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Effects of Microfluidization Treatment and Transglutaminase Cross-Linking on Physicochemical, Functional, and Conformational Properties of Peanut Protein Isolate

Xiao Hu, Mouming Zhao, Weizheng Sun, Guangli Zhao, and Jiaoyan Ren*

College of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510640, Guangdong, China

ABSTRACT: Peanut protein isolate (PPI) was treated by high-pressure microfluidization (40, 80, 120, and 160 MPa) and/or transglutaminase (TGase) cross-linking. It was found that individual microfluidization at 120 MPa was more effective in improving the solubility, emulsifying properties, and surface hydrophobicity of PPI than at other pressures (e.g., 40, 80, or 160 MPa). Individual TGase cross-linking also effectively changed the physicochemical and functional properties of PPI. Microfluidization (120 MPa) or TGase cross-linking caused the unfolding of PPI structure, resulting in the decrease of α-helix and β-turns levels and the increase of β-sheet and random coil levels, as proved by Fourier transform infrared (FTIR) and circular dichroism (CD) spectra. Compared with individual treatments, microfluidization followed by TGase cross-linking significantly ($p < 0.05$) improved the emulsion stability during long-term storage (20 days). Moreover, the combined treatments led to looser structure of PPI and resulted in more obvious changes in physicochemical properties.

KEYWORDS: peanut protein isolate, microfluidization, transglutaminase cross-linking, emulsifying properties, secondary structure

INTRODUCTION

Peanuts (*Arachis hypogaea* L.) are one of the most important oilseed crops in the world, and most peanuts are used for extraction of edible oil. Although the remaining defatted peanut meal, a protein-rich and inexpensive byproduct from the oil extraction, is largely underutilized as an animal feed, this meal is still an interesting source of vegetable proteins that can be a good alternative in food formulation. Therefore, improving the functional properties of peanut protein from the defatted meal would be crucial to increase the use of this protein resource by the food industry.

Microfluidization, a promising new technological approach, uses the combined forces of ultrahigh pressure, high-velocity impact, high-frequency vibration, instantaneous pressure drop, intense shear, and cavitation.[1,2] Thus, it differs from high hydrostatic pressure and conventional valve homogenization.[3] High-pressure microfluidization has been applied to the preparation of nanoemulsions[4] and enhancement of the relative activity of polyphenol oxidase,[2] etc. The use of microfluidization to modulate properties of whey proteins was reported in two recent publications.[5,6] Microfluidization has a variety advantages including no exogenous chemicals, low treatment temperature, little nutritional component loss, and very short processing time. However, limited work has been done for using microfluidization to improve the functional properties of peanut proteins.

Transglutaminase (TGase; EC 2.3.2.13) is an enzyme capable of catalyzing acyl-transfer reactions between the γ-carboxyamide of peptides or protein-bound glutamine residues (acyl donors) and various primary amines (acyl acceptors). When the ε-amino groups of lysine residues act as acyl acceptors, ε-(γ-glutamyl)lysine isopeptide bonds are formed, resulting in both intra- and intermolecular cross-linking.[7] TGase has been used to modify the functional properties of many food proteins in seafood, meat, dairy, and so on.[8]

Generally, vegetable proteins have compact quaternary and tertiary structures and have resistance to proteolysis. So far, many attempts have been made to unfold or disrupt the globular protein structure by physical pretreatments to improve their accessibility to proteases.[9] Chen et al.[10] found that extrusion pretreatment caused a marked improvement in the accessibility of soybean protein isolate to enzymatic hydrolysis and resulted in improved solubility and emulsifying properties. Microfluidization treatment has been proven to be able to expose the inner group of peanut protein and lead to the change of physicochemical properties and protein structure,[11] which might also have the potential to alter the accessibility of peanut protein isolate (PPI) to TGase. Little work has been done so far to investigate this possibility. In the present work, the effects of microfluidization and/or TGase cross-linking on the physicochemical (free sulfhydryl contents, surface hydrophobicity), functional (solubility, emulsifying properties), and conformational properties (secondary structure) of PPI were studied.

MATERIALS AND METHODS

Materials. Low temperature defatted peanut flour was purchased from Tianshen Bioprotein Co., Ltd. (Linyi, China). TGase (1000 units/g) was donated by Yiming Biological Products Co., Ltd. (Taixing, China). *o*-Phthaldialdehyde (OPA) and 1,8-anilinonaphthalenesulfonate (ANS) were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was obtained from Huamei Biotechnology Co., Ltd. (Luoyang, China). Low molecular weight protein markers were purchased from Dinguo Biotechnology Co., Ltd. (Shanghai, China). Corn oil was obtained from a local supermarket and used directly without

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further purification. All other chemicals used in the present study were at least of analytical grade.

Preparation of Various Peanut Protein Isolate (PPI) Products. PPI was obtained from the low-temperature defatted peanut flour according to the process described in a previous paper.[12] Briefly, the flour was fully dispersed in a 10-fold volume of deionized water for 2 h at room temperature, and the pH of the dispersion was adjusted to 8.0 with 1 M NaOH. The resultant dispersion was centrifuged in a CR22G high-speed centrifuge (Hitachi Co., Tokyo, Japan) at 8000*g* for 30 min at 20 °C to remove the insoluble material. The supernatant was collected and adjusted to pH 4.5 with 1 M HCl to precipitate the proteins. The precipitate was obtained by centrifugation at 8000*g* for 10 min at 20 °C and redispersed in deionized water. Finally, the dispersion was adjusted to pH 7.0 and freeze-dried using an Alpha 1-4 LD plus freeze-dryer (Marin Christ, Osterode, Germany) to obtain the PPI.

Microfluidization-treated PPI (MT-PPI) was obtained by dispersing the PPI in deionized water (containing 2% protein, w/w). The dispersion was adjusted to pH 7.0, microfluidized using an M-110EH microfluidizer (Microfluidics Co., Newton, MA) operating at 40, 80, 120, or 160 MPa (one pass), respectively, and then, freeze-dried and kept at room temperature until use.

