basic polypeptide (20 kDa) linked by a single disulfide bridge (Staswick, Hermodson, & Nielsen, 1981). The isoelectric points (pI) of glycinin and β -conglycinin are 6.4 and 4.8, respectively (Iwabuchi & Yamauchi, 1987). Thus, protein solubility (PS) of soy protein isolate at acidic pH (pH 3.0–5.0) is very low, which limits their application in acidic food, e.g., acidic beverage.

The exploitation of protein–polysaccharide interactions offers opportunities for the design of new ingredients with applications in the food industries (Dickinson, 2008). There has been a considerable amount of research on the formation and properties of

complex coacervates produced by mixing proteins with anionic polysaccharides (Weinbreck, de Vries, Schrooyen, & de Kruif, 2003). Coacervates have also been formed between proteins and cationic polysaccharides. Chitosan (CS), a cationic polysaccharide, is a β -1,4-linked polysaccharide of 2-amino-2-deoxy-D-glucopyranose. Owing to its non-toxicity, biodegradability and biocompatibility, chitosan is currently employed in a wide range of applications in the food, biotechnology, pharmaceutical and gene therapy fields (Harish Prashanth & Tharanathan, 2007). Previous studies have shown that chitosan can interact with proteins to form either soluble or insoluble complex (Guzey & McClements, 2006). These interactions may be either physical (e.g. electrostatic) or covalent (e.g. Maillard) in origin. Complex coacervation is usually driven by the attractive forces of oppositely charged polymers (Elmer, Karaca, Low, & Nickerson, 2011). In certain condition, polysaccharide and protein with homologous charge can form soluble complex, this interaction occurs between the opposite charge groups (Seyrek, Dubin, Tribet, & Gamble, 2003). Guzey et al. (2006) suggest that soluble complex formed between β -lg and chitosan at pH 4.0 and insoluble complex formed at pH values 6.0 and 7.0. Hong and McClements (2007) investigated the hydrogel particles by thermal treatment of β -lactoglobulin and chitosan mixtures.

Previous works mainly focus on the interaction between chitosan and whey protein. In contrast, the formation and properties of protein–chitosan complexes of plant oligomeric proteins, e.g.,

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soy protein, are investigated to a much lesser extent than those of whey proteins. Hydrothermal cooking (HTC) has been used to efficiently improve protein extraction and to refunctionalize heat-denatured soy proteins in extruded-exelled meals or alcohol-denature soy protein and increases in solubility by dissolution of protein aggregates (Wang, Wang, & Johnson, 2004, 2005). Unfortunately, few researchers investigate heat treatment at low pH. In our laboratory, we found that the biopolymer complex based on soy globulin could be formed by heating them in the presence of chitosan in hydrothermal system (Liu et al., 2011). However, the information about the effects of the hydrothermal cooking in the presence of chitosan on the physicochemical and functional properties of soy protein at acidic pH is very limited. The effects of different properties of chitosan on the properties of SPI–CS complexes are also limited. The objective of this research is to investigate the complex of SPI and chitosan in a hydrothermal system (121 °C, 15 min) at low pH. The effects of chitosan-to-protein ratios as well as molecular weights and charge densities of chitosan on protein solubility (PS), emulsifying properties and ζ -potential of heated SPI–CS complex are also evaluated. This type of study will help to use SPI as a potential food ingredient, especially in acidic food.

2. Materials and methods

2.1. Materials

The defatted soybean flakes were purchased from Shandong Xinjiahua Industrial and Commercial Co. Ltd., China. The protein content of soy flour was $55.0 \pm 0.5\%$ (determined by Kjeldahl method with nitrogen conversion factor of 6.25; on dry basis) and the nitrogen solubility index 84.0%. The chitosan (Moisture 8.0%; Ash content 0.7%) used for experiment at different molecular weights (MW, 50, 100 and 200 kDa) and different charge densities (CD, 20, 60 and 100 mV) was purchased from Shandong Aokang Industrial and Commercial Co. Ltd., China. All chemicals used were of analytical or higher grade.

2.2. Characterization of chitosans

The molecular weights of chitosan were confirmed by gel permeation chromatography (GPC). GPC was carried out with a high performance liquid chromatography system (Waters) equipped with ultrahydrogel linear column set and refractive index detector. Chitosan samples were prepared by dissolving dried chitosan in 100 mM acetic acid/sodium acetate buffer (pH 5.4) at 0.2% w/v and filtering through the microfilter membrane with pore diameter at 0.45 μm before injecting into the GPC column. The 20 μl of chitosan solution was injected into the column at room temperature. The eluent was 100 mM acetic acid/sodium acetate buffer at pH 5.4 with 0.6 ml/min flow rate. Dextran standards eluted with the same buffer were used for a calibration curve. All data provided by the GPC system were collected and analyzed using the Waters Workstation software package. The charge density of chitosan can be controlled by the fraction of deacetylated units along the chain and offers a way to control the interaction with polyanions (Maurstad, Danielsen, & Stokke, 2007). The ζ -potential titration was used to determine the charge densities of chitosan according to the previous studies (Hong et al., 2007).

2.3. Preparation of soybean protein isolates (SPIs)

SPI was prepared according to the method of Iwabuchi and Yamauchi (1987), with minor modifications. The defatted soy flakes were dispersed in distilled water (1:15, w/v) and the pH of

the dispersion adjusted to 8.0 with 2 M NaOH. The resulting dispersion was gently stirred at room temperature for 2 h and then was filtered (180-mesh size), the filtrate was then centrifuged at $9000 \times g$ (25 °C, 30 min) in a CR22G centrifuge (Hitachi Koki Co., Hitachinake, Japan). The pellet was discarded and the supernatant was adjusted to pH 4.8 at 4 °C with 2 M HCl and then centrifuged at $6000 \times g$ (20 °C, 20 min). The precipitate obtained was dissolved in distilled water, homogenized and adjusted to pH 7.0, then dialyzed against distilled water at 4 °C for 24 h. The dialyzate was lyophilized to yield SPI.

