J Am Oil Chem Soc (2010) 87:997–1004
DOI 10.1007/s11746-010-1583-0

ORIGINAL PAPER

An Improved Isolation Method of Soy β-Conglycinin Subunits and Their Characterization

De-Bao Yuan · Wei Min · Xiao-Quan Yang · Chuan-He Tang · Ke-Li Huang · Jian Guo · Jin-Mei Wang · Na-Na Wu · Heng-Guang Zheng · Jun-Ru QI

Received: 30 October 2009 / Revised: 15 March 2010 / Accepted: 24 March 2010 / Published online: 14 April 2010
© AOCS 2010

Abstract This study aimed at establishing an effective preparative isolation method of soy β-conglycinin constituent subunits and characterizing some of their physicochemical properties and their heat-induced aggregation. These subunits were isolated in relatively large amounts and in high purity by dissociating β-conglycinin in 6 M urea and using a combination of DEAE-Sepharose fast flow column chromatography and immobilized metal ion affinity chromatography (IMAC). At a pH deviating from isoelectric point (p*I*), zeta potentials of α′ and α subunits were much larger than that of β subunit, while in the latter case, the hydrophobic groups were more buried within the proteins. Dynamic light scattering analysis indicated that the extent of heat-induced aggregation of β subunit was much higher than that of α′ and α subunits, and the aggregation was also more affected by the increase in ionic strength. Atomic force microscopy analysis indicated that more ordered and stranded aggregates were formed for α′ and α subunits. These results confirm a close relationship between physicochemical properties and heat-induced aggregation of β-conglycinin subunits.

D.-B. Yuan · W. Min · X.-Q. Yang (✉) · C.-H. Tang · K.-L. Huang · J. Guo · J.-M. Wang · N.-N. Wu · J.-R. QI
Department of Food Science and Technology, South China University of Technology, Guangzhou 510640, People's Republic of China
e-mail: fexqyang@163.com

H.-G. Zheng
Institute of Food Science and Agriculture Engineering, Fujian Academy of Agricultural Sciences, Fuzhou 350003, People's Republic of China

Keywords Subunits of β-conglycinin · Isolation · Physicochemical characterization · Thermal aggregation · AFM

Abbreviations

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
IMAC	Immobilized Metal Ion Affinity Chromatography
HPSEC	High-performance size-exclusion chromatography
AFM	Atomic force microscope
p*I*	Isoelectric point
2-ME	β-Mercaptoethanol
EDTA	Ethylenediamine tetraacetic acid
SDS	Sodium dodecyl sulfate
BSA	Bovine serum albumin
ANS	1-Anilinonaphthalene-8-sulfonic acid

Introduction

Soybean proteins are utilized in many food formulations, for their good nutritional and physicochemical functions. β-Conglycinin is one of the major components, accounting for about 30% of the total seed proteins [1, 2]. It exists as a trimer with a molecular mass of 150–200 kDa, and contains three major subunits: α′, α, and β. Studies on β-conglycinin at the subunit levels have attracted much attention, since they can play some special roles in health care and food application [3–6].

Non-glycosylated individual subunits and deletion mutants (designated as α_c and α'_c) lacking the extension regions were expressed in *Escherichia coli* [7]. Lovati et al.

[3] adopted preparative isoelectric focusing technique to obtain homogeneous β subunit and mixtures of α and α'. Maruyama et al. [8, 9] purified homogeneous trimers consisting of α, α', or β from mutant soybean cultivars. Duranti et al. [10] describe a relatively large-scale isolation method of α' subunit by IMAC from dissociated β-conglycinin. Several other references have described the isolation of the three subunits from conventional soybean cultivars [11, 12]. The validity of the preceding methods to isolate the individual subunits was either inconsistent or restricted to laboratory scale [10], and thus, an universal method for obtaining a relatively large amount of homogeneous subunits from conventional soybean cultivars is needed [13, 14]. To the best of our knowledge, the physicochemical properties and heat-induced aggregation of these subunits, as well as their possible relationship are poorly understood.

In the present work, the combination of a DEAE-Sepharose Fast Flow column and an IMAC column was applied to obtain individual β-conglycinin subunits in relatively large quantity and high purity from commercial defatted flakes. Some physiochemical properties including zeta potential and surface hydrophobicity of these individual subunits were evaluated. Additionally, their thermal aggregation behavior and the morphology of the formed aggregates were also compared.

