Preparation of Arachin/Conarachin Rich Fractions and Analysis of Their Physicochemical and Functional Properties

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Abstract: The arachin/conarachin-rich fractions were isolated from the defatted peanut flour by cryoprecipitation, and the physicochemical and functional properties of both fractions were studied. The protein contents of arachin/conarachin fractions prepared by cryoprecipitation were 91.27 and 84.87%, respectively, while the corresponding protein purities were 93.1 and 71.4%, respectively. The study results indicated that the arachin-rich fraction prepared by this method had a lower degree of thermal denaturation and higher thermal stability compared with the conarachin-rich fraction and peanut protein isolate (PPI). Conarachin-rich fraction and PPI had a more loose tertiary conformation and surface hydrophobicity compared to the arachin-rich fraction. The arachin-rich fraction showed the highest solubility in the entire pH range except at pH 6.0; however, the solubility of the conarachin-rich fraction was the most susceptible to change in pH value between 3.0 and 6.0. The arachin-rich fraction showed the highest emulsifying activity and stability, followed by conarachin-rich fraction and PPI. The conarachin-rich fraction exhibited the best heat-induced gelation properties at neutral pH, followed by the arachin-rich fraction and PPI.

Key words: arachin-rich fraction; conarachin-rich fraction; cryoprecipitation; cryoproteins; functional properties; conformational properties

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花生球/伴球蛋白富集组分的制备及其物化与功能特性研究

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摘要：本文以低温脱脂花生粉为原料，采用冷沉法分离制备了花生球蛋白和伴球蛋白富集组分，并研究了两种组分的物化和功能特性。通过冷沉法所制备花生球/伴球蛋白富集组分的蛋白含量分别为 91.27%和 84.87%，蛋白的纯度分别为 93.1%和 71.4%。研究结果表明，该法制备的花生球蛋白富集组分相比伴花生球蛋白富集组分和花生分离蛋白，具有更低的热变性程度和更高的热稳定性。而伴花生球蛋白富集组分和花生分离蛋白相比花生球蛋白富集组分具有更加松散的三级构象和更高的表面疏水性。花生球蛋白富集组分展示了更高的溶解性（pH 6.0 处除外）。乳化活性和稳定性按从高到低的排序为花生球蛋白富集组分、伴花生球蛋白富集组分和花生分离蛋白。伴花生球蛋白富集组分的溶解性在 pH 值 3.0~6.0 的范围内变化最为敏感并表现出在中性条件下最佳的凝胶特性。

关键词：花生球蛋白富集组分；伴花生球蛋白富集组分；结构特性；功能特性

Peanut proteins are important protein resources with high nutrition value while these proteins still remain underutilized[1]. At present, peanut protein isolate (PPI) could be easily prepared by alkali dissolution and acid precipitation. Whereas, PPI showed poor functional properties relative to native peanut protein by ammonium sulfate, such as solubility, emulsifying and gel properties,which might be due to the high extent of denaturation during extract procession[2]. The limited emzymatic hydrolysis by Alcalase could improve the protein solubility and gel-forming ability of PPI but impair the emulsifying activity[3]. However, the limited emzymatic hydrolysis at similar conditions could increase the emulsifying and foaming properties of native-arachin fraction[4]. The Maillard reaction between PPI and dextran has been used to improve functional

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properties, especially solubility and surface properties of proteins[5]. As shown previous reports, conarachin was more easily formed conjugates with dextran than arachin. Conjugation with dextran might further enhance emulsifying and foaming properties of PPI, if arachin fractions participate in glycosylation as well as conarachin fractions. These researches suggested that the better functional properties might be gained by modifying isolated arachin/conarachin fraction with low denaturation. However, at present, the extraction and purification methods for arachin and conarachin are not able to afford a large scale preparation[6,7], which greatly impaired the importance of researches on modifying arachin/conarachin fraction.

Cryoprecipitation is a thermally reversible precipitation phenomenon of specific proteins. The glycinin and β-conglycinin fractions of soy proteins could be separated simultaneously base on this property and the laboratory process had been successfully scaled up to the pilot-plant scale[8,9]. Basha and Pancholy have ever employed this property for purification of peanut cryoproteins and found that a great majority of arachin fraction exhibit cryoprecipitation[10]. However, very little information is available on the isolation of arachin and conarachin fractions by cryoprecipitation. Therefore, in this paper, a study is initiated to isolate the peanut protein fractions by cryoprecipitation and to determine their physicochemical and functional properties.