2.4. Preparation of samples

SPI (4% w/v) and CS were dispersed in 100 mM acetic acid/sodium acetate buffer (pH 3.0) overnight under moderate stirring. The two stock solutions were mixed at appropriate ratios. The obtained dispersions were heated at pH 3.0 in the autoclave (121 °C, 0.1 MPa, 15 min), then cooled immediately to 22 °C in iced water. Finally, the dispersions were lyophilized by Christ Delta 1-24 LSC (Marin Christ Co. Germany) to yield SPI–CS complex powders to evaluate its reconstituted ability.

The SPI and SPI–CS mixture preparation without and with heat treatment named SPI, heated SPI, SPI–CS mixture and heated SPI–CS complex, respectively. The heated SPI–CS complexes with various weight ratios, molecular weights and charge quantities of chitosan were named 0.025 g g⁻¹, 0.05 g g⁻¹, 0.1 g g⁻¹ and 0.2 g g⁻¹; 50 kDa, 100 kDa and 200 kDa; 20 mV, 60 mV and 100 mV, respectively.

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

SDS-PAGE was performed on a discontinuous buffered system according to the method of Laemmli (1970) with 12% separating gel and 4% stacking gel. SPI, heated SPI, SPI–CS mixture and heated SPI–CS complex were assessed. Samples were mixed with reducing sample buffer (10% SDS, 2.5% β -mercaptoethanol) and non-reducing sample buffer (10% SDS without β -mercaptoethanol) to give a concentration of 4 mg/ml. Reducing sample solutions were heated for 5 min in boiling water and non-reducing sample solutions were heated at 55–60 °C for 1 h before electrophoresis. Each sample (10 μl) was applied to each lane. At beginning, electrophoresis was performed at 10 mA, and then raised to 20 mA when samples entered the separating gel. The gel was stained with 0.25% Coomassie brilliant blue (R-250) in 50% trichloroacetic acid, and destained in 0.5 M sodium chloride solution.

2.6. Fourier transform infrared spectroscopy measurements (FTIR)

FTIR measurements were performed on flakes of sample powder mixed with KBr. The sample quantity (about 5 mg) in KBr (200 mg) was chosen in order to optimize the flake transmittance and to obtain a well detectable absorption in the spectral region. All spectra were recorded at ambient temperature, with a resolution of 4 cm⁻¹, using a Bruker VEC70R-33 interferometer (Bruker Optics, Ettlingen, Germany) working under vacuum to avoid intense spectral components due to atmospheric CO₂ and H₂O.

2.7. Solubility profile

Aqueous solutions (10 mg/ml, w/v) of samples were stirred for 30 min and adjusted to the desired pH and centrifuged at $10,000 \times g$ (25 °C, 20 min). After appropriate dilution, the protein content of the supernatants was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) using bovine serum

albumin (BSA) as standard. The solubility was expressed as grams of soluble protein/100 g of protein. All measurements were conducted in duplicate.

2.8. ζ -Potential

The ζ -potential of the sample was determined using a Nanosizer ZS instrument (Malvern Instruments, Worcestershire, UK) in combination with a multipurpose autotitrator (model MPT-2, Malvern Instruments, Worcestershire, UK). Aqueous solutions (5 mg/ml, w/v) of samples were filled into a disposable ζ -potential folded capillary cell (DTS1060). Titration was performed with 0.25 and/or 0.025 M NaOH under constant stirring. The instrument determined the electrophoretic mobility. The Henry equation was then applied for calculating the ζ -potential. A dielectric constant of 78.5 and a viscosity of 0.8872 mPa s were also used for continuous phase. All measurements were conducted in duplicate.

2.9. Emulsifying activity index (EAI) and emulsion stability index (ESI)

EAI and ESI were determined according to the method of Pearce and Kinsella (1978) and Yin, Tang, Wen, Yang, and Li (2008). The buffer used in this paper was 100 mM acetic acid/sodium acetate buffer (pH 4.0). Measurements were performed in duplicate.

2.10. Pepsin and trypsin hydrolyses

The pepsin and trypsin hydrolyses of samples were determined according to the method of Wang, Tang, Yang, and Gao (2008). The hydrolysis was followed by sampling a various times, from 0 to 240 min during proteolysis. The determination of nitrogen release during hydrolysis was also the same with Wang et al. (2008). Measurements were performed in duplicate.

2.11. Statistical analysis

An analysis of variance (ANOVA) of the data was performed using the SAS version 9.0 software (SAS Institute, Cary, NC). Significant differences were determined by Duncan's multiple range test and accepted at $P < 0.05$ (Duncan, 1955).

3. Results and discussion

Molecular weights (MW) and charge densities (CD) of chitosan were measured by gel permeation chromatography (GPC) and ζ -potential, respectively. The MW of chitosan used, determined by GPC technique was generally consistent (within 3% error) with its MW in package label (data not shown). The ζ -potential at pH 3.0 was used to represent the CD of chitosan. The ζ -potential at pH 3.0 of chitosan 20 mV, chitosan 60 mV and chitosan 100 mV was +22.6 mV, +63 mV and +98.2 mV, respectively.

At slightly acidic pH (<6.5) chitosan is positively charged and SPI at low pH (3.0–4.0) is also positive charge predominant. However, there would be still a number of negatively charged groups (such as carboxyl groups) on the protein which could be proved by the partly negative charge distribution showed by ζ -potential distribution (data not shown). According to preliminary experiment, three pH values (3.0, 3.5 and 4.0) were chosen at the heating step (in the autoclave 121 °C, 15 min). The heat treatment at pH 3.5 and/or 4.0 led to the formation of insoluble protein aggregates or insoluble SPI–CS complex. Consequently, all samples were heated at pH 3.0 to form heated SPI–CS complexes in following experiments.