Materials and Methods

Materials

Low heat-treated soy flakes were provided by Shandong Xinjiahua Industrial & Commercial Co., Ltd. China. These flakes were obtained by flash desolventization and then heated to around 60 °C under vacuum. The flakes were ground in a Straub mill (Model 4E, Straub Co., Philadelphia, PA, USA) to pass through 80 mesh sieve. The soy flour obtained was stored in sealed containers at 4 °C until used. The protein content of soy flour was 55.5 ± 0.4% (determined by Kjeldahl method with nitrogen conversion factor of 6.25; on dry basis) and nitrogen solubility index 84.0%. The resins (DEAE-Sepharose Fast Flow, Chelating Sepharose Fast Flow) and two columns (4.5 × 25 cm, 6 × 35 cm) were purchased from Pharmacia Co., Ltd. All other chemicals were of analytical or better grade.

Preparation of β-Conglycinin and Isolation of Its Subunits

Preparation of β-Conglycinin

Soybean β-conglycinin was isolated according to the method by Nagano et al. [15].

Preparation of β Subunit and Mixture of α and α'

Separation of individual subunits from β-conglycinin was achieved by dissociating the trimeric form with urea using DEAE-Sepharose Fast Flow chromatography in combination with IMAC. First, β-conglycinin sample (5 g) in 60 mL 0.05 M borate buffer (pH 9.0) containing 6.0 M urea (the standard buffer) was loaded on the column (6 × 35 cm) previously equilibrated with the same buffer. Elution was performed with the standard buffer (3 L) containing NaCl in a step gradient of 0–0.3 M at a flow rate of 5 mL/min. The purified β subunit and a mixture of α and α' subunits were obtained by collecting specific protein fractions. The collected fractions were dialyzed against purified water at 4 °C and then lyophilized. The yields of β subunit and the mixture of α' and α subunits were about 1.0 and 3.1 g, respectively.

Preparation of α and α' Subunits

Mixtures of α and α' subunits (5 g), dissolved in 60 mL Tris–HCl buffer (0.05 M, pH 7.0, containing 0.5 M NaCl and 6 M urea) were loaded onto the IMAC column (4.5 × 25 cm) coupled with Cu^{2+} previously equilibrated with the same buffer. The column was thoroughly washed with the same buffer to elute the unbound fraction at a rate of 5 mL/min. Elution was performed with the same buffer (1.6 L) containing imidazole in a linear gradient of 0–0.1 M at a flow rate of 5 mL/min. Purified α and α' subunits can be obtained through collecting specific protein fractions. The collected fractions were dialyzed against purified water at 4 °C and then lyophilized. The yield of α' and α subunits was about 2.4 and 2.2 g, respectively.

Protein Measurement

Proteins in the samples were determined according to the Lowry method [16] using BSA as a standard.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE experiments were performed according to the discontinuous buffer system of Laemmli [17] at 5% stacking gel and 12.5% separating gel using gel electrophoresis apparatus DYCZ-30 (Beijing LIUYI Instrument Factory, China). The protein samples were directly dissolved in the sample buffer, namely 0.125 M Tris–HCl buffer (pH 8.0) containing 1.0% (w/v) SDS, 0.05% (w/v) bromophenol blue, 30% (v/v) glycerol and 5% (v/v) 2-ME. Then the samples were vortexed for about 5 s, heated at 100 °C for 5 min, and centrifuged (10,000 *g*, 10 min). The samples (containing about 5 μg protein for each) were

loaded, and then the electrophoresis was run at 20 mA in the stacking gel and at 40 mA in separating gel until the tracking dye reached the bottom of the gel. Lastly, the gel was then dyed with Coomassie Blue R250 and destained. The band patterns were then photographed and analyzed with Quantity One software version 4.4 (Bio-Rad Laboratories Inc., USA). The relative quantity of each subunit (protein band) was calculated from its respective percent area on the densitograms against the areas of total subunits.

Zeta Potential Measurement

Zeta potential measurement was performed with the aid of a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK) equipped with a multipurpose autotitrator (model MPT-2, Malvern Instruments, Worcestershire, UK). Freshly prepared protein dispersions were diluted to a protein concentration of 1 mg/mL with deionized water and filtered through a 0.45 μm HA Millipore membrane prior to analysis. A titration curve was obtained through monitoring the zeta potential of samples as a function of pH (2.0–10.0) with an increment of 0.5. The pH corresponding to zero zeta potential was the apparent isoelectric point of each sample. The pH of the samples was adjusted using 0.05 M HCl and 0.05 M NaOH. Each data set was obtained with duplicate measurements.