1 Materials and methods

1.1 Materials

Defatted Peanut flour was obtained from Shandong lanshan Group Co., Ltd. (Shandong, China). Folin-Ciocalteu phenol reagent (F-9252) reagent was purchased from Sigma (St Louis, MO, USA). Bovine serum albumin (BSA) and 1,8-aniline naphthalene sulphonate (ANS) were obtained from Fitzgerald Industries International Inc. (Concord, MA, USA). All the other chemicals used in the present study were of analytical grade.

1.2 Preparation of PPI from defatted peanut flour

Defatted peanut flour was dispersed in deionized water (1:20, *m/V*), and the pH of the dispersion was adjusted to 8.0 with 2 M NaOH. The resultant dispersion was gently stirred at 25 ℃ for 1 h, then centrifuged at 8000 r/min and 20 ℃ for 30 min in a CR22GⅡ high-speed refrigerated centrifuge (Hitachi Koki Co., Ltd, Japan). The pellet was discarded, and the supernatant was adjusted to pH 4.5 with 2 M HCl and then centrifuged at 5000 r/min and 20 ℃ for 20 min. The obtained precipitate was re-dispersed in deionized water. The dispersion was homogenized and adjusted to pH 7.0 with 2 M NaOH, then followed by freeze-drying to produce PPI product. The protein content of PPI was 91.32%, which was determined by NDA 701 Dumas nitrogen analyzer (Velp Scientifica, Italy).

1.3 Preparation of arachin/conarachin rich fractions

Defatted peanut flour was dispersed in 0.2 M phosphate buffer (pH 8.0, 1:10, *m/V*). The resultant dispersion was gently stirred at 25 ℃ for 1 h, then centrifuged at 8000 r/min and 20 ℃ for 30 min in CR22GⅡ high-speed refrigerated centrifuge (Hitachi Koki Co., Ltd, Japan). The clear supernatant was allowed to stand at 2 ℃ over night, and the cryoprecipitates were collected by centrifugation at 8000 r/min for 20 min at 2 ℃. The obtained precipitate which contained mostly arachin fraction was re-dispersed in deionized water and then followed by freeze-drying to produce arachin-rich fraction product. The supernatant was adjusted to pH 4.5 with 2 M HCl and then centrifuged at 8000 r/min and 20 ℃ for 20 min. The obtained precipitate which contained mostly conarachin fraction was re-dispersed in deionized water. The dispersion was homogenized and adjusted to pH 7.0 with 2 M NaOH, then followed by freeze-drying to produce conarachin-rich fraction product. The protein content of arachin/conarachin-rich fraciton were 91.27 and 84.87%, respectively, which was determined by NDA 701 Dumas nitrogen analyzer (Velp Scientifica, Italy).

1.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a discontinuous buffered system according to the method of Laemmli using a 12% separating gel and 4% stacking gel[11].

1.5 Differential scanning calorimetry (DSC)

Aqueous dispersions (20%, *m/V*) of the protein samples were prepared in 10 mM phosphate buffer (pH 7.0). The experiments were performed on a TA Q200-DSC thermal analyser (TA Instruments, New Castle, DE, USA). Aliquots (10 μL) of the protein dispersions were accurately injected into aluminium liquid pans. The pans were hermetically sealed and heated in the calorimeter from 25 to 120 ℃ at a rate of 10 ℃/min. A sealed empty pan was used as reference. Peak or denaturation temperature (T_d) and enthalpy change of denaturation (ΔH) were computed from the thermograms by Universal Analyser 2000, version 4.5A (TA Instrument-Waters LLC, New Castle, DE, USA). All the experiments were conducted in triplicate.

