**Multilevel structural responses of β -conglycinin and glycinin under acidic or
alkaline heat treatment**

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Abstract

The structural responses of soy protein isolate under various processing conditions are of practically important for broadening its functionalities. In this work, multilevel structural responses of β -conglycinin and glycinin under acidic or alkaline heat treatment were investigated. Our results suggested that heat treatment under acidic (i.e., pH 2.5) or alkaline (i.e., pH 8.5) conditions induced multilevel structural responses of β -conglycinin and glycinin: under acidic heat treatment, both β -conglycinin and glycinin underwent hydrolysis and experienced disruption and reorganization in ordered secondary structure. This process was accompanied with changes in tertiary structure where previously buried regions were exposed to the aqueous phase to different extent. Small-angle x-ray scattering (SAXS) results indicated that the protein conformations evolved from globular ones to elongated ellipsoids, with a more remarkable elongation effect in glycinin than β -conglycinin. Upon solvent evaporation, β -conglycinin favored aggregation with a low “lateral to vertical ratio”, glycinin tended to aggregate in a high “lateral to vertical ratio” style. In contrast, alkaline heat treatment did not induce hydrolysis but disturbed the secondary structure instead. The protein monomers maintained the globular conformation and assembled into irregular large aggregates during solvent evaporation. Under either treatment, glycinin responded more sensitively than β -conglycinin at all structure levels. The observed multi-level structural responses can be used to guide the rational modification of soy protein isolate with controlled conformation.

Keywords: β -conglycinin; glycinin; heat treatment; structural changes; morphology; SAXS

1. Introduction

Due to its nutritional value and versatile functional properties, soy protein isolate (SPI) has been widely applied in meat, beverage and nutraceutical products (Jideani, 2011). Functional properties of SPI in food applications have involved water absorption, thickening, gelling, emulsifying and foaming activities (Moure, Sineiro, Dominguez, & Parajo, 2006). These properties vary with protein component, protein concentration, ionic strength, dielectric constant of the medium, interactions with other macromolecules, as well as processing conditions such as pH, temperature, pressure, enzymatic treatment, etc. (Cavallieri, Garcez, Takeuchi, & da Cunha, 2010; Howard & Udenigwe, 2013; Kim, Weller, Hanna, & Gennadios, 2002). In recent years, research efforts in broadening functionalities of SPI (Chen, Zhao, Sun, Ren, & Cui, 2013; Fernandez-Avila, Escriu, & Trujillo, 2015; Gan, Cheng, & Easa, 2008; Shen & Tang, 2012) have increased the need of clarifying its multi-level structural responses under various processing conditions.

As the two major components of SPI (Iwabuchi & Yamauchi, 1987), glycinin is a hexameric 11S globulin consisting of acidic and basic subunits, and β -conglycinin is a 7S trimeric glycoprotein composed of α , α' and β subunits. They contribute distinctively to macroscale properties of SPI. Functional properties, such as heat denaturation, surface hydrophobicity, emulsifying capacity, aggregation and gelation, were found to be sensitive to the 7S/11S ratio (Marcano, Varela, & Fiszman, 2015; Miriani, Keerati-u-rai, Corredig, Iametti, & Bonomi, 2011; Salleh et al., 2002; Xu, Liu, & Zhang, 2015; Maruyama et al., 2002). To be specific, it was reported that 11S mainly contributed to the heat stability, coagulation or gelation of native SPI (Tang, Wu, Chen, & Yang, 2006). The on-set gelation of native SPI and the viscoelasticity of the corresponding gels

62 depended upon the relative ratio of 11S to 7S (Tang et al., 2006), and the emulsifying
63 capacity of 11S differed from 7S due to their different subunits with different portions of
64 extended regions (Maruyama et al., 2002). Furthermore, interactions between two
65 components were also found to play a role in regulating overall functionalities. The
66 existence of 7S, as reported by Utsumi et al. (Utsumi, Damodaran, & Kinsella, 1984) and
67 Guo et al. (Guo et al., 2012), inhibited the dissociation and aggregation of 11S subunits.
68 The B subunit of 11S and β subunit of 7S underwent interaction under heat treatment
69 (Wang, Li, Jiang, Qi, & Zhou, 2014), and the formation of soluble complexes thereafter
70 greatly impacted the gelling properties. Therefore, it is worth distinguishing the
71 individual contribution of each SPI fraction when probing the structure and the related
72 functionalities of SPI under specific processing conditions.