Fluorescence Spectroscopy

The extrinsic fluorescence spectra of 1-anilinonaphthalene-8-sulfonic acid (ANS), a fluorescence probe, were recorded using a Shimadzu RF-5301 PC fluorophotometer (20 ± 0.5 °C), with a constant excitation and emission slit of 5 nm. The excitation and emission wavelengths were 390 and 400–600 nm, respectively. Stock solutions of 8×10^{-3} M ANS^{-}, and 0.15 mg/mL (w/v) protein were prepared in phosphate buffer (pH 7, ionic strength $\mu = 0.01$). And ANS^{-} stock solution (20 μL) was added to 4 mL of protein solution. Samples were vortexed for about 5 s.

Dynamic Light Scattering (DLS) Analysis

Samples (β-conglycinin and its β, α and α' subunits) were prepared in buffers (pH 7.6 and $\mu = 0.08$ or 0.5) according to the method of Maruyama et al. [13] with some modifications. The sample solutions were diluted to a protein concentration of 1 mg/mL following filtration with Millipore membrane (0.45 μm). The particle size distributions of the sample solutions, after heating at 90 °C for 5 min were determined with a Zetasizer Nano ZS (Malvern Instrument Ltd., Malvern, and Worcestershire, UK). The analysis was performed using a scattering angle of 173°. Each determination was conducted in duplicate.

Atomic Force Microscopy (AFM)

Protein samples (1 mg/mL) were prepared by the method of Maruyama et al. [13]. The samples (pH 7.6 and $\mu = 0.08$) were heated at 90 °C for 5 min. After heating, the dispersions were passed through a membrane filter (0.22 μm) for AFM analysis. The images of the formed aggregates were obtained according to the method of Mills et al. [18], with a few modifications. The heated solutions were diluted to a final concentration of 50 μg/mL of protein using the same buffer. A drop of sample solution (5 μL) was deposited onto freshly cleaved mica and allowed to dry in air for 10 min at 20 °C. The samples were then placed into the cell of an atomic force microscope (AFM) of Multimode SPM with a controller of Nanoscope IIIa (Veeco Instruments, New York, USA). The AFM was equipped with a piezoelectric scanner with an xy range of up to 60 μm and oxide-sharpened V-shaped Si3N4 100 Wm long cantilevers (Digital Instruments, CA, USA) with a quoted spring constant of 0.38 N/m. Images were obtained typically at a normal force of <1 nN with a scan frequency of 1.0 Hz. Image analysis was performed using NIH Image (National Institutes of Health, USA) and ImagePro (Media Cybernetics, USA). Data were analyzed and combined for several images collected under a given set of experimental conditions.

Results and Discussion

Isolation and Purity Analysis of Soy β-Conglycinin Subunits

As expected, the β-conglycinin was mainly composed of three constituent subunits (β, α and α') as shown in Fig. 1a (lane 1). The purity of this β-conglycinin was above 95%, as estimated by the densitometric scanning technique (Data not shown). The presence of 6.0 M urea resulted in the dissociation of the β-conglycinin into its monomers. Figure 1b shows the ion-exchange chromatographic profile of β-conglycinin on DEAE-Sepharose fast flow column in the presence of 6.0 M urea, using stepwise NaCl solution as the elution solvent. In this profile, five major peaks (denoted as 1–5, respectively) were observed. The protein constituents of these five peaks were analyzed by SDS-PAGE, as also included in Fig. 1a. The first elution peak (peak 1, lanes 2–3) was a mixture of several un-adsorbed components such as small basic polypeptides, some aggregates, and some minor un-identified proteins, while peak 2(lanes 4–10) was mainly attributed to β subunit. A

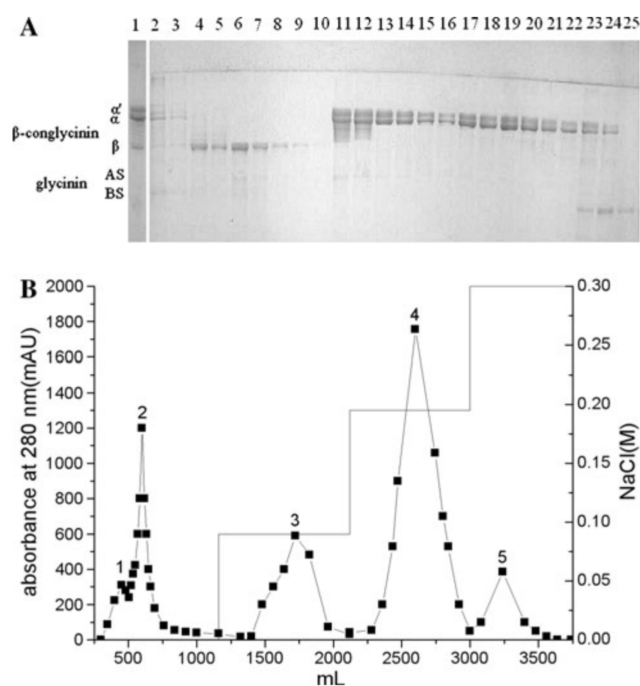

Fig. 1 SDS-PAGE analyses of β-conglycinin and fractions obtained by DEAE-chromatography of β-conglycinin (**a**) plus DEAE-chromatography of β-conglycinin (**b**). In **a** *lane 1* is β-conglycinin, *lanes 2–3*, peak **1**; *lanes 4–10*, peak **2**; *lanes 11–12*, peak **3**; *lanes 13–22*, peak **4** and *lanes 23–25*, peak **5** obtained by DEAE-chromatography (**b**)