Two samples, PPI cross-linked by TGase (TC-PPI) and PPI treated by combined microfluidization and TGase cross-linking (MTCTC-PPI), were prepared according to the method of Tang et al.[13] with slight modifications. The PPI and MT-PPI were respectively dispersed in deionized water (containing 2% protein, w/w), and the pH was adjusted to 7.0. The dispersions were preincubated at 37 °C in a water bath. Then TGase was added at an enzyme to protein substrates ratio (E/S ratio) of 0.5:100 (w/w) and incubated at 37 °C for 90 min. The reactions were stopped by immediately cooling to 4 °C in an ice water bath and then freeze-dried. As for the preparation of samples for SDS-PAGE analysis, the reactions were stopped by directly mixing with the sample buffer.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE samples were performed according to the method of Laemmli[14] with slight modifications. A 12% acrylamide separating gel and a 5% acrylamide stacking gel containing 0.1% SDS were used in a DYCZ-30 electrophoresis apparatus (Liuyi Instrument Factory, Beijing, China). The samples were mixed with 0.06 M Tris-HCl buffer (pH 8.8), containing 2% SDS, 5% 2-mercaptoethanol, 25% glycerol, and 0.1% bromophenol blue. The solutions were then heated for 5 min in boiling water and centrifuged at 10000*g* for 3 min before electrophoresis. Fifteen microliters of each sample was loaded onto the gel. A cocktail protein standard containing rabbit phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43.0 kDa), bovine carbonic anhydrase (31.0 kDa), and trypsin inhibitor (20.1 kDa) was also run. Last, the electrophoresis gel were stained with 0.25% Coomassie Brilliant Blue-R250 in 50% trichloroacetic acid and then destained in methanol/acetic acid/water (1:1:8, v/v/v).

High-Performance Size Exclusion Chromatography (HPSEC). The HPSEC experiment was performed using a Waters 600 HPLC system (Waters, Milford, MA) fitted with a TSK-GEL G4000SW column (0.75 × 60 cm, Tokyo, Japan). The column was eluted with 50 mM phosphate buffer (pH 7.2) containing 0.2 M NaCl. Each sample was mixed with 50 mM phosphate buffer (pH 7.2) containing 0.2 M NaCl to obtain the protein solutions (1%, w/v). All protein solutions were filtered through 0.45 μm membranes (Millipore, Billerica, MA) before use. A 20 μL aliquot of protein solution was loaded on the column and eluted at a rate of 1 mL/min. The UV absorbance at 280 nm was used to monitor protein elution.

Determination of Free Amino Groups. Free amino groups in samples were quantified spectrophotometrically using a modified OPA method.[15] The OPA reagent was prepared by mixing 40 mg of OPA (dissolved in 1 mL of methanol), 2.5 mL of 20% (w/w) sodium dodecyl sulfate (SDS), 25 mL of 0.1 M sodium tetraborate, and 100 μL of 2-mercaptoethanol and then diluting to a final volume of 50 mL with deionized water. Two hundred microliters of sample solution (0.4%, w/v) was incubated with 4 mL of OPA reagent at 35 °C in a water bath for 2 min. The absorbance at 340 nm was measured by a UV-2300 spectrophotometer (Tianmei Co., Shanghai, China).

Total and Exposed Free Sulfhydryl (SH) Contents. Free SH contents of protein samples were determined according to the method of Ellman,[16] as modified by Beveridge et al.,[17] with some further modifications. Ellman's reagent was prepared by dissolving 4 mg of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) reagent in 1 mL of Tris−glycine buffer (0.086 M Tris, 0.09 M glycine, and 0.004 M EDTA, pH 8.0). Fifteen milligrams protein samples were suspended in 5.0 mL of Tris−glycine buffer with (total sulfhydryl) or without 8 M urea (exposed sulfhydryl). Then, 50 μL of Ellman's reagent was added. The resultant suspensions were incubated for 1 h at room temperature, with occasional shaking. The absorbance of the supernatant was determined at 412 nm against the reagent buffer as the blank. The SH contents were calculated as follows: μM SH/g = 73.53 × A_{412}/C, where A_{412} is the absorbance at 412 nm, C is the sample concentration (mg/mL), and 73.53 is derived from $10^6/(1.36 \times 10^4)$; 1.36×10^4 is the molar absorptivity,[16] and 10^6 is for the conversion from molar basis to μM/mL basis and from mg solids to g solids.

Surface Hydrophobicity (H_0). H_0 was determined using ANS as fluorescence probe in the absence of SDS according to the method of Haskard et al.[18] Protein dispersions were diluted (0.2, 0.1, 0.05, 0.02, 0.01, and 0.005%) in phosphate buffer (0.01 M, pH 7.0). ANS solution (8.0 mM) was also prepared in the same phosphate buffer. A 20 μL ANS solution was added to 4 mL of each dilution, and the fluorescence intensity (FI) was measured at 470 nm (emission) using an excitation at 390 nm by F7000 fluorescence spectrophotometer (Hitachi Co.). The initial slope of fluorescence intensity versus protein concentration plot was used as an index of H_0.

Protein Solubility (PS). Protein solubility was determined by dispersing the protein samples in deionized water to obtain the protein solution (1%, w/w). The pH value of the protein solution was adjusted to 7.0 with 0.5 M HCl or NaOH. After 30 min of stirring, the pH was readjusted if necessary. Then it was centrifuged at 8000*g* for 20 min at 20 °C in a CR22G centrifuge (Hitachi Co., Japan). The content of protein for the resulting solution was analyzed according to the Lowry method[19] using BSA as the standard. The PS was expressed as grams of soluble proteinp per 100 g of protein.