73 Heat treatment is a common processing method for SPI based products to achieve
74 desired solubility, emulsifying or textural properties (Damodaran, 1988; Iwabuchi,
75 Watanabe, & Yamauchi, 1991). Under heat treatment, protein subunits underwent
76 dissociation or aggregation processes, and the kinetics of both pathways were highly
77 sensitive to medium pH (Sokolova, Kealley, Hanley, Rekas, & Gilbert, 2010). Currently,
78 the combined impacts of heat treatment under defined pH values on the conformation and
79 functional properties of each SPI fraction have not been clearly delineated. Among the
80 few available research articles, it was reported that 7S and 11S showed different
81 aggregation behaviors under heat treatment (60 - 100 °C) at pH 7.0, where the former
82 formed soluble aggregates while the latter ended up with insoluble aggregates (Guo et al.,
83 2012). During heat denaturation at pH 7.6, the disulfide bridge linking acidic and basic
84 polypeptides of 11S was broken and 11S dissociated partly into the 7S form under heat

treatment at pH 3.8 (Lakemond, de Jongh, Hessing, Gruppen, & Voragen, 2000). The combination of pH-shifting (incubated at pH 1.5 followed by refolding at pH 7.0) and mild heat treatment (50 or 60 °C) enforced the conversion of sulphhydryl groups into disulfides, thus strengthened the disulfide-mediated aggregation of SPI (Jiang, Xiong, & Chen, 2010). All of these available literatures only revealed changes on limited structural levels. Up to now, the structural responses of β -conglycinin and glycinin under acidic or alkaline incubation condition combined with heat treatment have not been fully clarified. Limited attempts have been made to clarify their multi-level structural changes, in terms of secondary, tertiary structure as well as self-assembled morphology at macroscale.

In this study, we conducted heat treatment (i.e., 80 °C) under acidic (i.e., pH = 2.5) or alkaline (i.e., pH = 8.5) conditions. We are particularly interested in the respective effects of the above-mentioned treatments upon conformational changes of 7S and 11S. In order to obtain detailed information about structural changes in 7S and 11S, samples were subjected to the small-angle X-ray scattering (SAXS) analyses. Tertiary and secondary conformational changes were monitored through tryptophan intrinsic fluorescence spectra and circular dichroism (CD), respectively. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to detect the extent of protein hydrolysis during heat treatment. Macroscale morphological observation was then performed via atomic force microscopy (AFM). Knowledge obtained in this work will improve our understanding of structure-property relationship of glycinin and β -conglycinin and facilitate their functionality oriented structural modification process.

2. Materials and Methods

2.1 Materials

Soy β -conglycinin (7S) and glycinin (11S) were prepared using the methods described previously (Feng et al., 2015; Yuan et al., 2013). The protein contents of 11S and 7S as determined by Kjeldahl method were 93.6 % and 92.0%, respectively. Tris-alkali, sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylethylenediamine (TEMED), 2-mercaptoethanol (2-ME), bromophenol blue, Coomassie brilliant blue R-250 were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). PageRulerTM prestained protein ladder (#26619) was purchased from ThermoFisher Scientific, Inc. (Waltham, MA, USA). Milli-Q water was used throughout the experiments.

2.2 Acidic or Alkaline Incubation Combined With Heat Treatment

Two mg/mL of 7S and 11S were prepared by dissolving freeze-dried samples into deionized water. The stock solutions of both 7S and 11S were then divided into two sets, respectively. One set of them was adjusted to pH=2.5 with 1N HCl, the other set was adjusted to pH=8.5 with 1N NaOH. Sodium azide (0.02%, w/v) was added to inhibit microbial growth. For thermal treatment, protein solutions were poured into glass vials with lids and heated at 80 °C for preset time (1h or 5 h) in a temperature-controlled bath with temperature deviation of 0.1 °C. After heat treatment, samples were immediately cooled in an ice bath and stored at 4 °C until further use, typically within 2 days. Control groups were held at specific pH values at room temperature (20-25°C) without heat treatment for 5 h and then stored at 4 °C until further use. Triplicate preparations were made for each sample. Sample abbreviations corresponding to specific treatments were summarized in Table 1.