3.1. The appearance of SPI and heated SPI–CS complex solutions

Fig. 1 shows images of SPI and heated SPI–CS complex dispersions at various pH values. SPI formed precipitate at the bottom of the flasks at pH 4.0 and 5.0, whereas heated SPI–CS complex dispersions were transparent at pH 3.0 and/or 4.0, translucent at pH 5.0 and started to produce insoluble aggregate at pH 6.0. This result indicated that the solubility of SPI was improved by the heated complexing with chitosan (in the acidic pH region, pH 3.0–5.0). A similar result was observed for the complexation between soy globulins and chitosan in aqueous solution (Liu et al., 2011). Because higher solubility of the complex was shown for lower pH, application in food formulations, e.g. fruit juices, seems feasible.

3.2. The effects of heat treatment and freeze drying

3.2.1. Solubility and ζ -potential

The effect of heating on the solubility and net charge of the protein and the aggregates were evaluated in Fig. 2, as well as the effect of freeze drying the samples. The PS of SPI displayed a typical U-shape pH dependence, and heated SPI showed a similar solubility–pH profile (Fig. 2A). The presence of chitosan increased the solubility of soy protein at acid pH ranges (pH 4.0), accompanied a shift of isoelectric point (Fig. 2A and B). This result was related to the formation of soluble SPI–CS complex under unheated condition as reported by many researchers (Guzey et al., 2006; Hong et al., 2007). Heated SPI–CS complex exhibited completely different PS profiles as compared with that of SPI and SPI–CS mixture. It was completely soluble at acidic pH and gradually becomes insoluble at neutral and alkaline pH. Concomitantly, the isoelectric point of SPI was shifted toward alkaline pH due to the heated complexing with chitosan (Fig. 2B). The possible formed mechanism of heated SPI–CS complex would be discussed in detail at the end of this section. The drying process also shows potential effect on solubility of protein, so freeze drying was used to evaluate the SPI and heated SPI–CS complex's reconstituted ability. Fig. 2C clearly indicates that there were no significant differences with

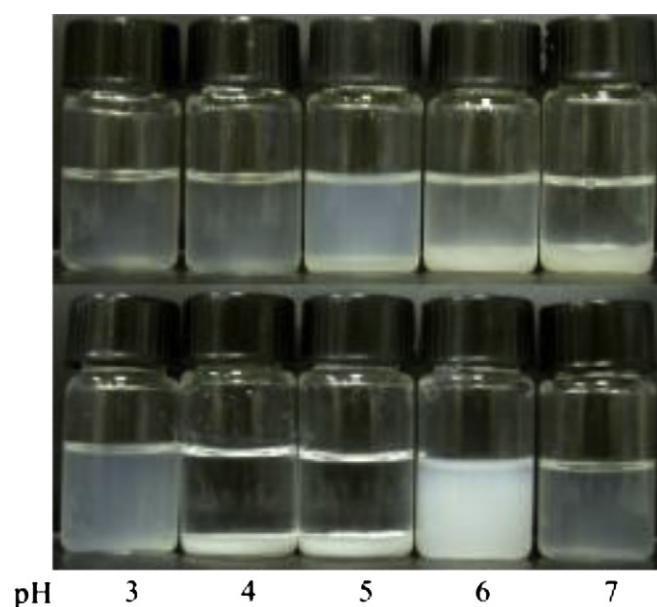


Fig. 1. Visual appearance of SPI (bottom row) and heated SPI–CS (top row) at various pH values. The solutions were placed 12 h at each pH before taking the picture.

