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Effect of reverse micelle on conformation of soy globulins: A Raman study

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ABSTRACT

Raman spectroscopy was applied to investigate the main and side chain conformational changes of 7S and 11S globulins from soybean proteins using aqueous buffer and reverse micelle extraction. The 7S and 11S globulins using two extraction methods displayed typical spectral features (such as amide I, III region and the side chain conformations of particular residues) due to characteristic amino acid composition and molecular conformation. In comparison, the degree of molecular disorder increased in both globulins using reverse micelle extraction and new bands appeared. The relative amount of different structures of 7S and 11S globulins could be estimated through accurate measurement of the band intensities. Finally, the increase of the $I_{850/830}$ intensity ratio of Raman tyrosine doublet in 11S globulin with reverse micelle extraction suggested a change towards a more exposed state of tyrosine residues, in good agreement with the more disordered conformation taken upon reverse micelle.

1. Introduction

Soy proteins are widely utilised in the manufacturing of many processed foods as functional ingredients due to their high nutritional value, functional properties and low cost. The soybean proteins contain four globulins, namely, 2S, 7S, 11S and 15S protein. These protein fractions are categorised by their sedimentation coefficients. The percentage content of 2S, 7S, 11S and 15S was found to 15%, 34%, 41.9% and 9.1%, respectively (Fukushima, 1991). The 7S and 11S globulins were shown to have different main and side chain conformation and functional properties (Mujoo, Trinh, & Ng, 2003). Adequate modification on soy proteins isolate may improve their functional properties, as a result, it is necessary to investigate 7S and 11S globulins from soybean proteins, in order to elucidate the gel and thermal properties of proteins.

The effects of heat, enzyme and ingredients on the main and side chain conformation of 7S and 11S globulins have been widely studied (Berjota et al., 1995; Indiani, Feis, Howes, Marzocchi, & Smulevich, 2000; Moon & Li-Chan, 2007). Adequate modification on structure of soy proteins has brought out the following outstanding functional properties that the native soy proteins do not possess (Mohamed, 2002). The 7S and 11S globulins have been considered in detail about their structures, stability and textural properties. While very limited information is available on the effect of the reverse micelles on structural properties of 7S and 11S globulins.

A considerable volume of work has been published on the proteins dissolved in reverse micelles (Correa, Durantini, & Silber, 1998; Matzke, Creagh, Haynes, Prausnitz, & Blanch, 1992; Souillac, Middaugh, & Rytting, 2002). Phase–phase extraction can also be utilised to recover proteins from the aqueous phase on a large scale through phase–phase extraction (Stephanie, Thorsten, Alan, Pawel, & Walter, 1991). Moreover, applying reverse micelles for the simultaneous production of soybean oil and protein is attractive since soybeans represent one of the major oilseeds for producing edible oils (Leser & Luisi, 1989).

In reverse micelles, the main driving forces responsible for the solute distribution between the organised assembly and the organic medium are hydrophobic effects, hydrogen bonding and electrostatic interactions, which may affect the structure of proteins (Correa et al., 1998). Besides, other factors, i.e., pH and ionic strength in reverse micelles (Matzke et al., 1992), may also affect structure of proteins. So it is important to study the effects of the reverse micelles on structural properties of 7S and 11S globulins of soybean.

Various techniques are currently available for obtaining the structural information of proteins; including X-ray diffraction, nuclear magnetic resonance (NMR) spectroscopy, and Fourier transform infrared spectra (FTIR). These methods have limitations. X-ray diffraction requires the preparation of perfect single crystal,

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which can be time-consuming or even impossible (Uson & Sheldrick, 1999); NMR spectroscopy is not easily applied to proteins larger than a few hundred residues (Couling, Fischer, Klenerman, & Huber, 1990); and FTIR can mainly yield information about protein secondary structure of amide I region (Souillac et al., 2002). Other analytical techniques of proteins in solution are circular dichroism (CD), ultraviolet absorption, and fluorescence spectroscopy. These methods are destructive, involve time-consuming sample preparation procedures, and require relatively large amounts of samples. Furthermore, the wet chemistry methods are not amenable to continuous monitoring for quality control, and difficult to be quantitatively relate structural changes of proteins in fluorescence yields. Thus, Raman spectroscopy would be the most convenient analytical method that could be used for solid proteins without any need for sample preparation, and provide a direct, non-destructive, and faster determination of the structure of proteins.

Raman spectroscopy has been proved to be a valuable method for the study of biological molecules with the availability of easily maintained and operated "bench-top" instruments; the technique is gaining in popularity (Jenkins, Larsen, & Williams, 2005). Raman spectroscopy can be applied to analyse samples directly, in air, at ambient temperature and pressure, wet or dry, and in many cases without destroying the sample.

The most conventional reverse micellar system studied was with sodium bis (2-ethylhexyl) sulfosuccinate (AOT) as amphiphic surfactant and with isooctane as organic solvent. The secondary structure of proteins dissolved in AOT reverse micelle using FTIR, has been studied in several papers (Chang, Liu, & Chen, 1994; Zhao, Chen, Xue, & Li, 2008), but not much work has reported about the effect of AOT reverse micelle on the main and side chain conformation of 7S and 11S globulins of soybean, especially combining with Raman spectroscopy.