2.3 Small-Angle X-ray Scattering (SAXS) Measurement

SAXS data were collected at Bio-CAT, 18-ID beam line section, at the Advanced

Photon Source, Argonne National Laboratory. Protein samples (2 mg/mL) in respective aqueous solutions were examined. The X-ray wavelength was 1.033 Å, and the experimental setup included a 3.5 m sample-to-detector length camera and another 0.3 m sample-to-detector length camera with the high-sensitivity CCD detector. A quartz capillary flow cell of 1.5 mm diameter was fitted to a brass block. This sample holder was maintained at 25 °C in all experiments. Each 2D scattered intensity distribution profile recorded by detector was integrated into the one-dimensional scattering function $I(Q)$, as a function of the scattering vector, Q , whose amplitude is given by $Q = (4\pi/\lambda) \sin(\theta/2)$, λ is the wavelength of the X ray beam, θ is the scattering angle. Ten curves were collected for each sample, and their averaged curves were utilized for further analysis. Identical measurement configurations were used for solvent backgrounds and sample solutions for proper background subtraction.

2.4 Small Angle X-ray Scattering Data Analysis

Kratky plots ($I(Q) \cdot Q^2$ versus Q) were plotted to distinguish the folded globular protein from their disordered states. The scattering intensity of a folded globular protein has a Gaussian-like shape at small Q range and decays approximately as $1/Q^4$ at high Q yielding a bell-shaped Kratky plot with a well defined maximum. For an unfolded protein, the Kratky plot presents a plateau over a specific range of Q , followed by a monotonic increase at large Q region.

Guinier analyses for compact sphere (Guinier & Fournet., 1955) were performed by following equations to obtain the radius of gyration, R_g , and the scattering intensity at zero angle, $I(0)$:

$$I(Q) = I(0) \exp \left(-\frac{R_g^2 Q^2}{3} \right) \quad (1)$$

The P(r) function or pair-distance distribution function (PDDF), which describes the paired-set of all distances between points within the object, was also performed according to equation (2) to detect conformational changes within the macromolecular structure and maximum distance, "Real Space" R_g was also extracted from P(r):

$$p(r) = \frac{1}{2\pi^2} \int_{Q=0}^{\infty} Q^2 I(Q) \frac{\sin Qr}{Qr} dQ \quad (2)$$

Debye-Bueche plot (Debye & Bueche, 1949) was plotted to calculate the correlation length, ξ , which helps to analyze the aggregation state in samples. By plotting $I(Q)^{-1/2}$ vs. Q^2 the slope of straight line (ξ^2) can be extracted:

$$I(Q) \propto (1 + (Q\xi)^2)^{-2} \quad (3)$$

For an ellipsoidal protein particle with a parallel radius of “a” and a perpendicular radius of “b”, the form factor can be expressed as:

$$P(Q) = \int_0^1 \left(\frac{3(\sin u - u \cos u)}{u^3} \right)^2 dx \quad (4)$$

$$u = Qb \left(\frac{ax}{b} + (1 + x)^2 \right) \quad (5)$$

2.5 Intrinsic Fluorescence Measurement

Intrinsic fluorescence of samples were determined at 23 °C with a FluoroMax-3 fluorescence spectrometer (Horiba, Kyoto, Japan). Protein solutions of all pH values were diluted to the final concentration of 0.2 mg/mL before loading into quartz cuvettes with path length of 1 cm. Samples were excited at 280 nm to maximize tryptophan and tyrosine quantum yield. Emission spectra were collected from 290 to 500 nm with a constant slit of 3 nm for both excitation and emission.