Determination of Particle Size. The oil/water (20:80, w/w) emulsions were prepared by adding the corn oil to the protein solutions (2%, w/w) with the aid of a mechanical stirrer (Shanghai Specimen Model Co., China), and the pH of the aqueous phase was adjusted to 7.0 with 1 M NaOH or HCl. The resulting crude emulsions were then homogenized using the APV-1000 homogenizer (APV Gaulin, Abvertslund, Denmark) to obtain aimed emulsions (30 MPa, two passes). Each emulsion sample was prepared in duplicate.

Particle size distribution of the emulsions was measured by the aid of a Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, U.K.). Measurements were carried out at room temperature, and the volume fraction of emulsion in the deionized water was approximately 1:1000. The mean particle size was characterized in terms of the surface area mean diameter d_{32} ($d_{32} = \Sigma n_i d_i^3/\Sigma n_i d_i^2$, where n_i is the number of particles with diameter d_i). Emulsion stability was evaluated by assessing changes in d_{32} values of the emulsions after storage (20 days) at room temperature.

Microstructure. The microstructure of the protein sample was determined according to the method of Luo et al.[20] The samples were deposited on silicon wafers and coated with a conductive material (gold) to ensure sufficient electron refraction. They were then mounted onto an aluminum stub with epoxy and coated with a gold platinum alloy in a

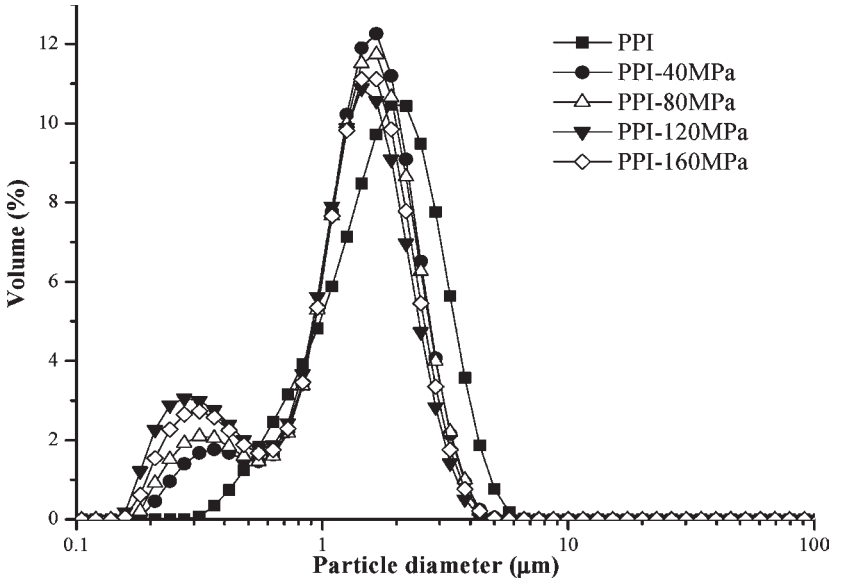

Figure 1. Particle size distribution of emulsions made with microfluidization-treated PPI samples.

sputter-coating device. The samples were observed with an S-3700N scanning electron microscope (Hitachi Co.) at an operating voltage of 10 kV.

Fourier Transform Infrared (FTIR) Spectra. FTIR spectra of protein samples were recorded using a Nicolet 8210E FTIR spectrometer (Nicolet, Madison, WI) equipped with a deuterated triglycine sulfate detector. The spectrometer was continuously purged with dry air from a Balston dryer (Balston, MA). One milligram of sample was mixed with 100 mg of KBr and ground gently with an agate pestle and mortar under an infrared lamp. The powder was pressed into a 13 mm diameter disk by applying 15 tons of pressure for 2 min. FTIR spectra were obtained in the wavenumber range from 400 to 4000 cm^{-1} during 128 scans, with 2 cm^{-1} resolution. A straight baseline passing through the ordinates at 1700 and 1600 cm^{-1} was adjusted as an additional parameter to obtain a best fit. The curve-fitting procedure was performed by stepwise iterative adjustment toward a minimum root-mean-square error of the different parameters determining the shape and position of the absorption peaks. It was carried out by assuming an initial mixed Gaussian lineshape function, with full width band at half-height (fwhh) of 10 cm^{-1} and a maximum resolution factor. Baseline corrections, normalization, derivation, and curve fitting were carried out by means of PeakFit version 4.12 software (SPSS Inc., Chicago, IL) and Origin 8.0 (OriginLab Corp., Northampton, MA) software.

Circular Dichroism (CD) Spectra. An MOS-450 CD spectrometer equipped with a Peltier element (Bio-Logic Science Instruments, Grenoble, France) was used for CD analysis. For steady state investigation, spectra were recorded at a protein concentration of 0.1 mg/mL using a 10 mm path length quartz cuvette at room temperature in the far-UV (190−250 nm) region. Secondary structure compositions of the samples were estimated from the far-UV CD spectra using the CONTIN/LL program in the CDPro software. Four secondary structures, α-helix, β-sheet, β-turns, and random coil, were calculated.

Statistical Analysis. The data of the experiments were subjected to one-way analysis of variance (ANOVA) using SPSS version 13.0 for Windows (SPSS Inc.). Significant differences between means ($p < 0.05$) were identified by the least significant difference (LSD) test.

RESULTS AND DISCUSSION

Microfluidization Treatment. The effects of different microfluidization treatments (40, 80, 120, and 160 MPa) on the functional properties and surface hydrophobicity of PPI are shown in Figure 1 and Table 1. Solubility is one of the most important characteristics of proteins because it can influence other functional properties. The solubility of PPI samples increased with increasing pressure from 40 to 120 MPa (Table 1), whereas further increase in pressure (from 120 to 160 MPa) caused a slight decrease of PPI solubility.