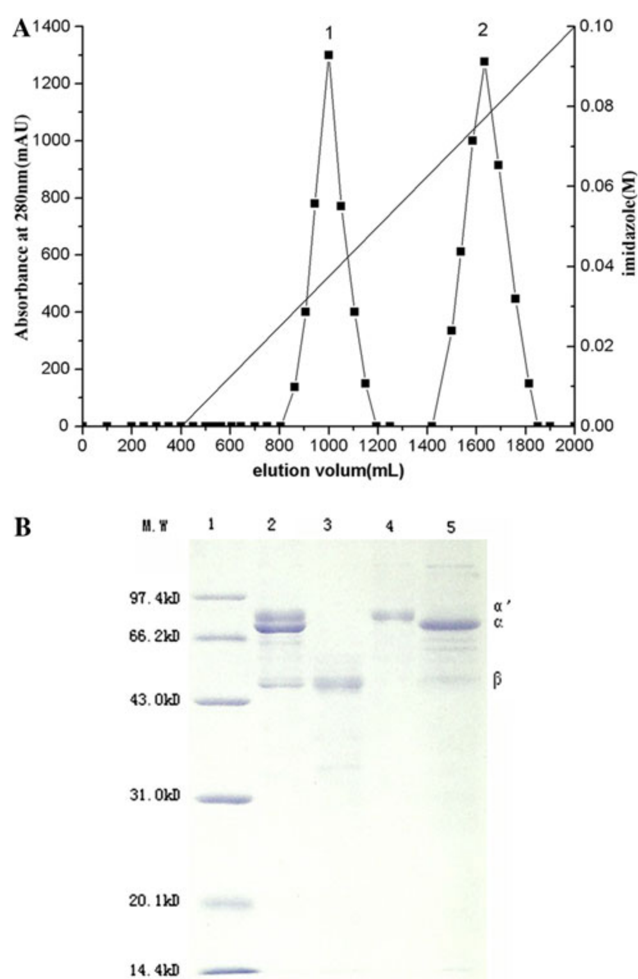

Fig. 2 IMAC elution profile of mixture of α and α' (**a**). Reducing SDS-PAGE profile for isolated subunits of β-conglycinin (**b**). In **b** *lane 1*, protein markers; *lane 2*, β-conglycinin; *lane 3*, β; *lane 4*, α'; *lane 5*, α

fraction rich in much contaminants (lane 11–12) mainly accounted for peak 3.The peak 4 (lanes 13–22) was composed of a mixture of α and α' subunits, while peak 5 (lanes 23–25) was a mixture of α and α' subunits contaminated by basic polypeptides of glycinin. The protein fractions of peak 2 were collected as the β subunit.

The protein fractions of peak 4 were collected for the further isolation of α- and α'-subunits by IMAC (Fig. 2a). In this profile, two peaks (denoted as 1 and 2) resulted from the linear elution of standard buffer containing 0.1 M imidazole. The first peak corresponded to the purified α subunit, while peak 2 was mainly attributed to α'-subunits (Fig. 2b).

Figure 2b shows all three purified subunits of β-conglycinin by SDS-PAGE under reducing condition. Their molecular weights (MWs) were calculated to be about 50.0, 67.0 and 71.0 kDa, respectively. The data are in good agreement with previous literature [11, 12]. Estimated by the densitometric scanning technique, the purity of individual polypeptides was 91.4, 95.0 and 92.1%, for β, α and α', respectively. The overall recovery of total protein fractions was almost 70% of the loaded sample (5 g). This recovery is slightly lower than that (around 80%) of previous work [11, 12], where the loaded amount of the sample was only 0.5–1.5 g. Clearly, the difference in the recovery could be attributed to the difference in loaded amount of the sample and the methods adopted, e.g. some minor contaminants were discarded in order to obtain high purity of subunits in the present process.

Overall, the predominant features of our isolation method are as follows: first, much larger samples can be loaded onto the DEAE-Sepharose Fast Flow column and then purified β-subunit and a mixture of α, α' with high purity can be obtained; second, Cu^{2+} in the IMAC column can absorb much more sample than Zn^{2+} and $Ni^{2+}$and the linear elution of imidazole makes the isolation of α and α' more controllable and stable.