2.6 Circular Dichroism

Far-ultraviolet circular dichroism (CD) spectra were collected at 25 °C using an AVIV model 400 circular dichroism spectrometer equipped with 0.1 cm path length

quartz cuvettes (model 110-OS, Hellma, USA). Protein samples of all pH values were diluted to final concentration of 0.2 mg/mL before put into quartz cuvettes. Each sample was scanned 5 times between 190 and 260 nm to obtain an averaged curve. The bandwidth, step resolution, and scan rate were 1.0 nm, 0.5 nm, and 100 nm/min, respectively. Identical cuvette was used for each sample and corresponding solution buffer. Recorded spectra were corrected by subtraction of the spectrum of a protein-free buffer. The residue ellipticity (θ) values were calculated as θ (deg cm² dmol) = (100dM)/LC, where d is the signal (millidegrees) obtained by the CD spectrometer, M is the average molecular weight (M_w) of amino acid residues (115), C is the protein concentration (in mg/mL) of the sample, and L is the cell path length (1 mm). Secondary structure estimation was processed by curve fitting with reference CD curves of α -helix, β -sheet, random coil, and β -turn.

2.7 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a discontinuous buffer system with 4% stacking gel (pH 6.8) and 12% separating gel (pH 8.8) using gel electrophoresis apparatus (Bio-Rad Laboratories Ltd., Hercules, CA, USA). The protein samples were prepared directly with loading buffer, which consisted of 0.125 M Tris-HCl (pH 8.0), 1.0% SDS (w/v), 0.05% bromophenol blue (w/v), 30% glycerol (v/v) and 5% 2-mercaptoethanol (v/v). The samples were boiled for 10 min before loading. Then, 20 μ L samples or pre-stained protein marker (M_w ranging from 10 to 220 kDa) were loaded. The electrophoresis was then operated at 80 V until the tracking dye reached the bottom of the gel. After electrophoresis, the gel was stained with Coomassie brilliant blue (0.25% Blue-R250) and destained in a methanol - water solution containing 10% acetic acid (methanol/ acetic

acid/water = 3:1:6, v/v/v). Gel was scanned by Bio-Rad Universal Hood II gel imaging system, and densitometry analysis was performed using Image J 2.1.4.7 software.

2.8 Atomic Force Microscopy Measurements (AFM)

Tapping mode AFM images were collected by a NanoScope IIIA Multimode AFM (Veeco Instruments Inc., Santa Barbara, CA, USA) equipped with a silicon-etched RTESP7 cantilever (Veeco Nanoprobe, Camarillo, CA, USA). Before tip engagement, the drive frequency of the silicon tip was tuned to a frequency range of 290–310 kHz. A 100 μ L aliquot of sample (after dilute to final concentration of 20 μ g/mL) was spread on a freshly cleaved mica disc, after 2-hour absorption, samples were washed with DI water and dried in air before test. During imaging, the surface was scanned at a rate of 0.7 to 1.0 Hz. All collected images were flattened before further analysis. Section and bearing analyses, which were incorporated in the Nanoscope 5.31 software, were utilized to obtain the quantitative information of protein morphology.

2.9 Statistical Analysis.

All treatments were run at least in triplicate. Experimental data were expressed as the mean $\pm$ SD.

3. Results and Discussion

3.1 Conformational Evolutions of 7S and 11S Revealed by SAXS

Small-angle X-ray scattering (SAXS) is one of the most suitable techniques in monitoring conformational changes in aggregation/dissociation and folding/unfolding processes of proteins in solution. In **Fig. 1**, Kratky plots of 7S and 11S under acidic incubation with different heating durations were shown and their corresponding pair-distance distribution function (PDDF) curves were inserted. For 7S incubated in acidic

condition without heat treatment, the Kratky plot presented a Gaussian distribution peak followed by a monotonically increased tail, indicating the preserved folded structure (**Fig. 1A**). When subjected to heat treatment combined with acidic incubation for 1 h, the intensity of Kratky plot decreased and the peak shifted toward the higher Q region, suggesting a partial unfolding process. Prolonged heat treatment for up to 5 h did not induce further changes in Kratky plot. As for 11S, incubation at acidic condition without heat treatment already induced partial unfolding (**Fig. 1B**), as compared to the sharp bell-shape Kratky plot for native glycinin at neutral pH reported elsewhere (Guo et al., 2012). Prolonged heat treatment caused a progressive unfolding in 11S as shown by the flat bell-like profile for 11S-2.5-1h and the plateau curve with up turning tail for 11S-2.5-5h.