Table 1. Protein Solubility (PS), Surface Area Mean Diameter (d_{32}), and Surface Hydrophobicity (H_0) of Samples[a]

protein sample	PS[b] (%)	d_{32} (μm)	H_0
PPI	69.2 ± 0.8 a	1.714 ± 0.007 e	6302.8 ± 26.7 a
PPI-40 MPa	71.9 ± 0.5 b	1.154 ± 0.010 d	6951.8 ± 63.0 b
PPI-80 MPa	76.0 ± 1.1 c	1.003 ± 0.008 c	7130.1 ± 86.3 c
PPI-120 MPa	80.1 ± 0.9 d	0.830 ± 0.011 a	7449.6 ± 69.7 d
PPI-160 MPa	78.4 ± 0.6 d	0.898 ± 0.007 b	7240.3 ± 78.5 c

[a] Results having different letters in one column are significantly different ($p < 0.05$). [b] PS was determined at pH 7.0.

The particle size distributions of the fresh emulsions made with samples are shown in Figure 1. A transition from the monomodal particle size distribution to the bimodal particle size distribution was observed after PPI was treated with microfluidization. The volume of small particles ($d < 1$ μm) in PPI emulsion was 14.2%, and it was obviously increased to 23.58, 26.1, and 35.2%, respectively, for PPI samples pretreated by microfluidization at 40, 80, and 120 MPa. However, a small decrease (from 35.2 to 31.1%) was found when microfluidization treatment pressure was further increased to 160 MPa.

The mean particle size d_{32} of the emulsions is shown in Table 1. The d_{32} value is considered to be related to emulsifying activity, and changes in d_{32} value during the storage could indicate the emulsion stability.[12] In comparison with PPI emulsion, microfluidization treatment led to a significant ($p < 0.05$) decrease in the d_{32} of the emulsions, indicating improved emulsifying activity and emulsion stability. Among all samples, the emulsion with PPI pretreated by 120 MPa microfluidization exhibited the lowest d_{32}, suggesting that the operating pressure at

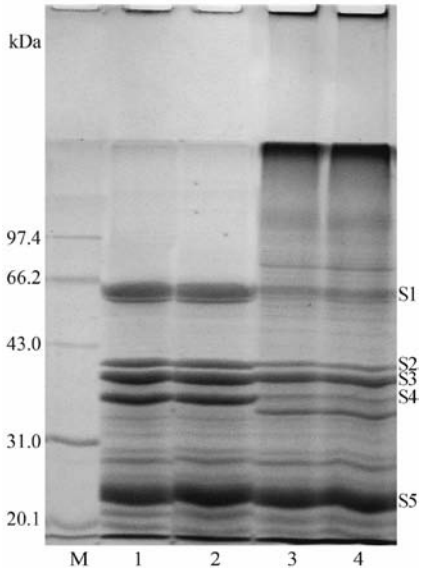

Figure 2. SDS-PAGE profile of protein samples. Lanes: M, molecular markers; 1, peanut protein isolate (PPI); 2, microfluidization (120 MPa)-treated PPI (MT-PPI); 3, TGase cross-linked PPI (TC-PPI); 4, PPI treated with combined treatments of microfluidization and TGase cross-linking (MTCTC-PPI).

120 MPa was more effective in obtaining better emulsifying properties of PPI compared with 40, 80, or 160 MPa treatment.

The surface hydrophobicity (H_0) values of protein samples, as determined using ANS emission fluorescence spectra at neutral pH, are shown in Table 1. It was found that microfluidization treatment at 40, 80, 120, or 160 MPa significantly ($p < 0.05$) increased the H_0 of PPI samples, which could be due to the exposure of hydrophobic groups initially buried in the interior of protein molecules. Basically, the H_0 increased with the increase of operating pressure from 40 to120 MPa, but higher treatment at 160 MPa caused the decrease H_0. The reason for the decrease is not clear, but the reaggregation of the exposed hydrophobic groups under much higher pressure might be one of the causes of the decrease.

Based on the above results, it could be concluded that microfluidization treatment at 120 MPa was more effective to improve the solubility, emulsifying properties and surface hydrophobicity of PPI than other treatments (e.g., 40, 80, or 160 MPa). Thus, 120 MPa was selected as a preferable condition for the microfluidization treatment of PPI, and the corresponding sample (as abbreviated to MT-PPI) was subjected to physicochemical, functional, and conformational properties study.

SDS-PAGE Assay. SDS-PAGE was performed to explore the subunit changes in samples by different treatments, as shown in Figure 2. It was found there were five major subunits in the untreated PPI (lane 1), which were referred to as S1−S5, respectively. Band S1 at around 64 kDa was identified as the major subunit of conarachin. Bands S2−S4 (approximately 42, 37, and 35 kDa, respectively) were the acidic subunits (AS) of arachin, and band S5 (around 22 kDa) was the basic subunit (BS) of arachin.[21,22] Comparison of PPI with MT-PPI (see lanes 1 and 2) revealed that the intensity of the subunits (S1−S5) in MT-PPI was similar with that in PPI, indicating that microfluidization treatment did not cause the dissociation of the PPI subunits. Similarly, Bouaouina et al.[23] found that under dynamic high-pressure treatment, whey proteins were not broken down into smaller entities.

After TGase cross-linking, new high molecular weight bands (>66.2 kDa) and an intense polymer band at the top of the stacking gel were observed in TC-PPI (lane 3), but there was no obviously observable change in the small-subunit bands (<31.0 kDa), showing that TGase treatment had no obvious effects on the basic subunit (S5 at around 22 kDa) of arachin. However, the band intensity of conarachin subunit S1 and arachin subunits S2−S4 (AS) in TC-PPI were much weaker than those in PPI. These results indicated that conarachin subunit S1 and acidic subunits of arachin (S2−S4) were better substrates for TGase enzyme, whereas the basic subunit of arachin (S5) was not sensitive to TGase.

For MTCTC-PPI (lane 4), TGase could also cross-link the microfluidization-treated PPI to form the high molecular weight bands, and the pattern of the subunit bands was similar to that of the TC-PPI (lane 3).

To sum up, microfluidization treatment has no influence on the subunits of PPI, whereas TGase treatment induced obvious changes of the conarachin subunit (S1) and acidic subunits of arachin (S2−S4). Besides, TGase could even work on PPI samples pretreated by microfluidization.