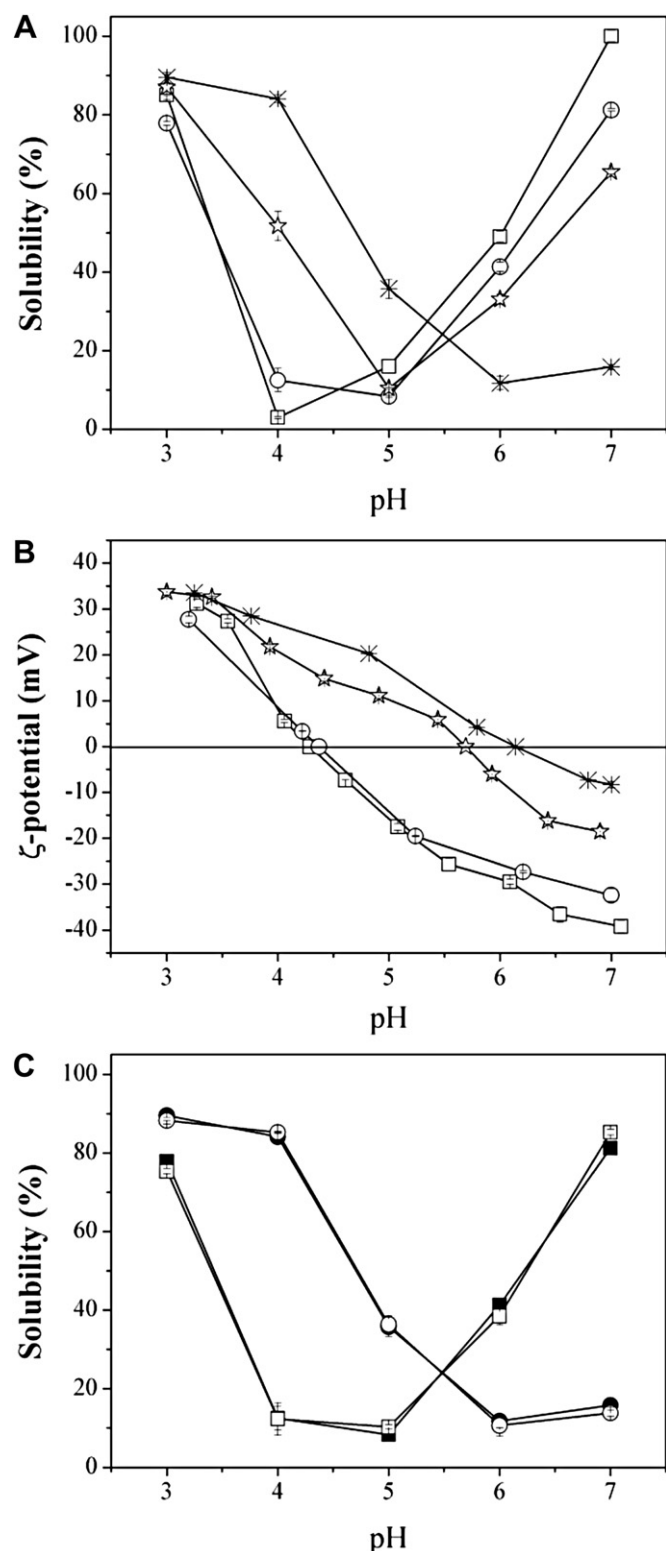


Fig. 2. The solubility and ζ -potential curve of the unheated and heated samples, heated samples before and after freeze drying (CS: SPI, 0.025 g g⁻¹). Panel A: the solubility curve of the unheated and heated samples; Panel B: the ζ -potential curve of the unheated and heated samples; Panel C: the solubility curve of the heated samples before and after freeze drying. Panels A and B: —□—, SPI; —○—, heated SPI; —×—, SPI-CS mixture; —×—, heated SPI-CS complex; Panel C: —■—, fresh heated SPI; —□—, freeze drying heated SPI; —●—, fresh heated SPI-CS complex; —○—, freeze drying heated SPI-CS complex. Each value is the mean and standard deviation of duplicate measurements.

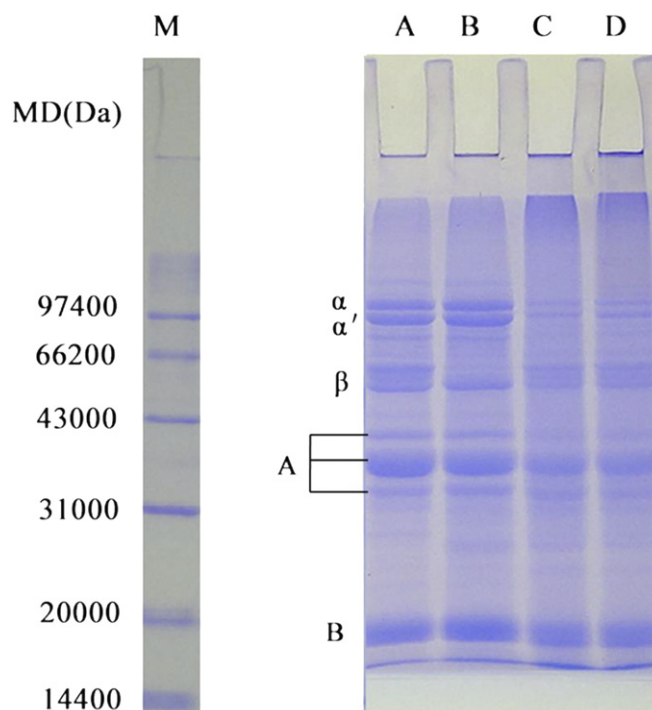


Fig. 3. SDS-PAGE of standard low molecular weight markers and samples under reducing (A–D) conditions. (M) marker, (A) SPI, (B) SPI–CS mixture, (C) heated SPI, (D) heated SPI–CS complex.

freeze drying, and shows that in all cases, the samples had good solubility after drying.

3.2.2. SDS-PAGE

SDS-PAGE was also performed to investigate the interaction of SPI and chitosan (Fig. 3). SPI displayed a typical electrophoretic profile under reducing (Lane A) conditions. β -Conglycinin (subunits of α , α' and β) and glycinin (subunits of A and B) were labeled in Fig. 3. The increase of band intensity at the top of stacking gel and decrease of band intensity of SPI fractions suggested that the formation of aggregates after heat treatment (Line C). These aggregates were partly formed via covalent bonds which cannot be completely reduced by mercaptoethanol (Line C). The addition of chitosan under unheated (Lane B) and heated (Lane D) conditions showed no new band formation, coupled with no Maillard-type aggregates were found in glycoprotein staining (data not shown). Under reducing conditions, disulphide bridging is disrupted, and the lack of difference between control and heated sample indicated that the main forces driving the interactions were not covalent in nature.

3.2.3. FTIR spectrum

The FTIR spectra of the SPI, heated SPI, heated SPI–CS complex and chitosan are shown in Fig. 4. The FTIR analysis of heated SPI–CS complex was based on the identification of bands related to the functional groups present in chitosan and soy protein (Beeke, Lasch, & Naumann, 2007; Pawlak & Mucha, 2003). According to Fig. 4A, the main characteristic absorption bands of chitosan appeared at 1656 cm⁻¹ (C=O stretching), 1598 cm⁻¹ (–NH angular deformation) and 1150–1040 cm⁻¹ (–C–O–C– in glycosidic linkage), which were in agreement with previous studies (Pawlak et al., 2003). The SPI spectrum showed the amide I band at 1646 cm⁻¹ and the amide II band at 1540 cm⁻¹. The amide I band was the most suitable band for the analysis of the protein's