To quantitatively describe the structural responses of 7S and 11S under acidic heat treatment, further data analysis derived from their scattering profiles were conducted. R_g values were first calculated through Guinier and pair distribution function $P(r)$ for globular particles to estimate protein dimension. Since the uncertainty in determination of R_g through these two methods would substantially increase as the protein underwent unfolding process, R_g was also calculated through Gaussian statistical analysis, where the protein was fitted to a flexible polymer chain model for unfolded proteins. As can be seen in **Table 2**, R_g for 7S calculated via three different methods showed negligible differences for each sample, and the results suggested that 7S first shrank in size and then extended during heat treatment. The halved $I(0)$ after one-hour heat treatment indicated that this process was most likely corresponding to the dissociation of 7S. Meanwhile, although similar R_g values were obtained through three different methods for 11S-2.5-0h, the R_g values of 11S after heat treatments (i.e. 11S-2.5-1h and 11S-2.5-5h) calculated

through Guinier and $P(r)$ differed significantly from those calculated through Gaussian function. Combined with the shapes of Kratky plots, we proposed that excessive extension in 11S might occur, thus the results from Gaussian estimation, which regarded the 11S as randomly stretched polymer would be more reliable than those derived from Guinier plot or $P(r)$. And the results revealed that the R_g of 11S increased dramatically from 58.3 Å to 143.9 Å and 166.4 Å after 1h and 5h heat treatment, confirming that further heat treatment caused more severe unfolding in dissociated 11S chains. To visualize the conformational changes of 7S and 11S under acidic heat treatments, the scattering profiles were modeled with an ellipsoidal form factor using a tool imbedded in Primus software provided by ATSAS 2.7.1. As can be seen from the dimensions calculated via ellipsoidal model (a and b values defined the size in perpendicular and parallel semi axis, respectively), 7S gradually elongated as the heat treatment prolonged, while the elongation extent was more remarkable for 11S.

When subjected to alkaline heat treatment, 7S and 11S exhibited totally different scattering profiles from those under acidic heat treatment. As presented in **Fig. 1C** and **Fig. 1D**, both 7S and 11S retained their folded structure under alkaline incubation, and heat treatment thereafter decreased the peak intensity and the peak position shifted to a lower Q region, a clear indication of protein aggregation with a substantially maintained folded structure. The aggregation process was further confirmed by the progressive increase in R_g , ξ and $I(0)$ values as shown in **Table 2**. Our results agreed with the previous work conducted by Guo, et al. (Guo et al., 2012), where they utilized SAXS as an analysis tool to investigate the aggregation behavior of β -conglycinin and glycinin at pH 7.0. Their results showed that the peak position of Kratky plots for both components

displayed a shift to low q range, and increase in both $I(0)$ and R_g were also observed. Besides, here we can also noticed that 11S responded to heat treatment by aggregation in a more rapid way, since the structure of 11S did not show remarkable changes after 1h heat treatment, whereas a progressive aggregation process was observed for 7S-8.5-1h and 7S-8.5-5h.

The present SAXS data were able to catch the general trends of acidic heat treatment induced unfolding and alkaline heat treatment induced aggregation of SPI fractions. Furthermore, SAXS was sensitive enough to distinguish detailed conformational changes for each individual fraction of SPI. Compared with 7S, 11S exhibited more excessive unfolding under acidic heat treatment and more rapid aggregation under alkaline heat treatment.

3.2 Tertiary Structural Response of 7S and 11S Detected by Fluorescence Spectroscopy

Tyr and Trp account for around 3% of the total amino acid of SPI, some of them located at in the hydrophobic interaction pocket among subunits, others distributed randomly throughout the protein domain (Adachi et al., 2003). And their fluorescence emission wavelengths are sensitive to the changes in their local environment. Thus fluorescence spectra were recorded to reveal tertiary structure changes of 7S and 11S under different treatments. A shift of the emission peak to a higher wavelength and a decrease in fluorescence intensity were observed in both 7S and 11S under acidic heat treatment (**Fig. 2 A, B**). To be specific, the $\lambda_{\max}$ of 7S incubated under pH 2.5 shifted from 351nm to 353 and 355nm as the heating time increased. Similar peak shifting was observed in 11S. The $\lambda_{\max}$ shifted from 352 to 354, 356 nm as heating duration extended

and the emission peak intensity experienced a more remarkable decrease in 11S than in 7S. The constant red shift indicated that the local environment of Tyr and Trp has changed from the relatively hydrophobic protein domain to more hydrophilic bulk water solution. Therefore, the more remarkable peak shift and intensity decrease that occurred in 11S suggested a higher exposure extent of the tryptophan or tyrosine-containing regions to the aqueous phase under heat treatment at acidic pH.