Physicochemical Characterization of Isolated Subunits

Zeta Potential Analysis

Figure 3 shows the zeta potential profiles of individual isolated subunits (β, α and α') as a function of pH. As

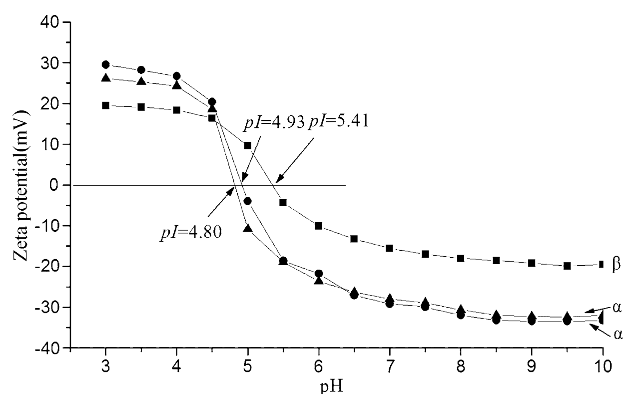

Fig. 3 Zeta potential profiles as a function of pH of three isolated subunits (β, α and α') of β-conglycinin

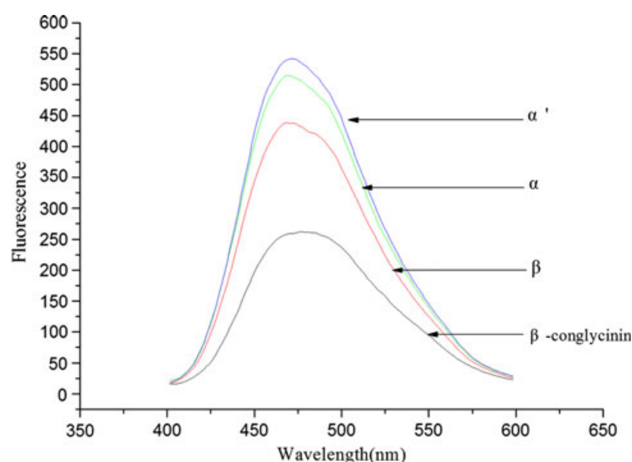

Fig. 4 Emission fluorescence spectra of ANS in the presence of β-conglycinin and its isolated subunits (α', α and β)

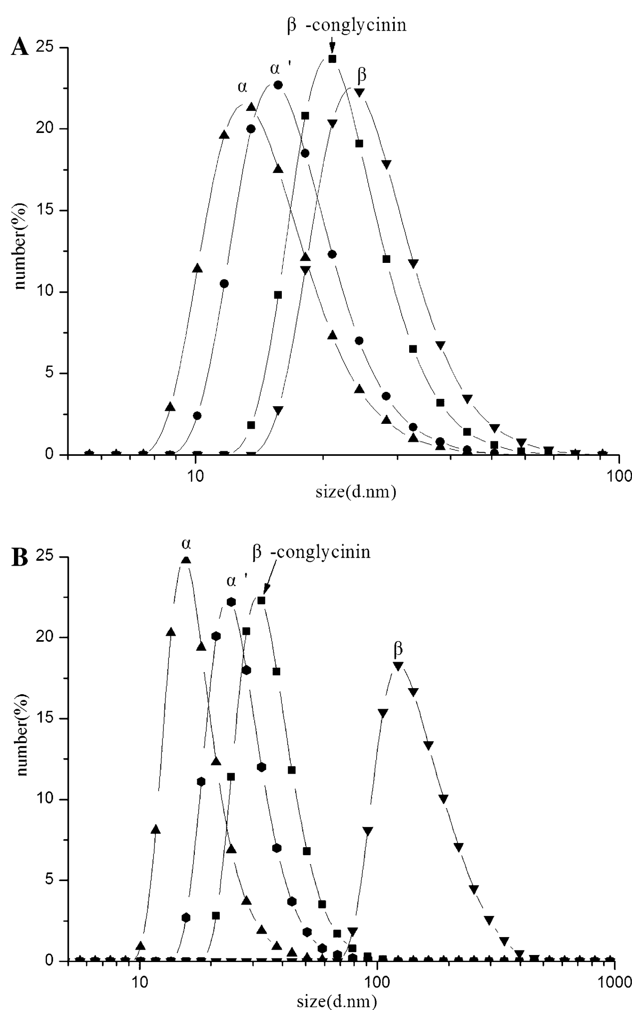

Fig. 5 Size distribution of the heat-induced aggregates of β-conglycinin and its isolated subunits, formed at 90 °C for 5 min at pH 7.6 at ionic strengths of 0.08 (**a**) and 0.5 (**b**)