1.6 Intrinsic fluorescence emission spectroscopy

Intrinsic emission fluorescence spectra of the protein samples were obtained by F-2500 fluorescence spectrophotometer (Hitachi Co., Ltd, Japan). Protein solutions (0.15 mg/mL) were prepared in 10 mM phosphate buffer (pH 7.0). To minimise the contribution of tyrosine residues to the emission spectra, the protein solutions were excited at 290 nm, and emission spectra were recorded from 300 to 400 nm at a constant slit of 5 nm for both excitation and emission. All the determinations were conducted in triplicate.

1.7 Measurement of surface hydrophobicity

Surface hydrophobicity was determined using 1,8-anilinonaphthalenesulfonate (ANS), as a fluorescent probe, according to the method of Mu, et al [12], with minor modifications. In brief, stock solutions of 8×10^{-3} M ANS^-, and 1.5% (*m/V*) protein were prepared in 10 mM phosphate buffer (pH 7.0). Five same samples containing 4 mL of buffer and 20 μL of ANS^- stock solution were added 10, 20, 30, 40 and 50 μL of 1.5% protein solution, respectively. Samples were shaken with a vortex mixer for 5 s. Fluorescence intensity (FI) was measured at 390 nm (excitation) and 470 nm (emission) at 20±0.5 ℃, with a constant excitation and emission slit of 5 nm. The FI for each sample with probe was then computed by subtracting the FI attributed to protein in buffer. The initial slope of the FI versus protein concentration plot was calculated by linear regression analysis and used as an index of surface hydrophobicity. Additionally, the extrinsic fluorescence spectroscopy of ANS in the presence of proteins was obtained at a protein concentration of 7.5×10^{-5} g/mL (or 20 μL of 1.5% protein solution mixed with 4 mL of the buffer). All the determinations were conducted in triplicate.

1.8 Protein solubility

Protein dispersions in deionised water (1%, *m/V*) were stirred magnetically for 30 min, and then the pH was adjusted to the desired value with 0.5 M HCl or 0.5 M NaOH. After 30 min of stirring, the pH was readjusted if necessary. Then the dispersions were centrifuged at 12000 g for 20 min at 20 ℃. After appropriate dilution, the protein content of the supernatants was determined by Lowry's method using bovine serum albumin (BSA) as the standard[13]. The protein solubility was expressed as grams of soluble proteins per 100 g of proteins. All the determinations were conducted in triplicate.

1.9 Emulsion preparation and determination of particle size

A protein solution (20 mg/mL) was prepared and the pH was adjusted to 7.0. Corn oil was then added to the protein solution and mixed to give a final mixture containing 20.0% (*m/m*) corn oil. The mixture of protein solution and corn oil was homogenised using an AH100D homogeniser (ATS Engineering Inc., Canada) operating at 30 MPa. Each emulsion sample was prepared in duplicate. The particle size distribution of the emulsions was measured immediately after emulsion preparation with the aid of a Microtrac S3500 (Microtrac Inc., USA). Measurements were carried out at room temperature.

1.10 Emulsion stability measurements

The stability of the emulsions against creaming was also measured, according to the method of Hu, et al[14], with minor modifications. Emulsion samples were poured into 10 mL glass tubes (height 135 mm, diameter 15 mm) and the height of the separated cream of the

emulsion layer in each tube was recorded after storage for 30 d at room temperature. The creaming of emulsions was determined as the ratio of the height of the cream layer to the total height of the emulsion. Prior to analysis, 0.2 g/L sodium azide was added to all samples to avoid microbial growth.

1.11 Low amplitude dynamic oscillatory measurements

Heat-induced gelation of the protein samples was followed by a small amplitude dynamic rheological technique, using a Physica MCR301 Rheometer (Anton Paar Co., Austria) equipped with a coaxial cylinder measuring system (d=27 mm). A sequence of sweeps on blends were conducted as follows: (1) a temperature increase ramp of 1 ℃/min from 20 to 90 ℃ and holding for 30 min at 90 ℃; (2) a temperature decrease ramp of 1 ℃/min from 90 to 20 ℃. The storage modulus (G′), loss modulus (G′′) and loss tangent (tan δ=G′′/G′) were recorded as a function of time. The measurements were performed at a constant strain of 0.01, which was within the linear region at a fixed frequency of 0.1 Hz. Three replications for each measurement were conducted.