In this paper, the dry-addition method was utilised to dissolve proteins in AOT reverse micelle. The objective of the work was to investigate the effects of AOT reverse micellar system on the main and side chain conformation of 7S and 11S globulins by Raman spectroscopy. The intensities of amide I band, amide III band, C–C bond, C–N bond and some amino acids in 7S and 11S globulins exhibited Raman vibration characteristics, and the change of vibrational bands is conveniently used for quick and accurate determination of the structure of 7S and 11S globulins. We compared the calculated results of the main and side chain derivative band areas of 7S and 11S globulins in reverse micellar solution extraction that from aqueous buffer (pH 7.5) solution extraction.

2. Materials and methods

2.1. Materials

AOT was bought from sigma chemical Company (>98%). Bicinchoninic acid (BCA) protein assay kit was purchased from USA Pierce Company. All other reagents were of analytical grade. Soybean (No. 8) was from local market, Beijing. The soy flour was sieved through a 100 screen to enrich protein contents, the soy flour consisted of 37.53% protein, 7.99% humidity, and 20.75% oil. All values are given in wt.% of the total flour weight.

2.2. Isolation of 7S and 11S globulins

AOT reverse micelle was used to isolate 7S and 11S globulins from soy flour (Vassiliki, Aristotelis, & Athanasios, 1993). Stock solution of 0.05 M AOT was obtained first by solubilising AOT in isooctane. The total volume of water was adjusted by the ratio $W_0 = [H_2O]/[AOT]$ using phosphate buffer pH 7.5 containing 0.05 M KCl, $W_0 = 18$. Then the forward extraction was prepared by adding soybean flour to the AOT system (1:20, w/w). Solubilisation was conducted in a magnetically agitated Erlenmeyer flask for 30 min at 45 °C. The resulting mixture was centrifuged at 6000 rpm for 10 min at room temperature, the clear supernatant solution was used for the next extraction step. Two-hundred millilitres of this solution was carefully laid on 200 ml of 1 M KCl phosphate buffer (50 mM) pH 7.5, magnetically agitated Erlenmeyer flask at ambient temperature for 1 h. The resulting mixture was centrifuged at 4000 rpm for 10 min. Then two phases, namely oil phase and aqueous phase, were separated by centrifugation at 3700 rpm for 5 min, respectively. The aqueous phase was stored for the experiment.

For preparing of the crude extract in an aqueous buffer solution, the defatted soybean flour was dispersed in 50 mM phosphate buffer (1:20, w/w) (pH 7.5). The suspension was then centrifuged at 12,000 rpm at 20 °C for 20 min. The supernatant solution was used for further investigations.

The 7S and 11S globulin fractions were isolated by modified method of Nagano, Hirotsuka, Mori, Kohyama, and Nishinari (1992). The protein solution with reverse micellar extraction method was dialysed at 4 °C for 48 h to remove impurities and excess reagents before 7S and 11S globulins were isolated. The 7S and 11S globulins were recovered by freeze-drying. Protein content of this lyophilised powder was 95.43% by the BCA method (Smith et al., 1985).

2.3. Collection and analysis of Raman spectra

Raman spectra were recorded on a HR-800-Raman Imaging Microscope (JY Company, France) equipped with a 532 nm (3 mW) argon ion laser excitation (Spectra Physics) requiring 25 mW power. The laser was focused on the solid samples which were placed on microscope slides. Each spectrum was obtained under the following conditions: 10 scans, 50 s exposure time, 2 cm^{-1} resolution. The average spectral data from the scan of samples in the Raman spectrophotometer was baseline corrected and normalised against the phenylalanine (Phe) band at 1003 cm^{-1}. Assignments of the bands in the Raman spectra to the specific vibrational modes of the main and side chain conformation were based on the literature (Tu, 1986). Secondary structure analysis based on the amide I and III region using the Raman Spectral Analysis Package (RSAP) programme was conducted with ingredient subtracted Raman spectral data. The Raman spectra (400–2200 cm^{-1}) of each sample were collected in triplicate, and the results were reported as the averages of these replicates.

2.4. Statistical analysis

All experiments were performed in triplicate. The differences in mean were calculated with *T*-test for means with 95% confidence limit ($P \leqslant 0.05$). SAS software (v.8.2, SAS Institute Inc., Cary, NC, USA) was applied to conduct statistical analysis of the data

3. Results and discussion

3.1. Isolation of 7S and 11S globulins

Fig. 1 shows the SDS–PAGE patterns of 7S and 11S globulins with using two methods (lanes 3 and 4: 7S and 11S globulins by aqueous buffer extraction; lanes 5 and 6: 7S and 11S globulins by AOT reverse micellar extraction). It could be concluded from experiment that the backward extraction solution of proteins with AOT reverse micellar extraction dialysed at 4 °C (24 h) was suitable to isolate 7S and 11S globulins by Nagano et al. (1992) method. The ionic strengthen (1 M) in backward extraction of proteins with AOT