Alkaline incubation combined with heat treatment induced slight fluorescence quenching in 7S and negligible peak shifting was observed for λ_{max} (**Fig. 2C**). These phenomena suggested that the microenvironment of tryptophan and tyrosine remained the same during alkaline heat treatment. As for 11S, after one-hour heat treatment, λ_{max} of 11S shifted from 350 to 352 nm suggesting limited degree of exposure, and no further peak shift was observed when heat treatment prolonged to 5 hours (**Fig. 2D**). Instead of decrease in emission peak intensity, 11S experienced a noticeable increase in peak intensity after alkaline heat treatment. This could be due to the excess scattering effect of aggregates produced during treatment. Combined with these two sets of data, the tertiary structures of 7S and 11S reacted differently towards acidic and alkaline heat treatments, while side chains progressively exposed under acidic heat treatment, alkaline heat treatment induced limited degree of exposure and aggregation took place. In both circumstances, 11S underwent more remarkable changes in tertiary structure.

3.3 Secondary Structure Changes Revealed by Circular Dichroism Spectroscopy

Circular dichroism scanning was conducted to detect evolution in secondary structures of 7S and 11S under acidic or alkaline heat treatment. As shown in **Fig. 3A**, the spectra of 7S after acid incubation (7S-2.5-0h) exhibited far-UV spectra with a negative

dichroic peaks at around 205 nm, a shoulder at 222 nm, a positive peak at 195 nm and a zero crossing at 200 nm. These features indicated the existence of highly ordered structure, such as α -helices and β -sheets (Kelly & Price, 2000). Heat treatment resulted in remarkable decrease in the magnitude of the negative dichroic and blue shift in zero-crossing position. The majority of decrease happened after 1h's heating, prolonged heating up to 5 h only induced slightly decrease thereafter. When subjected to heat treatment combined with alkaline incubation, the peak minimum progressively decreased and zero-crossing position of 7S gradually blue shifted (**Fig. 3C**). Compared to acidic heat treatment, secondary structural response of 7S was less sensitive toward alkaline heat treatment. Secondary structural changes occurred mainly within the first hour during acidic heat treatment, whereas a progressive change was observed throughout the five hours' alkaline heat treatment.

As for 11S, the solvent incubation induced secondary structure changes in 11S were reported previously when incubated under pH 1.5 and pH 12.0 (Jiang, Xiong, & Chen, 2011). When heated under pH 2.5, the decrease in the magnitude of negative band as well as the extent of blue shift in zero-crossing position of 11S was much more prominent than that for 7S (**Fig. 3B**). When heated under pH 8.5, 11S exhibited similar secondary structure changes as those in 7S. The secondary structural changes happened within the first hour's heating, and prolonged heat treatment did not induce further changes (**Fig. 3D**). Despite the various extent of changes in CD curves of 7S and 11S under acidic or alkaline heat treatments, a general trend was observed: the decrease in the magnitude of the negative band and blue shift in zero-crossing position. These phenomena suggested the disruption and reorganization of ordered secondary structures during the heat

treatment, probably associated with unfolding and/or hydrolysis process (Tang & Wang, 2010). From these set of data, we can also conclude that 11S demonstrated more sensitive secondary structure responses as compared to 7S under both acidic and alkaline heat treatment.