1.12 Statistical analysis

All the experiments were performed in triplicate and the data obtained were subjected to statistical analysis, using analysis of variance (ANOVA), and a least significant difference (LSD) or Tamhane T_2 with a confidence interval of 95% was used to compare the means.

2 Results and discussion

2.1 SDS-PAGE analysis

Fig.1 shows the SDS-PAGE profiles of PPI, arachin-rich and conarachin-rich fractions in the presence of β-mercaptoethanol. The composition and purity of arachin/conarachin were evaluated by SDS-PAGE (Fig. 1). According to the reports of Bhushan and Agarwal and chiou[15,6], the bands between 15 ku and 40 ku should belong to arachin subunits, the others are the subunits of conarachin. As shown in Fig.1, Lane b. PPI have all of the typical subunit bands of peanut protein. The arachin-rich fraction almost miss the 66 ku subunit of conarachin, however the lower molecular weight conarachin subunits between 10 ku and 15 ku were hard to be isolated from arachin fraction (Fig.1, Lane c). The subunits of arachin, in the conarachin-rich, were significantly decreased but still visibly. The relative content of arachin and conarachin was respectively close to 93.1% and 71.4% based on total protein content, as evaluated by comparing the optical density.

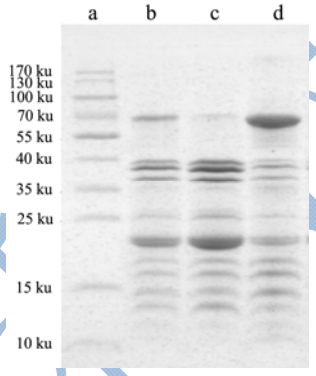

Fig.1 SDS-PAGE profiles of PPI and arachin-rich and conarachin-rich fractions

Note: Lane a, marker proteins; Lane b, PPI; Lane c, arachin-rich fraction; Lane d, conarachin-rich fraction.

These results were suggested that the cryoprecipitation might be a feasible method to isolate the arachin and conarachin fractions simultaneously. Whereas, the higher purity arachin-rich fraction could be gained by the cryoprecipitation but conarachin-rich fraction, which is agreement with the report of Wu et al[9], who had isolated to the glycinin and β-conglycinin fractions of soy protein by similar method which purities were 95.7% and 77.6%, respectively. This result might be due to two reasons: Firstly, not all the proteins of arachin exhibit cryoprecipitation, according to the report of Basha and Pancholy[10]. Secondly, the peanut cryoprotein is a kind of thermally reversible protein, which might cause to a part of cryoprecipitated arachin fraction redissolve on the process of isolation.

2.2 Thermal properties

The thermal properties of PPI and arachin/ conarachin-rich fractions were evaluated by DSC, and related DSC characteristics are summarized in Table 1. As shown in Table 1, the DSC profile of PPI showed two major endothermic peaks at 92.19 and 102.94 ℃, respectively. Similarly, the DSC profile of

conarachin-rich fraction also showed two endothermic peaks at 94.67 and 103.24 ℃, respectively. Both endothermic peaks of PPI and conarachin-rich fraction were corresponded to the thermal denaturation of arachin and conarachin, respectively. However, the DSC profile of arachin-rich fraction showed only one endothermic peak at 105.27 ℃ which corresponded to the thermal denaturation of arachin fraction. These result suggested that the arachin-rich fraction could gain the higher purity than conarchin-rich fraction by this isolate method, which was consistent with the result of SDS-PAGE (Fig.1).

Table 1 DSC characteristics and surface hydrophobicity (H_0) of PPI and arachin/conarachin-rich fraction samples

Sample	Conarachin		Arachin		H_0
	T_{d1}/℃	ΔH_1/(J/g)	T_{d2}/℃	ΔH_2/(J/g)	
PPI	92.19±0.51	0.025±0.005	102.94±0.14	5.31±0.38	474.9±3.45
arachin-rich	-	-	105.27±0.04	23.46±0.17	109.3±8.85
conarachin-rich	94.67±0.11	2.36±0.03	103.24±0.16	1.50±0.13	577.9±5.06

Note: Means±standard deviations of triplicate analyses are given.