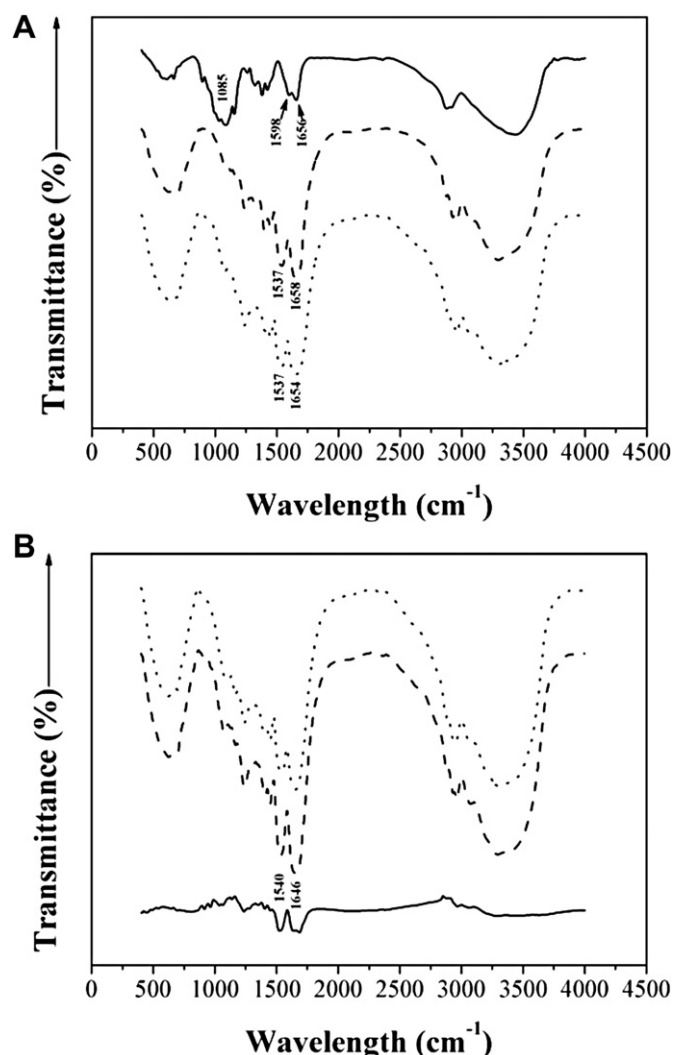


Fig. 4. The FTIR spectra of SPI, heated SPI, heated SPI–CS complex and chitosan. Panel A: The FTIR spectra of solid line, dashed line and dotted line are chitosan, SPI and heated SPI, respectively. Panel B: The FTIR spectra of dotted line, dashed line and solid line are calculated heated SPI–CS complex, experimental heated SPI–CS complex and difference (experimental–calculated), respectively.

secondary structure because different secondary structure elements absorb IR light in different wavelengths (Beekes et al., 2007). In addition, heat treatment increased the intensity of the characteristic absorption band of amides I and II band. Besides, displacement of amide I to higher wave-numbers ($1646\text{--}1654\text{ cm}^{-1}$) with respect to SPI was also noted. These shifts meant more disorder conformations obtained after heating (Lefevre & Subirade, 2001).

To better illustrate the development of specific interactions formed by heated complexation of the two biopolymers, a theoretically calculated FTIR spectrum was obtained from the weighted sum of the experimental spectrum of the Heated SPI and chitosan. This technique could be found in previous study (Pereda, Aranguren, & Marcovich, 2008). The FTIR spectra of the experimental, calculated and difference (experimental–calculated) are shown in Fig. 4B. If there are no interactions between components, the theoretical spectra must reproduce experimental ones. Some discrepancies between calculated spectra and experimental ones were observed. The slight enhancement in the intensity of some bands within the range $1500\text{--}1700\text{ cm}^{-1}$ that are related to amino and carbonyl moieties, evidenced that these groups interact mainly

through electrostatic interactions and hydrogen bonding. It can be inferred that the slight difference related to the slight interaction of the amino groups of the chitosan with the negatively charged carboxyl groups of the SPI in the complex. Similar results were observed for the caseinate–chitosan complex films (Pereda et al., 2008).

From the result above, it became clear that unheated SPI–CS mixture formed soluble complex at pH 4.0, improving the solubility of SPI. Heated SPI–CS complex shows improved acidic solubility than unheated SPI–CS mixture (Fig. 2A). The most probable reason we inferred was the chitosan played the role of chaperone when heat treated SPI–CS mixture. During heating, more negative charged groups upon protein exposed and interacted with amino groups of chitosan. That may be the reason for the slight difference of experimental and calculated FTIR spectra. Finally, heated SPI–CS complex possibly formed soluble aggregates which are driven by non-covalent forces (such as electrostatic interactions).

3.3. The effect of chitosan on stabilization of heated SPI–CS complexes

In this paper, the stabilizations of all kinds of heated SPI–CS complexes were compared in a hydrothermal condition. The equilibrium between repulsive interactions (electrostatic) and attractive interactions (van der Waals and hydrophobic) was used to explain the stabilization of heated SPI–CS complexes.

3.3.1. The effect of chitosan on solubility profile

Fig. 5 shows the effects of different chitosan mixing ratio, MW and CD on PS profiles of heated SPI–CS complexes as a function of pH. As mentioned before, heated SPI–CS complexes exhibited completely different PS profiles than control. These effects were dependent on the chitosan-to-protein ratios, MW and CD of chitosan.

The effects of chitosan-to-protein ratios on the solubility of heated SPI–CS complexes are shown in Fig. 5A. The PS at pH 2.0–5.0 was considerably increased depending on the polysaccharide/protein ratio, while the PS at pH above 6.0 was on the contrary markedly decreased. In detail, the PS of heated SPI–CS complexes at pH 4.0 and 5.0 was increased significantly ($P < 0.05$) from about 10% to 84–94% (pH 4.0), 30–60% (pH 5.0), as the chitosan-to-protein ratios increased from 0 to 0.2 g g^{-1} . However, the PS decreased from 80–90% to 17.1–25.0% at neutral and alkaline pH range (pH 7.0–10.0) with increased chitosan-to-protein ratios (Fig. 5A). Concomitantly, the pI shifted gradually toward a more alkaline pH upon heated complexing SPI with chitosan, which was in agreement with the ζ -potential data (Fig. 6A).