3.4 Primary Structure Changes Monitored via SDS-PAGE

To identify possible changes in primary structure, electrophoresis patterns of 7S and 11S treated under reducing conditions were displayed in **Fig. 4**. When incubated at pH 2.5 without heat treatment, 7S presented characteristic major bands corresponding to subunits of β -conglycinin (α' , 71kDa; α , 67kDa; β , 50 kDa). Electrophoresis bands of 7S-2.5-1h and 7S-2.5-5h revealed progressive decrease in intensity of major bands (quantitative data obtained by Image J, data not shown), and α' , α were more remarkably hydrolyzed than β subunit (**Fig. 4A**). For 11S, SDS-PAGE presented bands at 40, 36 kDa for acid polypeptides, 19 kDa for basic polypeptides (**Fig. 4A**). Decrease in band intensity, which was more remarkable in acid subunits than basic subunits, was observed for 11S under acidic heat treatment. New bands with $M_w < 20$ kDa were observed as heating duration prolonged, which demonstrated an extensive polypeptide hydrolysis phenomenon.

When treated under alkaline heating condition, subunits of 7S showed similar band profile as 7S-2.5-0h in SDS-PAGE. Electrophoresis bands of 7S subunits retained and band intensity did not decrease as alkaline heat treatment proceeded (**Fig. 4B**). Meanwhile, 11S-8.5-0h presented characteristic bands at 40, 36 and 19 kDa corresponding to acid and basic subunits. Heat treatment thereafter did not induce changes in position and intensity of individual subunit. In summary, while acidic heat

treatment leads to dissociation of 7S and 11S into individual subunits and finally breakage into polypeptide fragments, subunits were preserved under alkaline heat treatment.

3.5 Morphology Probed by AFM

Morphology of protein on mica substrate after solvent evaporation is quite different from its in situ structure in solution, but the latter largely determines the macroscale performance of the former. Representative tapping mode AFM height images of 7S and 11S after solvent evaporation are presented in **Fig. 5**. 7S casted from acidic incubation solution without heat treatment showed evenly distributed spherical morphology with averaged vertical height of 6.6 nm (**Fig. 5A**). Heterogeneous aggregates emerged in 7S-2.5-1h and the average vertical height increased to 23.4 nm, numerous small spherical particles that resembled native 7S without heat treatment were also observed. Morphology of 7S-2.5-5h after solvent evaporation formed stubby rod like aggregates, section analysis further revealed that the lateral dimension of large aggregates fell in the range of 300 to 1100 nm and the average vertical height increased to 35.4 nm. Therefore, during solvent evaporation, polypeptides of 7S after acidic heat treatment aligned and aggregated in both horizontal and vertical directions. After solvent evaporation, 11S-2.5-0h also displayed spherical morphology with an average vertical height of 7.6 nm. After one hour's acidic heat treatment, 11S displayed heterogeneous morphology with large spherical aggregates (> 80 nm). Apart from large aggregates, 11S-2.5-5h formed arc-shaped fibrils and the vertical height of the fibril arms ranged from 16.5 to 41.6 nm. Fibrils produced from globular proteins under acidic heat treatment have been extensively reported in previous researches (Jones, Adamcik, Handschin, Bolisetty, &

Mezzenga, 2010; Munialo, Martin, van der Linden, & de Jongh, 2014). However most of the works were conducted under pH equal or less than 2.0, with longer heating incubation time (usually 16 to 24 h). Our results suggested that globular soy protein fractions could self-assemble into fibril-like morphology even under milder acidic heat treatment (i.e., pH=2.5, heating duration = 5h). We also discovered that each fraction demonstrated different assembly preferences, while 7S favored aggregation with low “lateral to vertical ratio”; 11S tended to aggregate in a high “lateral to vertical ratio” style.

Representative AFM images of 7S and 11S after basic incubation (**Fig. 5B**) without heat treatment showed small, spherical morphology with averaged vertical heights of 9.2 and 6.7 nm, respectively. The overall morphology of 7S did not change too much as heat treatment proceeded, except that their vertical heights progressively increased to 12.6 nm and 17.0 nm, respectively. As for 11S, one-hour alkaline heat treatment caused heterogeneous increase in protein particle size, and the vertical size of large aggregates fell in the range of 25-75 nm. At the end of 5-hour alkaline heating incubation, 11S assembled into irregular shaped cluster with vertical height up to 100 nm. It is clear that 11S monomers displayed higher aggregation tendency than that of 7S after alkaline heat treatment.