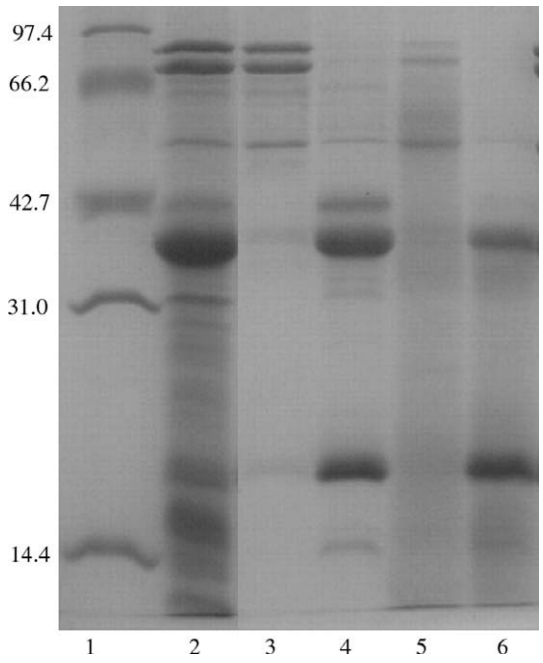

Fig. 1. SDS–polyacrylamide gel electrophoresis of 7S and 11S globulin fractions. Lane 1, molecular weight markers; lane 2, SPI; lanes 3 and 4, 7S and 11S globulins by aqueous buffer extraction; and lanes 5 and 6, 7S and 11S globulins by AOT reverse micellar extraction.

reverse micelle extraction would affect the isoelectric of 7S and 11S globulins, which would lead to hindering the separation of 7S and 11S globulins. According to Koshiyama (1972) method, the 11S globulin would be completely separated, when ionic strengthen was more than 0.5, the pH was adjusted to 2.0. However, it was difficult to isolate 7S and 11S globulins properly of soybean proteins using reverse micelle extraction by this method.

One mole of 11S globulin molecule consisted of at least 20 disulphide bands, 0.0082 M SBS was not enough to break all disulphide bands. Therefore 0.0094 M SBS was applied, which facilitated the separation of 11S globulin. The amount of reducing agent was required to give a good purity of 11S globulin and a high yield of 7S globulin (Fig. 1).

3.2. Analysis of Raman spectroscopy

The Raman spectra of proteins were sufficiently different from each other in detail to permit unambiguous identification by spectral database search algorithms (Jenkins et al., 2005). Similar to infrared absorption spectra, the rather crowded area from 400 to 2000 cm^{-1} Raman shift was known as the "fingerprint region" and contained the major of the Raman bands applied to uniquely identify proteins (Tu, 1986). The Raman spectra of 7S and 11S globulin samples with different extraction methods are shown in Fig. 2A–D. Assignment of some major Raman bands was made based on results from previous workers (Li-Chan, 1996; Tu, 1986). The "fingerprint region" of Raman spectra and characteristic frequencies of 7S and 11S globulins are shown in Table 1 (Tu, 1986). The intensity and location of the phenylalanine band at 1003 cm^{-1} band was not sensitive to conformation or microenvironment, and therefore could be used as an internal standard (Li-Chan, 1996). The intensity and location of the CH_2 and amide I band at around 1447 and 1665 cm^{-1} from 7S and 11S globulins