HPSEC Assay. HPSEC was performed to evaluate the changes in the protein samples in their native conformation (nondenaturing conditions, see Figure 3) that could not seen in the electrophoresis gel (Figure 2). In the profile of PPI, there were five major peaks, and these fractions were respectively referred to as P1−P5 according to the elution time. Compared with the profile of PPI, there was a slight decrease of the high molecular weight fraction P1 (approximately 440 kDa) in the profile of microfluidization-treated PPI (MT-PPI), suggesting that the high-pressure microfluidization induced a dissociation of large aggregates in PPI. For the profile of TC-PPI, a new fraction of high-molecular-weight polymer (>669 kDa) was formed after PPI was cross-linked by TGase. It was also observed that fractions P1 and P3 in PPI were both increased after TGase treatment, whereas the low molecular weight fractions P4 and P5 (<75 kDa) were slightly affected by TGase. Compared with the TC-PPI, more high molecular weight polymers with elution in shorter time were formed in MTCTC-PPI, and the peak area of low molecular weight fractions P4 and P5 in MTCTC-PPI was obviously larger than that in the TC-PPI. This might be due to the microfluidization treatment caused by the macromolecule dissociation, denaturation, and unfolded molecular structure in PPI, which could facilitate the cross-linking of the resultant by TGase to conjugate the low molecular weight components and form the more and smaller polymers than that in TC-PPI.

Free Amino Groups. TGase could induce the reaction between the ε-amino group on protein-bound lysine residues and the γ-carboxyamide group on protein-bound glutamine residues, leading to covalent cross-linking of the proteins. Owing to the involvement of the amino groups in the cross-linking reaction, a decrease in the number of these groups indicates the occurrence of TGase-catalyzed conjugation reaction. The number of free amino groups in protein samples could be indicated by the value of absorbance at 340 nm,[24] which was obtained by the OPA method. By comparison of PPI with MT-PPI (Table 2), the individual microfluidization treatment could not significantly ($p > 0.05$) change the number of the free amino groups in PPI. However, TGase cross-linking caused a significant ($p < 0.05$) decrease of the free amino groups, which was consistent with the previous reports that TGase treatment decreased the number of the free amino groups in soybean and cereal proteins.[25,26] The above result was also consistent with the

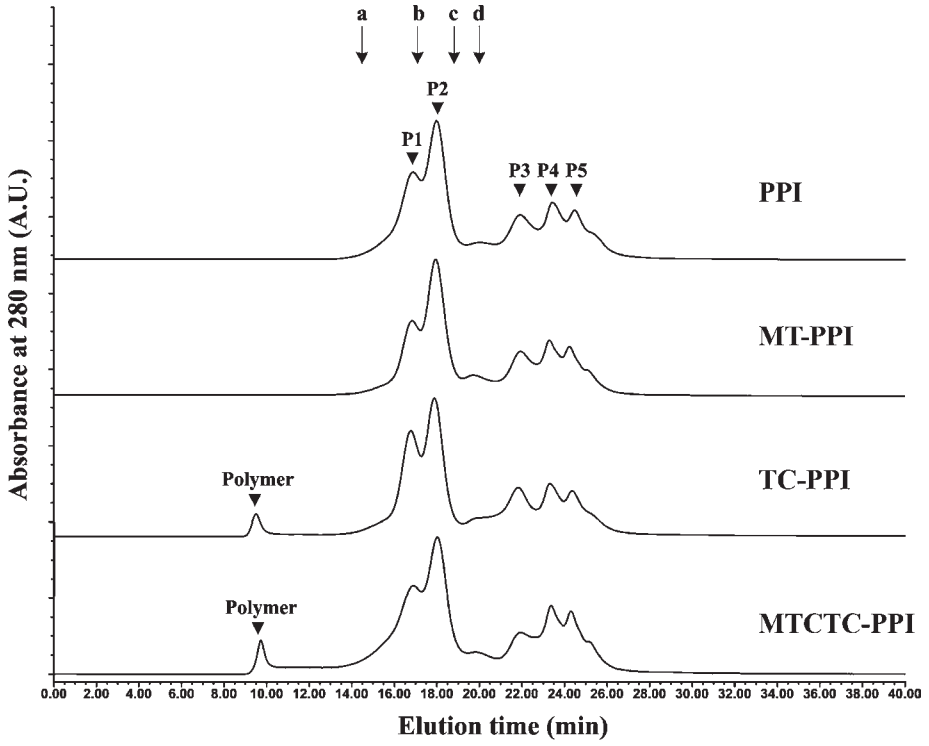

Figure 3. HPSEC profile of protein samples. Arrows a−d indicate protein markers of 669, 440, 158, and 75 kDa, respectively.

Table 2. Free Amino Groups (As Presented by A_{340}), Free SH Contents (Total and Exposed), Surface Hydrophobicity (H_0), and Protein Solubility (PS) of Protein Samples[a]

		free SH contents (μM/g protein)			
protein sample[b]	A_{340}	total	exposed	H_0	PS[c] (%)
PPI	0.401 ± 0.011 c	4.08 ± 0.06 d	2.11 ± 0.04 a	6302.8 ± 26.7 a	69.2 ± 0.8 c
MT-PPI	0.405 ± 0.012 c	3.79 ± 0.08 c	2.26 ± 0.02 b	7449.6 ± 69.7 b	80.1 ± 0.9 d
TC-PPI	0.351 ± 0.008 b	3.38 ± 0.05 b	2.31 ± 0.06 b	7725.1 ± 64.5 c	55.8 ± 1.2 a
MTCTC-PPI	0.324 ± 0.005 a	3.20 ± 0.05 a	2.54 ± 0.05 c	7942.0 ± 72.9 d	66.1 ± 1.1 b

[a] Results having different letters in one column are significantly different ($p < 0.05$). [b] PPI, peanut protein isolate; MT-PPI, microfluidization (120 MPa)-treated PPI; TC-PPI, TGase cross-linked PPI; and MTCTC-PPI, PPI treated with combined treatments of microfluidization and TGase cross-linking. [c] PS was determined at pH 7.0.

analysis of SDS-PAGE and HPSEC, as shown in Figures 2 and 3. The number of free amino groups in MTCTC-PPI was significantly ($p < 0.05$) lower than that in TC-PPI, suggesting that the pretreatment of microfluidization could facilitate the cross-linking of PPI with TGase. This might be attributed to the fact that high-pressure microfluidization induced the exposure of the lysine and glutamine residues initially buried in the interior of protein molecules and, thus, facilitated the TGase cross-linking reaction.