expected, the zeta potential of all samples gradually increased from a negative value to a positive value, when the pH was decreased from 10.0 to 3.0. This reflects that the interactive force pattern between the proteins is gradually changed from negative electrostatic repulsion to positive electrostatic repulsion. At lower pH, the carboxyl and amino groups would gradually become protonated. The amplitude of the zeta potential change for α and α' subunits was much larger than that of β subunit (Fig. 3). Additionally, the isoelectric point (p*I* 5.4; at which zeta potential is zero) of β subunit was much higher than that (4.8 and 4.9) of α' and α subunits. These values are similar to those reported by Thanh and Shibasaki [11]. The difference between α' (or α) and β subunit is consistent with the fact that the acidic amino acid content (aspartic acid/asparagines and glutamic acid/glutamine) in α' and α subunits is much higher than that of β subunit. These amino acids can impart acidic characteristics to the proteins [19].

Fluorescence Spectroscopic Analysis

Figure 4 shows the extrinsic fluorescence spectra of ANS in the presence of β-conglycinin and its isolated subunits. At a constant pH (e.g. pH 7.0), ANS usually binds to exposed hydrophobic surfaces in partially unfolded proteins with much higher affinity than to native or completely unfolded proteins, resulting in an increase in fluorescence emission compared with the emission of free ANS in aqueous solution [20]. The fluorescence intensity of all individual subunits was much higher than that of β-conglycinin (Fig. 4), indicating a higher extent of hydrophobic surfaces exposed to ANS. The application of 6.0 M urea during the isolation process may account for this phenomenon, since 6.0 M urea would result in partial or even complete structural unfolding of the proteins.

The fluorescence intensity for the α' and α subunits was higher than that for β subunit, which is in accordance with

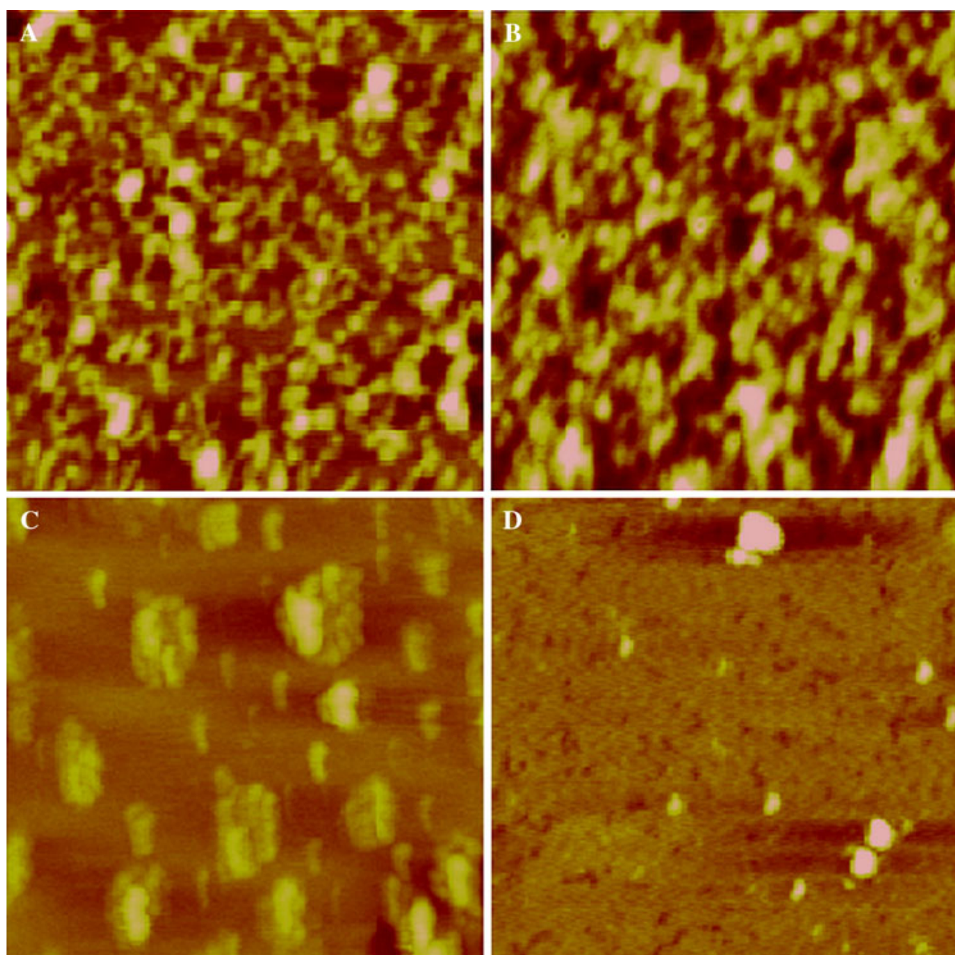

Fig. 6 AFM images of the heat-induced aggregates of β-conglycinin and its isolated subunits. **a** β-conglycinin; **b** α'; **c** α; **d** β. Samples were heated at 90 °C for 5 min, $\mu = 0.08$, 0.1% (w/v) protein. Scan sizes: 1.0×1.0 μm