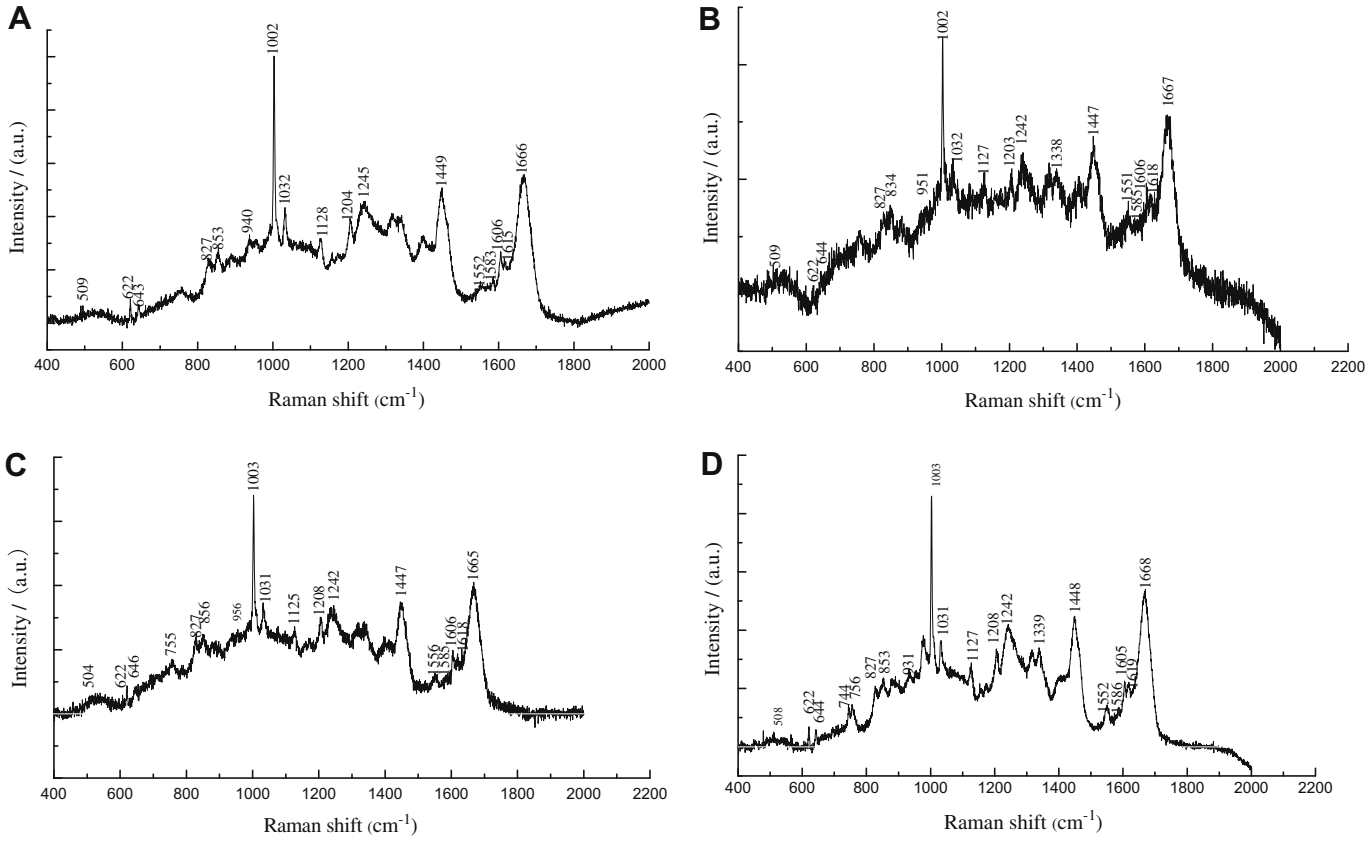

Fig. 2. Raman spectra of 7S and 11S globulins from soybean proteins. (A) Raman spectrum of 7S globulin using aqueous phase extraction. (B) Raman spectrum of 11S globulin using aqueous phase extraction. (C) Raman spectrum of 7S globulin using AOT reverse micellar phase extraction. (D) Raman spectrum of 11S globulin using AOT reverse micellar phase extraction.

Table 1
Characteristic Raman frequencies and tentative assignments of 7S and 11S globulins.

Raman spectrum (cm^{-1})				Tentative assignments
Globulins by aqueous buffer extraction		Globulins by AOT reverse micelle extraction		
7S	11S	7S	11S	
622	622	622	622	Phenylalanine (Phe)
643	644	646	644	Tyrosine (Tyr)
		755	744	Tryptophan (Trp)
			756	Trp
827	827	827	827	Tyr
853	853	856	853	Tyr
940	942	951	931	γ(C–C)
1002	1002	1003	1003	Phe
1032	1032	1031	1031	Phe
1128	1127	1125	1127	γ(C–N)
1204	1203	1208	1208	Tyr + Phe
1245	1242	1242	1242	Amide III bands
	1338		1339	Trp
1449	1447	1447	1448	γCH_2
1552	1551	1556	1552	Trp
1583	1585	1585	1586	Trp
1606	1606	1606	1605	Tyr + Trp
1615	1618	1618	1619	Amide I bands
1666	1667	1665	1669	Amide I bands

γ means vibrations.

using two extraction methods was not significantly different. The results indicated that the AOT reverse micelle system had no influence on the intensity and location of three significant peaks of 7S and 11S globulins. The intensity and location of three significant peaks agreed with the investigation of Berjota et al. (1995).

In comparison with the 7S and 11S globulins using aqueous buffer extraction, globulins using AOT reverse micellar extraction exhibited some new peaks at 755, 744 and 756 cm^{-1}, some bands shifted to higher frequency, some to lower, while some had shown no change (Table 1). The shift of bands in main and side chain conformation could be originated only from change of 7S and 11S globulin structures by the AOT reverse micelle. At least four factors contributed to the changes of side and main chain conformation environment of 7S and 11S globulins. First, the unusual properties of water localised in the interior of reverse micelles (Matzke et al., 1992) could bring about stronger interaction with the charged groups of amino acid on surface of the neighbouring 7S and 11S globulin molecule than that of the aqueous buffer extraction. The strong interaction of aqueous buffer solution with the molecule of 7S and 11S globulins might increase the effect of hydrogen bonding, which caused a decrease in band frequency of main and side chain conformation hydrogen bonding in model systems (Chang et al., 1994). Second, the interaction of AOT and 7S, 11S globulins could change not only the structure of 7S and 11S globulins, but also the band frequency in main and side chain conformation. Third, the contact of 7S and 11S globulin molecule with isooctane in the process of solubilisation, could also change the structure of 7S and 11S globulins. Last but not least, the state of water and water-head group interaction in AOT/isooctane microemulsion could also change the structure of 7S and 11S globulins (Christopher, Yarwood, Belton, & Hills, 1992). Furthermore, solvent (such as, isooctane) of low dielectric constant could also shift the band to a lower frequency. The co-effect of these factors had changed the main and side conformation environment, which either made most of the bands shift to lower frequencies or stay unchanged.