Total and Exposed Free SH Contents. Table 2 shows free SH (total and exposed) content of the protein samples. For the untreated PPI, the total free SH content was 4.08 μM/g protein and the exposed free SH content was 2.11 μM/g protein, showing that more than half of total free SH groups in PPI were exposed. It was found that individual treatment of microfluidization or TGase cross-linking significantly ($p < 0.05$) decreased the total free SH content, and the combined treatments induced the largest extent of the decrease (from 4.08 to 3.20 μM/g protein). The decrease in total free SH content was clearly due to oxidation of free sulfhydryl groups to form disulfide bonds,[27] and most of the oxidized free sulfhydryl groups might be initially buried in the inner part of the protein molecules. Meanwhile, a significant increase in exposed free SH content was observed after PPI was treated with microfluidization, TGase cross-linking, and the combined treatments. The increase of exposed free SH content in treated samples suggested that unfolded molecular structures might be formed by the treatments and seem to be in accordance with the decrease of total free SH content due to the increased possibility of oxidation between the free SH groups.

Surface Hydrophobicity (H_0). The H_0 values of protein samples, as determined using ANS emission fluorescence spectra at neutral pH, are also shown in Table 2. A significant ($p < 0.05$) increase in H_0 value was observed after microfluidization treatment due to molecules unfolding and subsequent exposure of initially buried groups. The result was similar to the previous report that dynamic high-pressure treatment could induce the increase in H_0 value of whey protein.[23] The increased H_0 value might be due to high-pressure microfluidization causing the disruption of large protein aggregates to expose the buried

hydrophobic clusters. Changes in the protein structures caused by pressure treatment had been suggested to be partly due to the cleavage of hydrogen bonds and van der Waals forces.[28,29] After PPI was treated with TGase, a significant ($p < 0.05$) increase in H_0 value was also observed and the increase was significant in comparison to MT-PPI sample. This indicated that TGase cross-linking resulted in the unfolding of PPI structure, which was consistent with the previous reports that TGase induced the unfolded structures in vicilin-rich kidney protein isolate,[30] whey protein isolate, and total milk protein.[31] It was noteworthy that the H_0 value of MTCTC-PPI was significantly ($p < 0.05$) higher than those of MT-PPI and TC-PPI, suggesting that the treatment of high-pressure microfluidization followed by TGase cross-linking caused further unfolding of the protein molecular structures. H_0 values were consistent with the data of free SH content and jointly confirmed that the unfolding of protein structure was brought to different extents by different treatments.

Protein Solubility. Table 2 also gives the protein solubility of protein samples at pH 7.0. The solubility of MT-PPI was significantly ($p < 0.05$) higher than that of the other samples. The enhanced solubility of MT-PPI could be attributed to the disruption of tight or impacted structure of proteins and the transformation of insoluble aggregates into soluble ones by means of the combined forces of high pressure, high-velocity impact, high-frequency vibration, and cavitation in the microfluidization process. Similarly, Puppo et al.[32] also found that the increased solubility (pH 3.0) of soybean protein isolate was caused by the hydrostatic high-pressure treatment at 200 MPa. For TC-PPI, TGase cross-linking induced a significant ($p < 0.05$) decrease in the solubility of PPI. Tang et al.[30] also found that TGase led to a decrease in the solubility of vicilin-rich kidney protein isolate, which could be attributed to the changes in the conformation of proteins and the formation of high molecular weight insoluble biopolymers. The solubility of MTCTC-PPI was significantly ($p < 0.05$) higher than that of TC-PPI, suggesting that TGase cross-linking of the microfluidization-treated PPI would facilitate the formation of soluble aggregates. This implied that physicochemical properties as well as the solubility of the substrates were closely related to the solubility of the TGase cross-linked PPI products.

Emulsion Particle Size. The particle size distributions of the fresh emulsions made with samples are shown in Figure 4A. For the PPI and MT-PPI emulsions, a transition from the monomodal particle size distribution to the bimodal particle size distribution and an obvious increase in the volume of small particles ($d < 1\ \mu$m) from approximately 14.2 to 35.2% were both observed after PPI was treated with microfluidization. After PPI was treated with TGase, the particle size distribution of the emulsion had a slight shift to the large particle diameter. In terms of the MTCTC-PPI emulsion, a bimodal particle size distribution was observed, and the volume of small particles ($d < 1\ \mu$m) in this emulsion was approximately 27.8%, which was slightly less than that in MT-PPI emulsion but much more than that in PPI and TC-PPI emulsions.