Effects of MW and CD of chitosan on the PS of heated SPI–CS complex were also investigated, as shown in Fig. 5B and C. Similar PS–pH of heated SPI–CS complexes was observed as the MW of chitosan increased from 50 to 200 kDa. In contrast, the changes in CD of chitosan significantly ($P < 0.05$) affected the PS–pH profiles. Complex obtained by heating SPI with chitosan +20 mV also presented typical U-shape PS–pH profile and the isoelectric point was shifted from 4.4 ± 0.1 (Heated SPI) to 5.5 ± 0.1 (Heated SPI–CS complex). However, heated SPI–CS complexes exhibited completely different PS–pH profiles, due to further increase in charge density of chitosan from +30 to +100 mV (Fig. 6C).

3.3.2. The effect of chitosan on charge properties

Fig. 6 shows effects of different chitosan mixing ratio, MW and CD on the ζ -potential of heated SPI–CS complexes as a function of pH. The ζ -potential of heated SPI decreased from $27.7 \pm 0.7\text{ mV}$ at pH 3.0 to $-37.5 \pm 0.6\text{ mV}$ at pH 9.0. A zero value of ζ -potential was

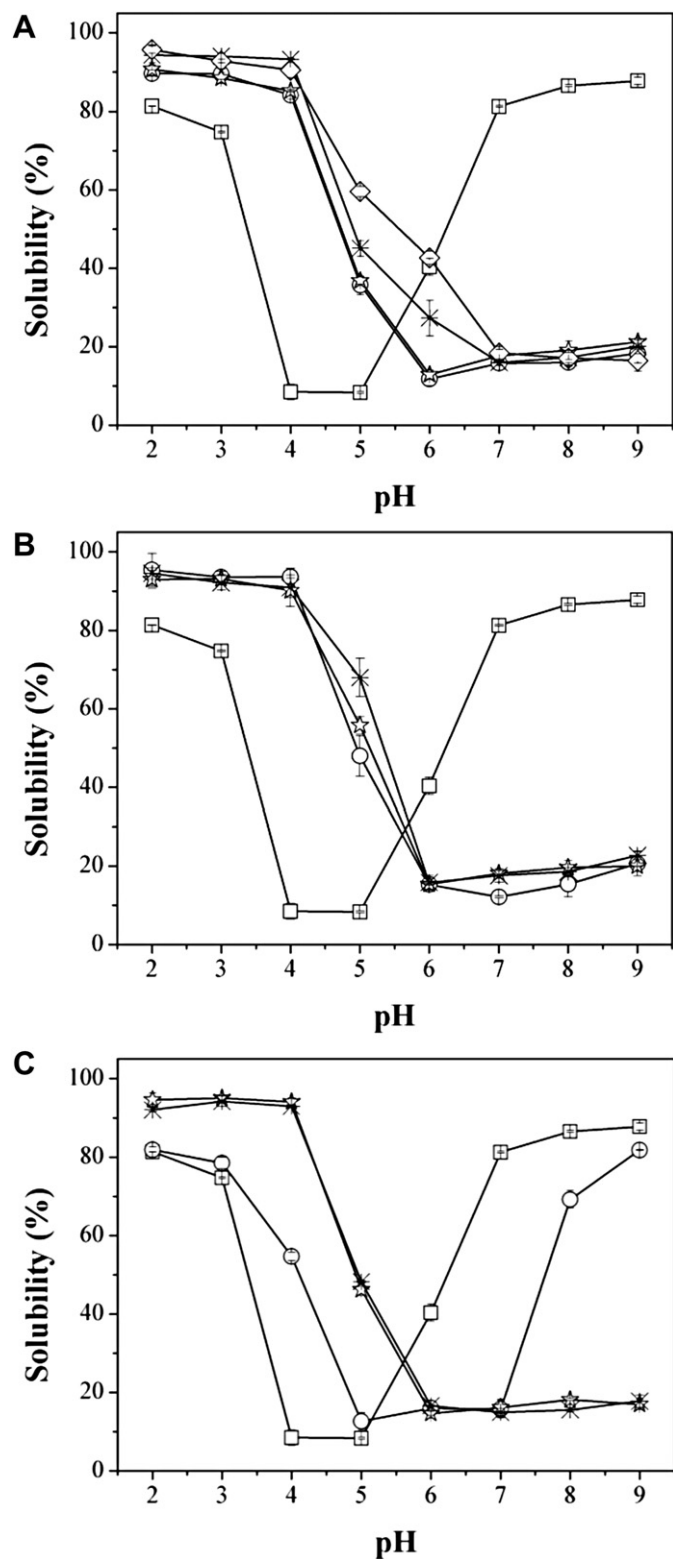


Fig. 5. The PS of heated SPI and heated SPI–CS complex as a function of pH. Panel A: Effect of chitosan-to-protein ratios (g g⁻¹), —□—, heated SPI; —○—, 0.025; —●—, 0.05; —×—, 0.1; —◇—, 0.2; Panel B: Effect of molecular weights (MW) of chitosan (CS: SPI, 0.1 g g⁻¹), —□—, heated SPI; —○—, 50 kDa; —●—, 100 kDa; —×—, 200 kDa; Panel C: Effect of charge densities (CD) of chitosan (CS: SPI, 0.1 g g⁻¹), —□—, heated SPI; —○—, 20 mV; —●—, 60 mV; —×—, 100 mV. Each value is the mean and standard deviation of duplicate measurements.