3.3. The main chain conformation analysis of 7S and 11S globulins

Raman spectra of main chain conformation of proteins were complex and various, since the C–C, C–N and –CONH– vibrations were the most important parts to study the main chain conformation of proteins, such as amide A and B bands, amide I, II, III, IV, V, VI and VII bands (Tu, 1986). Among several distinct vibrational modes of the –CO–NH– amide or peptide bond, the amide I and III bands were the most useful to determine the secondary structure of proteins (Li-Chan, 1996). Raman spectra of 7S and 11S globulins using aqueous buffer (Fig. 2A and B) and AOT reverse micellar extraction (Fig. 2C and D) showed distinct features. Detailed information could be obtained by analysing the deconvoluted spectra. In this paper, all band assignments and calculations of secondary structures for 7S and 11S globulins were based on the second-derivative Raman spectra.

The intensity analysis of the secondary structures in proteins could be achieved by many methods. Among which, Lippert method had been proved to be reliable (Lippert, 1976). According to the investigation of Tu (1986), the fingerprint region of the second structure of amide I and III bands was known. The bands and intensities of the secondary structures in amide I and III bands of 7S and 11S globulins using two extraction methods are shown in Table 2. The intensities of some secondary structures showed statistically significant ($P < 0.05$), some were not. For 7S globulin using AOT reverse micellar extraction, the frequencies of α-helix and β-sheet structures in amide I bands were unchanged, the bands of turn structure did not occur at 1681 and 1688 cm^{-1} in comparison with aqueous buffer extraction. The intensities of β-sheet and turn structures in amide I bands for 7S globulin using aqueous buffer and AOT reverse micelle extraction at 1667 and 1684 cm^{-1} were 6.981, 7.475 and 3.659, 4.160, respectively, which showed significant changes (Table 2). For 11S globulin using AOT reverse micellar extraction, the frequency of α-helix structure was occurred at 1646 cm^{-1}, β-sheet structure was unchanged. No unordered structure by aqueous buffer extraction (Table 2) was found in 7S and 11S globulins. While the bands by the reverse micellar extraction (Table 2) at 1662, 1661 and 1664 cm^{-1}, were assigned to unordered structure in the amide I region. In addition, the intensity of β-sheet structure in 7S and 11S globulins using two extraction methods was higher than α-helix, turn and unordered structures. The intensities of β-sheet structure of 11S globulin using aqueous buffer extraction at 1665 and 1668 cm^{-1} were 5.299 and 6.244, respectively; while the intensities of β-sheet structure of 11S globulin using AOT reverse micelle extraction were 7.577 and 7.368, respectively. The values were significant difference (Table 2). The intensities of turn structure of 11S globulin using two extraction methods at 1682 cm^{-1} were 3.862 and 4.820, respectively, which were significantly different ($P < 0.05$) (Table 2). These spectral features indicated that 7S and 11S globulins had a prevalent β-sheet structure. By second-derivative analysis, the percentages of α-helix, β-sheet, unordered and turn secondary structures in the amide I region of 7S and 11S globulins using two extraction methods are listed in Table 3. Based on least squares analysis of amide I region, in aqueous solution, the 7S globulin contained 19.2% α-helix, 44.6% β-sheet, 35.5% turn, and no unordered structures; 11S globulin contained 20.4% α-helix, 41.2% β-sheet, 29.2% turn, and no unordered structures (Table 3). Zhao, Chen, Xue, et al. (2008) reported 17.0% α-helix, 47.3% β-sheet, 35.9% turns, and no unordered structures for 11S globulin in aqueous buffer by FTIR spectroscopy, and 16.5% α-helix, 46.7% β-sheet, 35.9% turn and no unordered structures for 7S globulin. The observed secondary structures of 7S and 11S globulins using Raman spectroscopy (Table 3) showed the reported similar values in the literature for 7S and 11S globulins. While in AOT reverse micelle, the 7S globulin contained 15.6% α-helix, 41.7% β-sheet, 27.5% turn, and 14.7% unordered structures, and the 11S globulin contained 21.2% α-helix, 41.3% β-sheet, 19.7% turn, and 16.8% unordered structures (Table 3). In comparison, Zhao, Chen, Xue, et al. (2008) investigated the FTIR spectra of 7S and 11S globulins using reverse micelle extraction, and reported

Table 2
Raman bands and intensity of 7S and 11S globulins in amide I and amide III bands.