the data of homogenous trimers of these subunits, based on the mutant lines [13]. This seems to be contradictory to the fact that β subunit exhibits a much higher hydrophobic amino acid content than α' and α subunits. A reasonable explanation is that the β subunit with higher hydrophobic amino acid content, exhibits higher refolding ability to rebuild its tertiary structure, after the removal of 6.0 M urea, than α' and α subunits.

Heat-Induced Aggregation

DLS Analysis

The thermal aggregation of β-conglycinin and its isolated subunits was investigated using dynamic light scattering technique, by heating (90 °C, 5 min) at pH 7.6, $\mu = 0.08$ and 0.5. The particle size distribution of heat-induced aggregates is shown in Fig. 5. The size distribution of the formed aggregates varied considerably with the type of the subunits and the μ. At a μ of 0.08, the size distribution of the formed aggregates of α' and α subunits was similar, but much less than that of β subunit (Fig. 5a). Similar phenomena have been observed by Maruyama et al. [13], who found that β subunit is liable to form insoluble aggregates, or macro-aggregates. The difference can be attributed to the extensive regions of α and α' subunits which would play an important role in prevention of thermal aggregation [9]. As expected, the size distribution of the formed aggregates of β-conglycinin was intermediate.

The heat-induced aggregation of α and α' subunits, as well as β-conglycinin was slightly accelerated when the μ increased from 0.08 to 0.5, while the extent of aggregation of β subunit was remarkably increased (Fig. 5b). The difference in extent of aggregation between α (or α') and β subunits may also be related to the difference in their polypeptide structure. The order in extent of aggregation of individual subunits at neutral pH and ionic strength of 0.5 is consistent with the data reported by Maruyama et al. [13], using size exclusion chromatography combined with multi-angle laser light scattering technique. The screening effect of the electrostatic interaction at higher μ clearly

accounts for the increase in extent of heat-induced aggregation [21].

AFM Analysis

The shape and nature of the heat-induced aggregates of β-conglycinin and its isolated subunits, at μ of 0.08 were further investigated by AFM, as shown in Fig. 6. The formed aggregates of different samples exhibited different morphologic patterns. In the β-conglycinin case (Fig. 6a), much more ordered and stranded aggregates were formed than in the cases of individual subunits. Mills et al. [18] also observed similar stranded aggregates of β-conglycinin, when heated at the same condition. The morphologic pattern for the aggregates of α' subunit (Fig. 6b) was similar to that of β-conglycinin, though the aggregates in the former case were more unordered and worm-like. In the α subunit case (Fig. 6c), the morphology of the formed aggregates was bud-like (although the mass resulted from the uneven dispersal of the sample during its preparation can also be seen), while in the case of β subunit (Fig. 6d), there were no ordered aggregates observed. In the latter case, few aggregates were observed, due to the formation of insoluble precipitates which were removed by centrifugation during sample preparation. The phenomena seem to be consistent with previous reports that α' and α subunits form translucent gels while β subunit is associated with opaque gel [22]. By combination with the DLS data, it thus is suggested that the aggregation behavior of β-conglycinin subunits may be closely related to its physicochemical and structural properties. In the cases of α and α' subunits, the presence of the extension regions with acidic characteristic is favorable for the formation of the ordered and stranded aggregates.

Conclusions

The improved isolation method of the β-conglycinin constituent subunits will promote investigations based on these subunits. The results of the relationship between the physicochemical properties and heat-induced aggregation of individual subunits of β-conglycinin will aid us to study the aggregation behavior of soy protein. These isolated subunits exhibit to a variable extent inhibition of thermal aggregation of some heat-labile proteins, which is our next investigation.

Acknowledgments This work is one of the research projects of the Chinese National Natural Science Fund (20776050), sponsored by the NNSF of China. This research project was also financed by Natural Science Foundation of Guangdong Province (7006508) and by Research Fund for the Doctoral Program of Higher Education of China (20070561059).