Furthermore, arachin-rich fraction had a higher T_d value compared with arachin fraction of PPI and conarachin-rich fraction. It suggested that the extraction process by alkali dissolution and acid precipitation led to a noticeable decrease of thermal stability of PPI and conarachin-rich fraction. In addtion, the enthalpy change (ΔH) of the endothermic peak of PPI and conarachin-rich fraction were significantly lower ($p<0.05$) than that of arachin-rich fraction (Table 1). This result indicated that the cryoprecipitation method was a mild process to gain the arachin-rich fraction, which would almost not cause the denaturation of arachin fraction. However, alkali dissolution and acid precipitation induced unfolding of undenatured protein in PPI and conarachin-rich fraction[16].

2.3 Emission fluorescence spectroscopic analysis and surface hydrophobicity

The fluorescence emission spectra of ANS (a polarity-sensitive fluorescent probe) upon binding to the proteins in PPI, arachin/conarachin-rich fraction are shown in Fig.2A. A higher fluorescence intensity and lower emission maximum (λ_{max}) value is usually associated with a higher hydrophobicity for a globular protein. Thus, the conarachin-rich fraction had the lowest λ_{max} at about 466.5 nm with a highest fluorescence intensity, followed by PPI (λ_{max} 468.5 nm) and arachin-rich fraction (λ_{max} 480 nm) (Fig.2A), indicating the order of hydrophobicity of the ANS^- microenvironment upon protein binding. This is in good agreement with H_0 (Table 1), the conarachin-rich fraction had shown the highest surface hydrophobicity value, followed by PPI and arachin-rich fraction.

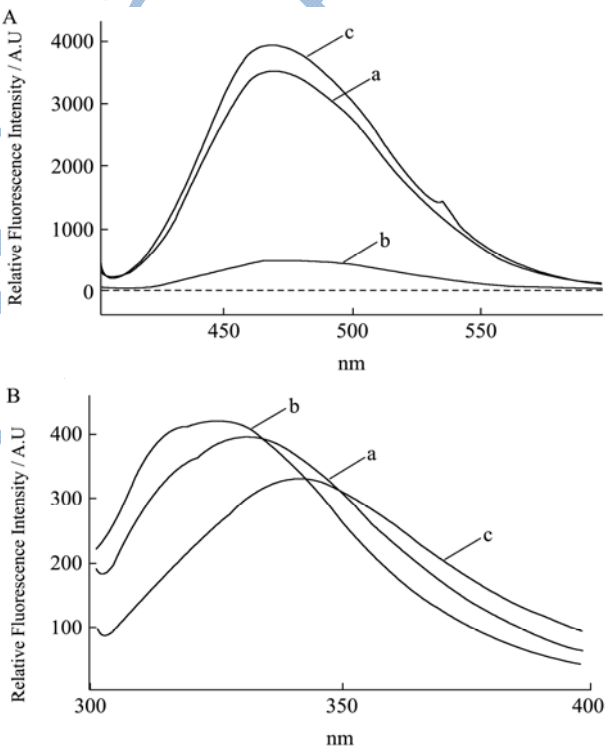

Fig.2 (A) The extrinsic emission fluorescence spectra of ANS in the presence of tested samples (B) The intrinsic emission fluorescence spectra of the proteins in tested samples

Note: a, PPI; b, arachin-rich fraction; c, conarachin-rich fraction.

The difference in the hydrophobic clusters on the molecular surfaces of the proteins in tested samples could be well reflected by intrinsic fluorescence spectra (Fig. 2B). The emission fluorescence spectrum can provide a sensitive means of detecting proteins and their conformation by determining the polarity of the environment of the tryptophan/tyrosine residues or their

specific interactions, since a lower fluorescence emission maximum value can usually indicated that chromophores in proteins become more buried in the hydrophobic core[17]. The proteins in arachin-rich showed a typical intrinsic fluorescence spectrum, with an emission maximum (λ_{max}) at around 326.5 nm[18], which was higher than the λ_{max} in PPI (332 nm) and conarachin-rich (341.5 nm). The data further indicated the Trp residues in PPI and conarachin-rich were more exposed to the hydrophilicity environment than that in arachin-rich. The proteins in arachin-rich had a more compacted tertiary conformation than those in PPI and conarachin-rich. These results were corresponding with the fluorescence emission spectra of ANS and surface hydrophobicity.