	Globulins			7S[a]	7S[b]	11S[a]	11S[b]
Amide I bands	Secondary structure of protein	α-Helix	Band (cm^{-1})	1647, 1650, 1653, 1656, 1660	1647, 1651, 1653, 1656, 1659	1646, 1647, 1651, 1654, 1658	1649, 1650, 1654, 1656
			Intensity	3.596^{c}, 4.070^{c}, 4.994^{c}, 5.943^{c}, 6.687^{c}	3.584^{c}, 4.162^{c}, 4.794^{c}, 5.624^{c}, 6.032^{c}	3.142, 3.221^{c}, 4.122^{c}, 4.291^{c}, 5.736^{c}	3.689^{c}, 4.181^{c}, 4.912^{d}, 5.720^{c}
		β-Sheet	Band (cm^{-1})	1665, 1667, 1670, 1674, 1678	1665, 1667, 1670, 1674, 1679	1665, 1668, 1672, 1676, 1678	1666, 1669, 1673, 1676, 1678
			Intensity	6.797^{c}, 6.981^{c}, 6.684^{c}, 6.124^{c}, 5.197^{c}	7.127^{c}, 7.475^{c}, 6.887^{c}, 6.117^{c}, 5.832^{c}	5.299^{c}, 6.244^{c}, 6.333^{c}, 6.393^{c}, 4.603^{c}	7.577^{d}, 7.368^{d}, 6.920^{c}, 6.651^{c}, 5.905^{c}
		Turn	Band (cm^{-1})	1642, 1644, 1681, 1684, 1686, 1688, 1690	1642, 1643, 1684, 1687, 1690	1641, 1643, 1682, 1686, 1689	1642, 1644, 1682, 1686, 1690
			Intensity	2.461^{c}, 2.877^{c}, 4.325, 3.659^{c}, 3.154^{c}, 2.779, 2.014^{c}	2.337^{c}, 2.963^{c}, 4.160^{c}, 3.210^{c}, 2.929^{c}	2.629^{c}, 2.811^{c}, 3.862^{c}, 2.989^{c}, 2.023^{c}	2.317^{c}, 2.998^{c}, 4.820^{d}, 3.714^{d}, 2.548^{d}
		Unordered	Band (cm^{-1})		1662		1661, 1664
			Intensity		6.598		7.002, 7.030
Amide III bands	Secondary structure of protein	α-Helix	Band (cm^{-1})	1263, 1266, 1267, 1269, 1272, 1275, 1278, 1280, 1283, 1285, 1288, 1292, 1294, 1297, 1300	1266, 1270, 1272, 1274, 1276, 1278, 1281, 1285, 1288, 1291, 1293, 1296, 1300	1263, 1267, 1269, 1273, 1276, 1281, 1284, 1286, 1289, 1292, 1295, 1299	1269, 1273, 1275, 1278, 1279, 1281, 1283, 1286, 1289, 1292, 1294, 1298
			Intensity	3.346, 2.847^{c}, 2.528^{d}, 2.151^{c}, 2.360^{d}, 1.929^{c}, 2.148^{d}, 1.912^{c}, 1.070^{c}, 1.463^{c}, 1.087^{c}, 1.063^{c}, 0.681^{c}, 0.777^{c}	2.725^{c}, 1.725^{c}, 1.856^{c}, 2.077^{c}, 1.900^{c}, 1.845^{c}, 1.856^{c}, 1.811^{c}, 1.110^{c}, 1.750^{d}, 1.055^{c}, 0.563^{c}, 1.423^{d}	2.772, 2.510^{c}, 1.593^{c}, 1.753^{c}, 1.575^{c}, 1.167^{c}, 2.008^{d}, 1.752^{c}, 1.190^{c}, 1.223^{d}, 1.185^{c}, 1.542^{d}	2.828^{c}, 2.295^{d}, 2.277^{d}, 2.085^{d}, 1.900^{c}, 1.836^{c}, 1.783^{c}, 1.951^{c}, 0.906^{c}, 1.061^{c}, 1.010^{c}
		β-Sheet	Band (cm^{-1})	1232, 1235, 1238	1230, 1233, 1236, 1239	1230, 1234, 1237, 1240	1233, 1236
			Intensity	5.143^{d}, 4.810^{c}, 4.953^{c}	3.275, 4.621^{c}, 4.656^{c}, 4.611^{c}	2.602, 2.866^{c}, 2.806^{c}, 4.060	4.553^{d}, 5.034^{d}
		Turn	Band (cm^{-1})	1306	1305	1306	1304
			Intensity	1.686^{c}	1.938^{c}	2.650^{d}	1.309^{c}
		Unordered	Band (cm^{-1})	1241, 1245, 1250, 1254, 1257	1242, 1248, 1251, 1253, 1258	1242, 1247, 1250, 1255, 1258, 1260	1242, 1245, 1250, 1255, 1257, 1259
			Intensity	5.520^{c}, 5.540^{d}, 4.779^{d}, 3.412^{c}, 3.560^{c}	4.985^{c}, 4.585^{c}, 3.923^{c}, 3.415^{c}, 3.046^{c}	3.500^{c}, 3.111^{c}, 3.263^{c}, 2.851^{c}, 2.542^{c}, 2.414^{c}	5.568^{d}, 5.047^{d}, 4.729^{d}, 3.796^{d}, 3.714^{d}, 4.027^{d}

Mean values are shown from triplicate analyses.
[a] Globulins by aqueous buffer extraction.
[b] Globulins by AOT reverse micelle extraction.
[c,d] Indicate significant ($P \leqslant 0.05$) difference within a row.

the relative contribution of α-helix, β-sheet, turns, and unordered structures to be 14.5%, 45.7%, 23.8%, 14.7% and 17.0%, 47.3%, 19.3%, 16.5%, respectively. The observed secondary structures of 7S and 11S globulins had similar values to those used Raman spectroscopy. The results suggested that the Raman spectroscopy was a reliable method to study the secondary structure of proteins.