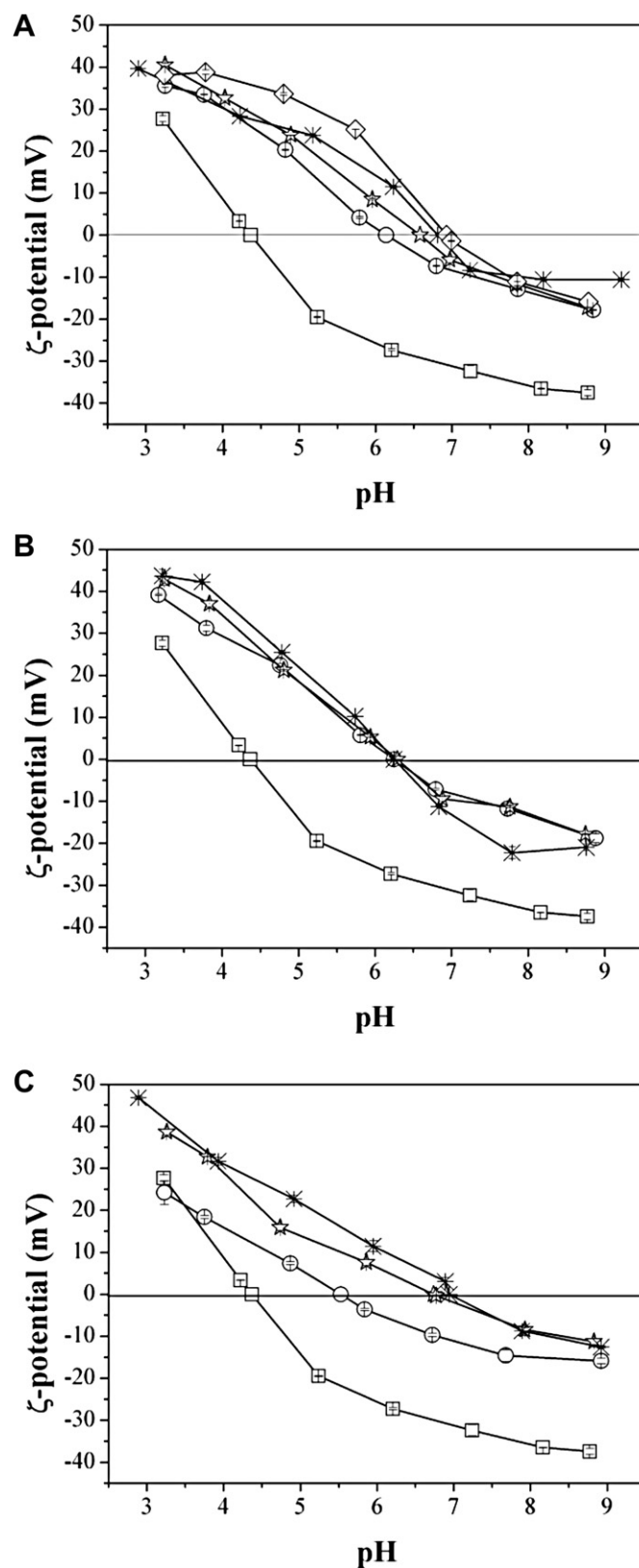


Fig. 6. The ζ-potential of heated SPI and heated SPI–CS complex as a function of pH. Panel A: Effect of chitosan-to-protein ratios (g g⁻¹), —□—, heated SPI; —○—, 0.025; —●—, 0.05; —×—, 0.1; —◇—, 0.2; Panel B: Effect of molecular weights (MW) of chitosan (CS: SPI, 0.1 g g⁻¹), —□—, heated SPI; —○—, 50 kDa; —●—, 100 kDa; —×—, 200 kDa; Panel C: Effect of charge densities (CD) of chitosan (CS: SPI, 0.1 g g⁻¹), —□—, heated SPI; —○—, 20 mV; —●—, 60 mV; —×—, 100 mV. Each value is the mean and standard deviation of duplicate measurements.

attained at $\text{pH } 4.4 \pm 0.1$, which is usually referred to as the isoelectric point (pI). The shape and magnitude of ζ -potential–pH profiles were markedly affected by heated complexing SPI with chitosan, depending on the mixing weight ratio.

The magnitude of ζ -potential increased gradually at pH lower than the pI (e.g., pH 2.0–4.0), while the ζ -potential changed from negative to positive number at pH 5.0 and 6.0 and decreased from -35 and -40 mV to about -10 mV, as the chitosan-to-protein ratios were increased from 0 to 0.2 g g^{-1} (Fig. 6A). Concomitantly, the pI of heated SPI–CS complexes was shifted from 4.4 ± 0.1 (Heated SPI) to 6.1 ± 0.1 , 6.6 ± 0.1 , 6.8 ± 0.1 and 6.9 ± 0.1 (0 – 0.2 g g^{-1}). Effects of MW and CD of chitosan on the ζ -potential of heated SPI–CS complex are also shown in Fig. 6B and C. The differences between 50 kDa, 100 kDa and 200 kDa were negligible. In detail, the isoelectric points of these three samples were about 6.3. In contrast, the change in CD of chitosan significantly ($P < 0.05$) affected the ζ -potential profiles. Fig. 6C showed that the more charge chitosan had, the higher the isoelectric point of the heated SPI–CS complex exhibited.

Overall, the heated SPI–CS complexes were mainly soluble at pH 2.0–4.0 and gradually insoluble as the pH increased. As the results of ζ -potential profiles, with pH elevation, the equilibrium of repulsive force and attractive force was broken by the decreased electrostatic repulsive. The attractive interactions between the heated SPI–CS complexes resulted in the insoluble and destabilization. In this paper, chitosan-to-protein ratios and CD of chitosan are valuable factors in influencing the stabilization of heated SPI–CS complex.

3.4. The effect of chitosan on emulsifying property

Table 1 shows emulsifying activity index (EAI) and emulsion stability index (ESI) values of heated SPI and SPI–CS complexes at pH 4.0. The EAI of SPI at test pH was about $7.7 \text{ m}^2/\text{g}$, which was in agreement with the results of Tsumura et al. (2005) and lower than the EAI of SPI at pH 7.0. This may be attributed to the decrease in protein solubility as pH shift from neutral to acidic conditions. Heat treatment showed insignificant ($P > 0.05$) effect on SPI at such pH (from 7.7 to $8.6 \text{ m}^2/\text{g}$). But the EAI of heated SPI at pH 4.0 was significantly ($P < 0.05$) increased from 8.6 to the value between 19 and 49 (m^2/g) by complexing SPI with chitosan (Table 1). This improvement was mostly dependent on the presence of chitosan. The EAI was increased gradually from 8.6 (control) to 19.7 and 49.8 (m^2/g) with increasing chitosan charge from 0 to 20 mV – 60 mV , but the changes of EAI between the samples effected by molecular weights of chitosan and chitosan-to-protein ratios were minor. Unexpectedly, complexation did not seem to affect the ESI significantly ($P > 0.05$) (Table 1).