2.4 Protein solubility

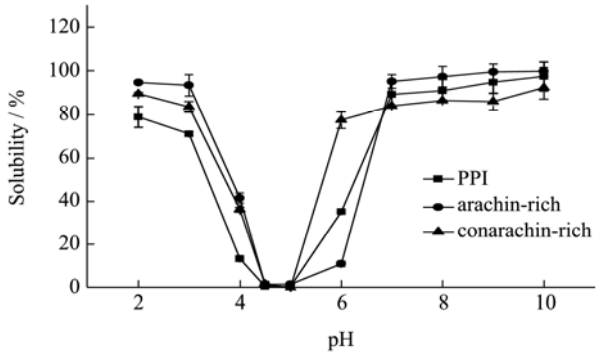

Fig.3 Solubility of PPI, arachin-rich fraction and conarachin-rich fraction

Note: Error bars indicate the standard deviations (n=3).

Fig.3 shows protein solubility profiles of PPI, arachin-rich and conarachin-rich, respectively, as a function of pH. The PPI and peanut protein fractions showed minimum solubility between pH 4.5 and 5.0, and more than 70% of the protein was solubilized at pH below 3.0 and above 7.0. The arachin-rich fraction showed the highest solubility at all pH value range except at pH 6.0. The PPI showed the lowest solubility at pH below 4.5. In addition, the noticeably difference of solubility among three protein samples were found at pH between 5.0 and 7.0. The solubility of conarachin-rich showed the sharpest increase at pH from 5.0 to 6.0, but for arachin-rich at pH from 6.0 to 7.0. The solubility of PPI was gradually increasing at pH from 5.0 to 7.0. Furthermore, the solubility profile of conarachin-rich showed a V-shaped pattern but the profiles of PPI and arachin-rich showed a typical U-shaped pattern, indicated that the solubility of conarachin-rich were the most susceptible to the change of pH value between 3.0 and 6.0.

The difference of protein solubility may be attributed to a difference in the process conditions, subunit composition, the extent of protein denaturation and intermolecular interactions. The higher solubility of arachin-fraction might be due to the mild extract procress by cryoprecipitation, the lower extent of denaturation and higher surface hydrophilic properties (Table 1). On the contrary, the relatively poor solubility properties of PPI and conarachin-rich fraction might be attributed to the higher extent of denaturation and surface hydrophobicity, which might promote intermolecular hydrophobic interaction to induce aggregation of proteins. The results are correspond with the reports of Liu, Zhao, et al[3].

2.5 Emulsion Particle Size and emulsion stability

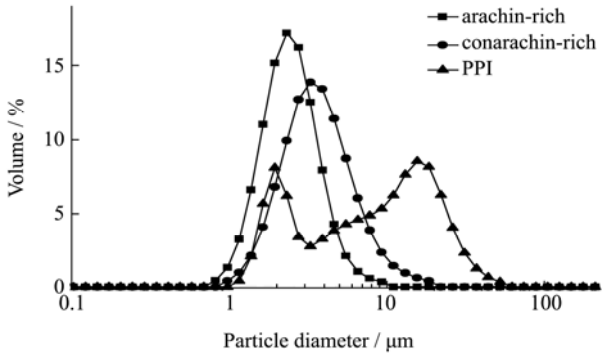

Fig.4 Particle size distribution of emulsions made with PPI, arachin-rich fraction, and conarachin-rich fraction

The particle size distributions of the fresh emulsions made with samples are shown in Fig.4. The arachin-rich fraction and conarachin-rich fraction emulsions showed a monomodal particle size distribution but a bimodal particle size distribution for PPI. The volume of small particles (d<10 μm) of arachin-rich, conarachin-rich fraction and PPI emulsions were 96.45, 75.98 and 51.32%, respectively. Results might be implied that the arachin-rich had the higher emulsifying activity in comparison to conarachin-rich fraction and PPI samples. To evaluate the stability of the different emulsions, the creaming of emulsions made with samples during 30 days of storage at room temperature were investigated. It was found that the creaming of emulsions made with

arachin-rich fraction, conarachin-rich fraction and PPI reached 28%, 34.7% and 53.3% after 30 d of storage, respectively. The results suggested that arachin-rich fraction can give emulsions more stability against creaming, followed by conarachin-rich fraction and PPI.