Compared with the structures of 7S and 11S globulins using aqueous buffer extraction, the percentage content of α-helix, β-sheet of 7S globulin and turn structures of 7S and 11S globulins in reverse micelle decreased, while the percentage content of 11S globulin α-helix and β-sheet structures did not change. The proportion of random coil structure was 14.7% and 16.8%, respectively. As shown in Table 3, the environment of AOT reverse micelles had influence on the α-helix, β-sheet, unordered and turn structures of 7S globulin, but the environment had no influence on the α-helix and β-sheet structures of 11S globulin except for unordered and turn structures. The results agreed with the investigation of Zhao, Chen, Xue, et al. (2008). Change of random coil and turn structures might have an effect on the conformation of 7S and 11S globulins, and affect some properties of 7S and 11S globulins, such as gel transparency, emulsification, which was evidenced by Zhao, Chen, Chen, et al. (2008).

It was necessary to investigate amide III region for 7S and 11S globulins using two extraction methods (Carey, 1982). As shown in Table 2, the intensities of α-helix, β-sheet and random coil structures for 7S globulin with AOT reverse micelle extraction were lower than those with aqueous buffer extraction except for turn structure, some difference were statistically significant ($P < 0.05$). The phenomenon attributed to some H-bonds of broken proteins and C–C bonds vibrations under the effect of AOT reverse micelle (Carey, 1982). The intensities of α-helix, β-sheet and random coil structures for 11S globulin with AOT reverse micelle extraction were higher than aqueous buffer extraction except for turn structure. The results also indicated that α-helix, β-sheet and random coil structures of 11S globulin were steady. By second-derivative analysis, the percentages of α-helix, β-sheet, unordered and turn structures in amide III region of 7S and 11S globulins are listed in Table 3. In contrast to the structures of 7S and 11S globulins using aqueous buffer extraction, the β-sheet and turn structure content of 7S globulin and unordered structure of 11S globulin using AOT reverse micelle extraction increased 30.6%, 33.0% and 33.7%, respectively, while the α-helix and unordered structure content of 7S globulin and α-helix, β-sheet and turn structures of 11S globulin decreased 6.3%, 13.4%, 6.3%, 33.0%, and 58.3%, respectively. The results indicated that the environment of AOT reverse micelle had influence on the secondary structures of 7S and 11S globulins in amide III region. In two extraction methods, the secondary structures of 7S and 11S globulins in amide III region were the prevalent α-helix and unordered structures.

Among other bands that characterised the Raman spectrum of 7S and 11S globulins, those falling at 890–945 cm^{-1} (α-helix region of γC–C), 945–960 cm^{-1} (unordered of γC–C), 1067–1130 cm^{-1} (γC–N), could be attributed to vibrations of alanine in 7S and 11S globulins. C–C bands of 7S and 11S globulins with aqueous buffer

Table 3
Quantification estimation of the secondary structure of 7S and 11S globulins in amide I and III bands by Raman spectra analysis.

Method	Secondary structure (%)							
	Globulin	Amide I band				Amide III band			
		α-Helix	β-Sheet	Unordered	Turn	α-Helix	β-Sheet	Unordered	Turn
Aqueous buffer extraction	7S	19.2 ± 3.1	44.6 ± 3.5	Zero	35.5 ± 2.1	38.1 ± 1.4	21.6 ± 1.1	37.9 ± 2.2	2.4 ± 0.6
Reverse micelle extraction	7S	15.6 ± 1.6	41.7 ± 3.3	14.7 ± 1.1	27.5 ± 2.7	35.7 ± 2.1	28.2 ± 1.3	32.8 ± 1.7	3.2 ± 0.6
Difference[a]		−18.7%	−6.4%	100%	−22.5%	−6.3%	+30.6%	−13.4%	+33.0%
Aqueous buffer extraction	11S	20.4 ± 2.1	41.2 ± 4.2	Zero	29.2 ± 2.3	36.3 ± 3.6	22.1 ± 1.8	36.8 ± 3.1	4.8 ± 0.2
Reverse micelle extraction	11S	21.2 ± 1.8	41.3 ± 3.9	16.8 ± 1.0	19.7 ± 1.5	34.0 ± 3.1	14.8 ± 1.1	49.2 ± 4.5	2.0 ± 0.1
Difference[a]		3.9%	0.4%	100%	−32.7%	−6.3%	−33.0%	+33.7%	−58.3%