The mean particle size d_{32} of the emulsions during storage for 0 and 20 days is shown in Figure 4B. The d_{32} of PPI emulsion increased from 1.714 μm (day 0) to 2.585 μm (day 20). However, after microfluidization treatment, a significant decrease ($p < 0.05$) in the d_{32} (day 0) was observed in comparison to native PPI sample, which implied that emulsifying activity was increased. After 20 days storage, the d_{32} of MT-PPI was still significantly lower than that of PPI, indicating that emulsion

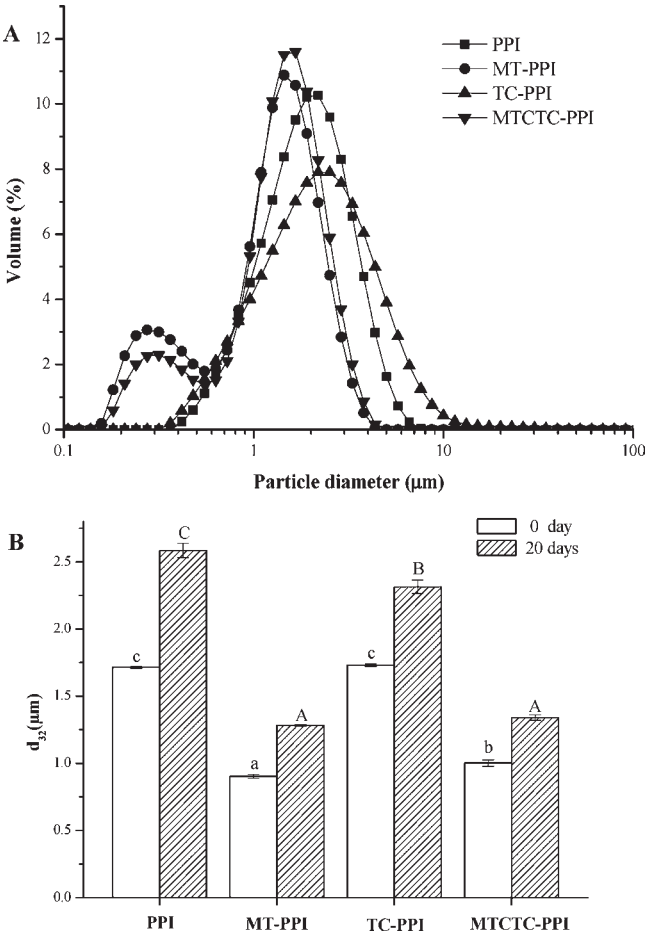

Figure 4. Particle size distribution (A) and mean particle size d_{32} (B) of emulsions made with protein samples. Different letters a−c on the tops of columns indicate significant ($p < 0.05$) differences among samples at day 0 and different letters A−C on the tops of columns indicate significant ($p < 0.05$) differences among samples at day 20.

stability was improved. This might be due to the fact that the solubility and surface hydrophobicity (H_0) of PPI were both significantly ($p < 0.05$) enhanced by microfluidization, which was very important for the improvement of the emulsifying properties. Similarly, high-pressure treatment (above 200 MPa) was found to cause a decrease of the d_{32} in lupin protein based emulsions and seemed to be beneficial to the stability.[33] Compared with the PPI emulsion, TGase cross-linking induced a slight increase ($p > 0.05$) in d_{32} at day 0 but a significant decrease ($p < 0.05$) in d_{32} at day 20, implying that TGase cross-linking had no beneficial effect on the emulsifying activity of PPI but improved the emulsion stability. This result was consistent with previous research on the emulsifying properties of TGase-treated other plant proteins such as cowpea, wheat, barley, and soy flours.[34,35] The unimproved or decreased emulsifying activity might be due to the fact that TGase induced the decrease in solubility and limited the immediate absorbence of protein onto the oil−water interface to form the small droplets in the emulsion. The improved emulsion stability seemed to be due to an increased resistance of the cross-linked proteins on the oil−water interface to deformation or rupture, and the surface layer rigidity could be enhanced by the presence of covalent cross-links.[34,36] By comparison of the changes in the d_{32} of all the samples, the smallest change in the

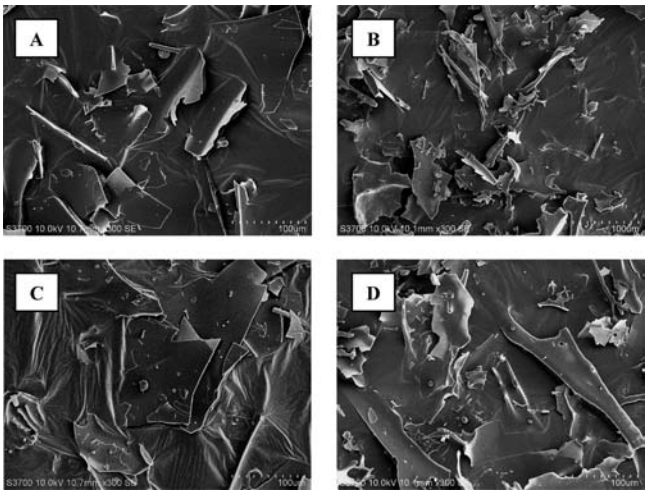

Figure 5. Microstructure of protein samples determined by scanning electron microscopy at a magnification factor of 300-fold: (A) PPI; (B) MT-PPI; (C) TC-PPI; (D) MTCTC-PPI.

d_{32} during the storage was observed in the MTCTC-PPI emulsion, suggesting that combined microfluidization and TGase cross-linking was more efficient in improving the emulsion stability of PPI than the individual treatment. This might be due to the combined treatments resulting in the preferable solubility and H_0 of PPI and the formation of more unfolded protein structures.

Microstructure. The microstructure of the protein samples was observed by scanning electron microscopy at a magnification factor of 300-fold to evaluate the effect of different treatments on the PPI, as shown in Figure 5. PPI (Figure 5A) showed a complex heterogeneous structure consisting of clumps in different sizes and shapes. For MT-PPI (Figure 5B), fragmentized clumps were observed because the high-pressure microfluidization treatment could cause the disruption of the large clumps into a small size. Compared with PPI (Figure 5A), TGase cross-linking induced the decrease of small clumps and the formation of larger clumps in TC-PPI (Figure 5C). In the case of MTCTC-PPI (Figure 5D), TGase cross-linked MT-PPI also led to the increase of clump size. The observations were consistent with the SDS-PAGE and HPSEC analyses (Figures 2 and 3).