References

1. Utsumi S (1992) Plant food protein engineering. In: Kinsella JE (ed) Advances in food and nutrition research. Academic Press, San Diego, CA, pp 89–208
2. Utsumi S, Matsumura Y, Mori T (1997) Structure-function relationships of soy proteins. In: Damodaran S, Paraf A (eds) Food proteins and their applications. Marcel Dekker, New York, pp 257–291
3. Lovati MR, Manzoni C, Gianazza E, Sirtori CR (1998) Soybean protein products as regulators of liver low-density lipoprotein receptors. I. Identification of active β-conglycinin subunits. J Agric Food Chem 46:2474–2480
4. Manzoni C, Lovati MR, Gianazza E, Sirtori CR, Morita Y (1998) Soybean protein products as regulators of liver low-density lipoprotein receptors. II. α-α' rich commercial soy concentrate and α' deficient mutant differently affect low-density lipoprotein receptor activation. J Agric Food Chem 46:2481–2484
5. Manzoni C, Duranti M, Eberini I, Scharnagl H, Maerz W, Castiglioni S, Lovati MR (2003) Subcellular localization of soybean 7S globulin in HepG2 cells and LDL receptor up-regulation by its constituent subunit. J Nutr 133:2149–2155
6. Krishnan HB, Kim WS, Jang S, Keller MS (2009) All three subunits of soybean β-conglycinin are potential food allergens. J Agric Food Chem 57:938–943
7. Maruyama N, Katsube T, Wada Y, Oh MH, Barba dela Rosa AP, Okuda E, Nakagawa S, Utsumi S (1998) The Roles of the N-Linked glycans and extension regions of soybean β-conglycinin in folding, assembly and structural features. Eur J Biochem 258:854–862
8. Maruyama N, Sato R, Wada Y, Matsumura Y, Goto H, Okuda E, Nakagawa, Utsumi S (1999) Structure-Physicochemical function relationships of soybean β-conglycinin constituent subunits. J Agric Food Chem 47:5278–5284
9. Maruyama N, Salleh MRM, Takahashi K, Yagasaki K, Goto H, Hontani N, Nakagawa S, Utsumi S (2002) Structure-physicochemical function relationships of soybean β-conglycinin heterotrimers. J Agric Food Chem 50:4323–4326
10. Duranti M, Lovati MR, Dani V, Barbiroli A, Scarafoni A, Castiglioni S (2004) The α' subunit from soybean 7S globulin lowers plasma lipids and up-regulates liver β-VLDL receptors in rats fed a hypercholesterolemic diet. J Nutr 252:1334–1339
11. Thanh VH, Shibasaki K (1977) β-Conglycinin from soybeans. Isolation and immunological and physicochemical properties of the monomeric forms. Biochim Biophys Acta 490:370–384
12. Zheng HG, Yang XQ, Ahmad I, Min W, Zhu JH, Yuan DB (2009) Soybean β-conglycinin constituent subunits: Isolation, solubility and amino acid composition. Food Res Int 42:998–1003
13. Maruyama N, Salleh MRM, Takahashi K, Yagasaki K, Goto H, Hontani N, Nakagawa S, Utsumi S (2002) The effect of the N-Linked glycans on structural features and physicochemical functions of soybean β-conglycinin homotrimers. J Am Oil Chem Soc 79:139–144
14. Salleh MRM, Maruyama N, Takahashi K, Yagasaki K, Higasa T, Matsumura Y, Utsumi S (2004) Gelling properties of soybean β-conglycinin having different subunit compositions. Biosci Biotechnol Biochem 68:1091–1096
15. Nagano T, Hirotsuka M, Mori H, Kohyama K, Nishinarit K (1992) Dynamic viscoelastic study on the gelation of 7S globulin from soybeans. J Agric Food Chem 40:941–944
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin-phenol reagent. J Biol Chem 193:265–275
17. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227:680–685

18. Mills ENC, Huang L, Noel TR, Gunning P, Morris VJ (2001) Formation of thermally induced aggregates of the soya globulin β-conglycinin. Biochim Biophys Acta 1547:339–350
19. Carbonaro M, Cappelloni M, Nicoli S, Lucarini M, Carnovale E (1997) Solubility-digestibility relationship of legume proteins. J Agric Food Chem 45:3387–3394
20. Pallarès I, Vendrell J, Avilès FX, Ventura S (2004) Amyloid fibril formation by a partially structured intermediate state of a-chymotrypsin. J Mol Biol 342:321–331
21. Langton M, Hermansson AM (1992) Fine-stranded and particulate gels of β-lactoglobulin and whey protein at varying pH. Food Hydrocolloid 5:523–539
22. Dong JG, Zhang SR, RAO AG (2007) Modified soy proteins and methods of use. US patent 071235