According to the reports of Li-Chan & Nakai SandWood[19], solubility and surface hydrophobicity are two major factors in determining protein emulsifying properties. The conarachin-rich fraction and PPI were all prepared by alkali dissolution and acid precipitation. The conarachin-rich fraction had the better emulsifying properties relative to the PPI. This might be due to the higher surface hydrophobicity and the more loose tertiary conformation of conarachin-rich fraction than PPI (Table 1 and Fig.2B), which could induce proteins to absorb and unfolding at the oil-water interface more easier than PPI. However, in this case, arachin-rich fraction had the lowest surface hydrophobicity but best emulsifying activity and stability. This result might be due to the lower denaturation extent and higher solubility of arachin-rich fraction prepared by cryoprecipitation. Similar results have been obtained by Liu et al.[2], who have found that PPI had the higher surface hydrophobicity but lower emulsifying properties than native peanut protein by ammonium sulfate extraction, which be attributed to the higher extent of protein aggregation by the extract process of alkali dissolution and acid precipitation.

2.6 Heat-induced gelation properties

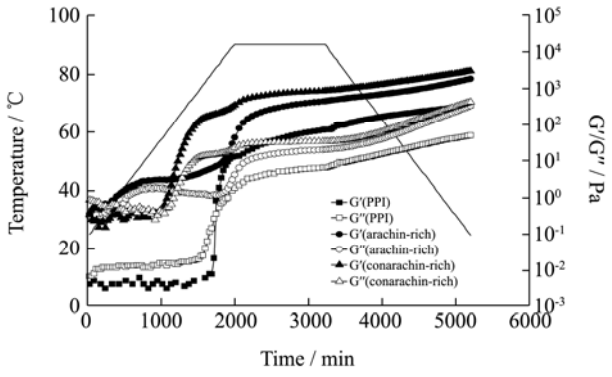

Fig.5 Heat-induced gelation profiles of PPI, arachin-rich fraction, and conarachin-rich fraction in 0.01 M phosphate buffer (pH 7.0)

Note: The protein concentration of the dispersions was about 20% (*m/V*).

As shown in Fig.5, the heat-induced gelation of PPI, arachin-rich and conarachin-rich fractions at neutral pH were evaluated by the low amplitude dynamic oscillatory measurements. For the PPI and arachin-rich fraction, the sharp increase in G´ (elastic modulus, an indication of the network formation) were observed during the holding period at 90 ℃ and increased continuously during cooling. However, the conarachin-rich fraction exhibited a remarkable increase in modulus before temperatures increased to 90 ℃ and a gradual increase during the holding and cooling period. Finally, the conarachin-rich fraction gel exhibited the highest G´ at the end of cooling, followed by arachin-rich fraction and PPI (Fig.5).

It has been reported that the denaturation of protein proved to be a prerequisite for gel formation[20]. On the basis of the SDS-PAGE results (Fig.1), the conarachin was a main component of conarachin-rich fraction sample (accounting for over 70%), while the arachin was the main component of PPI and arachin-rich fraction. The corresponding T_d of conarachin fractions in conarachin-rich fraction was lower than that of arachin fraction (Table 1), which might promote the more adequate denaturation and then form the gel of conarachin-rich fraction at the temperature below 90 ℃. Furthermore, the conarachin-rich fraction had the more surface hydrophobic groups and more loose tertiary conformation than PPI and arachin-rich fraction (Table 1and Fig.2B), which might be greatly contributed to the formation of gel.

3 Conclusion

The cryoprecipitation was a feasible method for isolating arachin/conarachin-rich fraction instead of saturated ammonium sulfate and might be a potential process that could be scaled up to the pilot-plant scale. Arachin/conarachin-rich fractions prepared by cryoprecipitation had the better functional properties in comparison with PPI. In addition, arachin/conarachin -rich fraction showed different conformational and physiochemical characters, which might be lead to remarkably different results in same modification conditions. Further investigations on Maillard reaction between arachin/conarachin-rich fractions and polysaccharide will be carried out to achieve more improvements in functional properties.

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