Analysis of Fourier Transform Infrared (FTIR) Spectra. Figure 6 shows stacked infrared amide I spectra (1600−1700 cm^{-1}) of PPI samples, after deconvolution. Nine major bands associated with conformation of proteins were distinctly observed in the amide I region of this deconvoluted curve, indicating that the secondary structure of PPI samples was very complex, due to the diversity of the protein components. These bands were mainly attributed to the C═O stretching vibration and to a small extent to C—N stretching vibration of the peptide bonds, which had widely been used to reflect the secondary structure of proteins.[37] On the basis of previous literature, these major bands in the amide I region of PPI samples can be assigned as follows: The bands located at 1697 and 1664 cm^{-1} arose from turns and bends. The bands located at 1686, 1675, 1631, and 1621 cm^{-1} were due to β-sheet structures. The band at 1652 cm^{-1} corresponded to α-helix structure. The band at 1641 cm^{-1} was assigned to random coil structure. The band around 1611 cm^{-1} was generated from aromatic ring vibration of tyrosine residues.[38,39]

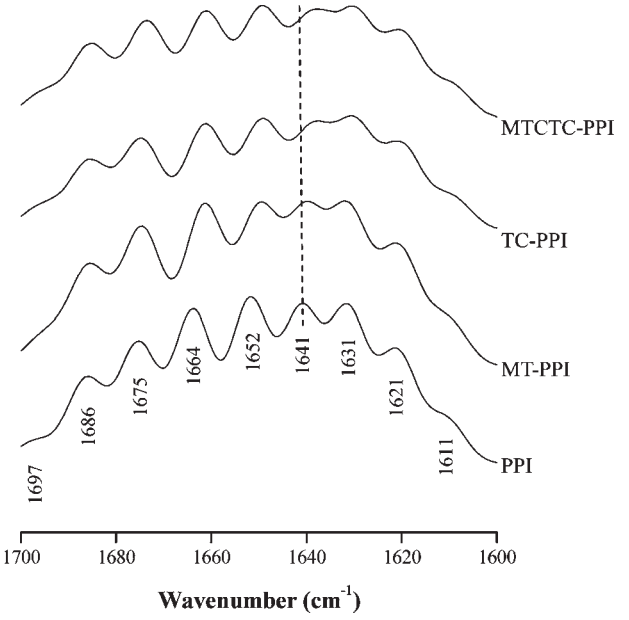

Figure 6. Deconvoluted FTIR spectra of protein samples. The dotted line within the figure indicates observable wavenumber shift of the corresponding bands.

Table 3. Secondary Structure Compositions of Protein Samples As Calculated from Far-UV CD Spectra Using the CONTIN/LL Program in CDPro Software

	secondary structure composition (%)			
protein sample	α-helix	β-sheet	β-turns	random coil
PPI	6.6	32.5	20.6	40.3
MT-PPI	5.9	33.0	20.1	41.0
TC-PPI	5.1	33.1	18.8	43.0
MTCTC-PPI	4.7	33.6	18.3	43.4

Compared with PPI, marked changes in intensity and wavenumber of various amide I bands were observed in MT-PPI, TC-PPI, and MTCTC-PPI, respectively. Different extents of decrease in the intensity of the band at 1652 cm^{-1} were observed in all treated PPI samples, reflecting the fact that α-helix structure in PPI was decreased by microfluidization, TGase cross-linking, and the combined treatments. For TC-PPI and MTCTC-PPI, the intensity of the bands at 1664 cm^{-1} attributed to the turns and bends was evidently decreased, as compared to PPI and MT-PPI. This reflected turns and bends in PPI or MT-PPI being involved in TGase-induced changes of secondary structures. As to MT-PPI, microfluidization treatment led to a red-shift (about 1 cm^{-1}) of the deconvoluted amide I band peaks of PPI. This indicated that the protein conformation of PPI was unfolded after microfluidization treatment. As a comparison, the red-shifts in TC-PPI and MTCTC-PPI were more obvious (about 2 cm^{-1}), reflecting the fact that more unfolded protein structures were formed. This phenomenon was consistent with the data of H_0.

Analysis of Circular Dichroism (CD) Spectra. The secondary structure of PPI samples was further evaluated using CD spectra, as shown in Table 3. Dispersions of PPI contained 6.6% α-helix, 32.5% β-sheet, 20.6% β-turns, and 40.3% random coil. High-pressure microfluidization treatment of PPI induced a decrease

in α-helix (from 6.6 to 5.9%) and β-turns (from 20.6 to 20.1%) and an increase in β-sheet (from 32.5 to 33.0%) and random coil (from 40.3 to 41.0%). Similar changes in the secondary structure were found in soybean protein isolate treated with high pressure.[32] Compared with native PPI, individual TGase cross-linking treatment could also decrease the α-helix and β-turns in PPI and increase the β-sheet and random coil. In comparison with individual microfluidization and TGase cross-linking, the combined treatments seemed to be more efficient for modifying the secondary structure of PPI. Partial denaturation and a greater amount of disordered structure in proteins could induce better adsorption at the oil−water interface.[40,41] Thus, the increase of random coil in the treated PPI samples contributed to distinct improvement of the emulsifying properties. The data of CD spectra were consistent with the data of FTIR spectra.

In conclusion, individual microfluidization (120 MPa) or TGase cross-linking treatment could improve the emulsifying properties of PPI. As a comparison, microfluidization was more effective for improving the functional properties (e.g., solubility or emulsifying properties) of PPI, whereas TGase cross-linking has more effect on the physicochemical and conformational properties (e.g., surface hydrophobicity or secondary structure) of PPI. Besides, it was found that microfluidization pretreatment could facilitate TGase-induced conjugation of PPI. Compared with individual treatment, microfluidization followed by TGase cross-linking could result in more unfolded structures of PPI and lead to more greatly improved emulsion stability.

AUTHOR INFORMATION

Corresponding Author

*Phone: +011-86-20-22236089. Fax: +011-86-20-87113914. E-mail: jyren@scut.edu.cn.

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