4. Conclusion

In summary, under acidic heat treatment, both 7S and 11S underwent hydrolysis in polypeptides, and their secondary structures experienced the disruption and reorganization of the ordered structures, e.g. α -helix and β -sheet, the buried tryptophan and tyrosine side chains exposed along with the unfolding process. The protein conformations in solution evolved from globular ones to elongated ellipsoids. The

stretched polypeptide fragments then favored the lateral assembly during solvent evaporation, which resulted in short or long fibril morphologies. Mild alkaline heat treatment did not induce hydrolysis in 7S or 11S but disturbed their secondary structure. The protein monomers maintained their globular confirmations and assembled in both lateral and vertical directions during solvent evaporation, which caused the formation of large aggregates. Under both treatments, 11S responded more sensitively than 7S most likely due to their fundamental differences in composition and association mode of subunits. Lastly, we would like to point out that the practical application of SPI as food ingredients in an acidic or alkaline aqueous solution is limited. By necessity, the treated SPI solutions are to be neutralized and dried. The observed multi-level structural responses under acidic and alkaline heat treatment can be used to guide the rational modification of SPI with controlled conformation.

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538

539 **Table 1.** Sample abbreviations corresponding to specific treatments

Sample name	Protein fraction	pH	Heating time (h)	Sample name	Protein fraction	pH	Heating time (h)
7S-2.5-0h	7S	2.5	0	11S-2.5-0h	11S	2.5	0
7S-2.5-1h	7S	2.5	1	11S-2.5-1h	11S	2.5	1
7S-2.5-5h	7S	2.5	5	11S-2.5-5h	11S	2.5	5
7S-8.5-0h	7S	8.5	0	11S-8.5-0h	11S	8.5	0
7S-8.5-1h	7S	8.5	1	11S-8.5-1h	11S	8.5	1
7S-8.5-5h	7S	8.5	5	11S-8.5-5h	11S	8.5	5

540

541 **Table 2.** Structural parameters of 7S and 11S under acidic or alkaline heat treatment
542 derived from SAXS data

Sample	Rg (Å)			Ellipsoid (Å)		ξ (Å)	I(0)
	Guinier	GNOM	Gaussian	a	b		
7S-2.5-0h	49.76	49.3	49.6	69.3	69.3	24.67	204
7S-2.5-1h	46.40	44.5	40.2	38.0	91.0	20.10	102
7S-2.5-5h	53.17	51.7	55.3	32.9	105.2	21.14	113
11S-2.5-0h	53.91	56.23	58.3	49.0	49.0	23.72	90.8
11S-2.5-1h	52.24	53.16	143.9	32.0	162.3	20.59	52.0
11S-2.5-5h	47.7	50.85	166.4	13.0	133.7	17.46	16.8
7S-8.5-0h	50.9	51.2	-	61.5	61.5	21.79	198
7S-8.5-1h	68.1	67.4	-	58.2	102.6	28.32	281
7S-8.5-5h	69.7	69.4	-	62.6	119.4	30.35	309
11S-8.5-0h	54.63	50.14	-	57.0	62.4	21.54	223
11S-8.5-1h	67.5	65.35	-	90.6	140.0	40.69	367
11S-8.5-5h	66.8	65.27	-	91.0	143.4	39.92	297

543

544 **Figure captions:**

545 **Fig. 1.** Kratky plots of (A) 7S and (B) 11S under acidic heat treatment, as well as (C) 7S
546 and (D) 11S under alkaline heat treatment. Inserted plots are corresponding PDDF for
547 each set of samples.

548 **Fig. 2.** Intrinsic fluorescence emission spectra of (A) 7S and (B) 11S under acidic heat
549 treatment, as well as (C) 7S and (D) 11S under alkaline heat treatment.

550 **Fig. 3.** Far UV-CD spectra of (A) 7S and (B) 11S under acidic heat treatment, as well as
551 (C) 7S and (D) 11S under alkaline heat treatment.

552 **Fig. 4.** (A) SDS-PAGE of 7S and 11S under acidic heat treatment; (B) SDS-PAGE of 7S
553 and 11S under alkaline heat treatment.

554 **Fig.5.** AFM height images of 7S and 11S after (A) acidic heat treatment and (B) alkaline
555 heat treatment.

Fig.1

Fig.2

Fig.3

Fig.4

Fig.5

Heating at pH 2.5

7S

Heating at pH 8.5

11S