Table 1
Emulsifying activity index (EAI) and emulsion stability index (ESI) of heated SPI and heated SPI–CS complexes.

Sample	EAI (m^2/g)	ESI (min)
SPI	$7.7 \pm 1.5^{\text{d}}$	$22.2 \pm 4.3^{\text{a}}$
Heated SPI	$8.6 \pm 0.8^{\text{d}}$	$22.8 \pm 5.2^{\text{a}}$
0.025 g g^{-1}	$37.7 \pm 7.3^{\text{ab}}$	$28.8 \pm 8.8^{\text{a}}$
0.05 g g^{-1}	$31.1 \pm 0.2^{\text{bc}}$	$23.7 \pm 3.4^{\text{a}}$
0.1 g g^{-1}	$38.6 \pm 1.6^{\text{ab}}$	$19.6 \pm 0.7^{\text{a}}$
0.2 g g^{-1}	$45.0 \pm 5.5^{\text{ab}}$	$18.6 \pm 0.7^{\text{a}}$
50 kDa	$39.1 \pm 3.7^{\text{ab}}$	$25.5 \pm 1.2^{\text{a}}$
100 kDa	$45.0 \pm 8.3^{\text{ab}}$	$23.2 \pm 2.2^{\text{a}}$
200 kDa	$40.8 \pm 4.7^{\text{ab}}$	$23.2 \pm 1.9^{\text{a}}$
20 mV	$19.7 \pm 0.9^{\text{cd}}$	$21.5 \pm 0.7^{\text{a}}$
60 mV	$49.8 \pm 0.8^{\text{a}}$	$18.2 \pm 0.4^{\text{a}}$

Superscript letters (a–d) indicate significant difference ($P < 0.05$) in EAI and insignificant difference ($P > 0.05$) in ESI among all samples.

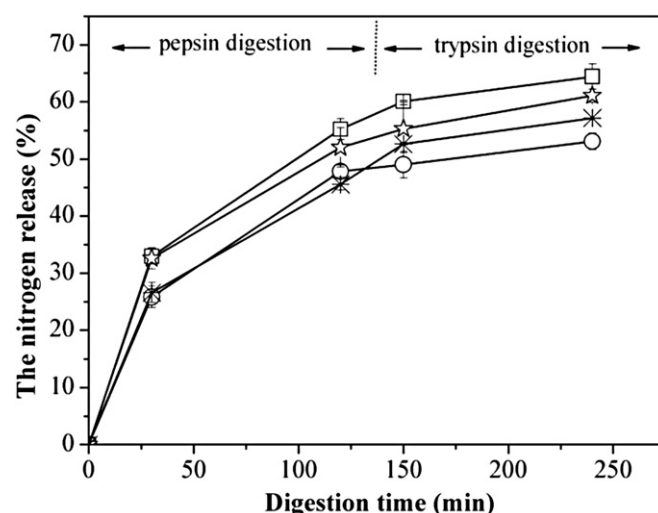


Fig. 7. The hydrolysis of SPI and SPI–CS mixture before and after heating in the sequential pepsin and trypsin digestion. The SPI–CS mixture was prepared at the chitosan-to-protein ratio of 0.1 g g^{-1} . —□—, SPI; —○—, heated SPI; —*—, SPI–CS mixture; —×—, heated SPI–CS complex. Values were expressed as means and standard deviations of duplicate measurements.

3.5. Pepsin and trypsin hydrolyses

The pepsin and trypsin hydrolyses of SPI and SPI–CS mixture before and after heating are shown in Fig. 7. During the pepsin hydrolysis, nitrogen release increased fast at this hydrolysis stage (0–30 min) then slowed down during 30–120 min. In the further trypsin hydrolysis the % nitrogen release of SPI increased gradually and slowly from the initial value of 55% to a maximum value of 64% which is similar to previous experiment (Wang et al., 2008). Both heat treatment and adding chitosan reduced the digestibility of SPI. These results were attributed to the reduction of enzymatic reaction sites by heat treatment or chitosan enwrapping. Adverse effect of heat treatment on digestibility of legume proteins has already been reported (Carbonaro, Cappelloni, Nicoli, Lucarini, & Carnovale, 1997). Previous studies on the effect of thermal denaturation on digestibility of purified 7S and 11S globulins showed that autoclaving reduced digestibility of both proteins. However, the digestibility obtained by heating SPI–CS complex slight higher than heating SPI alone.

4. Conclusions

The soluble SPI–CS complex was obtained by heating the biopolymer solutions at pH 3.0 in the autoclave (121°C , 15 min). Heated SPI–CS complex exhibited completely different PS–pH profiles compared to that of SPI and unheated SPI–CS mixture. SDS–PAGE as well as FTIR and ζ -potential analyses indicated that the heated SPI–CS complex is probably more like a soluble SPI–CS aggregate which is driven by electrostatic interaction. However, the mechanism of complex formation is not yet clear and needs further study. Moreover, the chitosan-to-protein ratios and CD of chitosan were valuable factors influencing the protein solubility and ζ -potential of heated SPI–CS complex. Concomitantly, the EAI of SPI at pH 4.0 was significantly improved and the ESI was not affected (significantly). Additionally, the heated SPI and/or SPI–CS complex exhibited slightly decreased pepsin and trypsin hydrolyses. The results presented here support the potential use of soluble SPI–CS complex in food formulations, especially in acidic food, in view of its excellent functional properties.

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