[a] The difference of the percentage of secondary structure of 7S and 11S globulins.

extraction were 940 and 942 cm^{-1} (Fig. 2A and C), respectively, C–N bands were 1128 and 1127 cm^{-1} (Fig. 2A and C), respectively. While C–C and C–N bands of 7S and 11S globulins with AOT reverse micelle extraction were 951, 931 cm^{-1} and 1125, 1127 cm^{-1} (Fig. 1B and D), respectively. Be comparable with two extraction methods, the results showed that C–C band of 7S globulin using AOT reverse micelle extraction was shifted to higher wave frequency. It indicated that unordered structure increased (Lord & Yu, 1970), C–N band was shifted to lower wave number. However, C–C band of 11S globulin using AOT reverse micelle extraction was shifted to lower wave number, which indicated that α-helix conformation increased (Koening, 1978); C–N band did not change. Vibration of 1450 cm^{-1} (CH_2) did not change in two extraction methods. There were no significant differences in the intensities at 1450 cm^{-1} (CH_2). It indicated that the covalent bonds of 7S and 11S globulins using AOT reverse micelle extraction were not destroyed.

3.4. The side chain conformation analysis of 7S and 11S globulins

The Raman tyrosine (Tyr) doublet intensity at about 850–830 cm^{-1} was not significantly different in 7S globulin using two extraction methods (Fig. 2A and C), but by far less intense in 11S globulin using aqueous buffer extraction (Fig. 2B) than AOT reverse micelle extraction (Fig. 2D), which accounted for lower content of tyrosine in 11S globulin from aqueous buffer extraction. Much more interesting was the trend of the $I_{850/830}$ intensity ratio, which had been widely applied to describe the average hydrogen bonding state of the tyrosine phenoxyl groups in globular proteins (Lord & Yu, 1970; Tu, 1986). In fact, the $I_{850/830}$ ratio achieved its minimum value of about 0.3 when tyrosine residues were buried and the phenolic OH group acted as a strong hydrogen bond donor to an electronegative acceptor, such as carboxyl oxygen (Lord & Yu, 1970). When tyrosines were exposed at the surface of the proteins, the phenolic OH acted as both a donor and an acceptor of moderate hydrogen bonds, and the $I_{850/830}$ was approximately 1.25 (Lord & Yu, 1970). If the phenoxyl oxygen was an acceptor of a strong hydrogen bond from an electropositive group, such as a lysyl NH^+ group, and did not participate in significant hydrogen bond donation, the $I_{850/830}$ approached a presumed maximum value of 2.5. Referring to the spectra of 7S and 11S globulins using aqueous buffer extraction (Fig. 2A and B), the values of $I_{850/830}$ intensity ratio were 1.13 and 1.36, respectively. The spectra of 7S and 11S globulins using AOT reverse micelle extraction (Fig. 2C and D), the values of $I_{850/830}$ intensity ratio were 1.10 and 1.53, respectively. The lower values of 7S globulin, which approached that characteristic of phenolic OH groups acting as both donor and acceptor of moderate hydrogen bonds (Monti et al., 2007), could be better explained in terms of different amino acid composition rather than balance between buried and exposed tyrosine residues. The tyrosine residues in 7S and 11S globulins using two extraction methods could act as donor and acceptor of H-bonds. The values of $I_{850/830}$ intensity ratio of 11S globulin increased from 1.36 (aqueous buffer extraction) to 1.53 (AOT reverse micelle extraction), suggesting a change towards a more exposed state of tyrosine residues (Howell & Li-Chan, 1996). This feature was in good agreement with a more disordered conformation taken by the 11S globulin using AOT reverse micelle extraction.

Within the complex fingerprint of many peaks, some organic compounds possessed one or two rather strong bands that can be more confidently assigned to molecular structural features. The most recognised of these was probably the strong ring-breathing vibration in mono-substituted aromatic compounds, found at around 1000 cm^{-1}. Not surprisingly, this was one of the strongest Raman peaks in the spectrum of phenylalanine (Phe) (Lord & Yu, 1970). The bands of phenylalanine in 7S globulin using aqueous buffer and AOT reverse micelle extraction were 622, 1002 and 1003 cm^{-1} (Fig. 2A and C) in Raman, respectively, 622, 1032 and 1031 cm^{-1} (Fig. 2B and D) in 11S globulin, respectively. The location of 7S and 11S globulin using two extraction methods was unchanged. The intensities of phenylalanine bands for 7S globulin by aqueous buffer extraction were 0.075, 1.000 and 0.288, while 0.099, 0.720 and 0.277 by AOT reverse micelle extraction; the intensity of phenylalanine bands for 11S globulin by aqueous buffer extraction was 0.046, 1.000 and 0.194, while 0.106, 0.707, 0.174 by AOT reverse micelle extraction. The intensities of other bands for 7S and 11S globulins using AOT reverse micelle extraction except for the intensity of phenylalanine at 622 cm^{-1} decreased, which indicated that mono-substituted phenylcycle of phenylalanine using AOT reverse micelle might be destroyed.

Tryptophan (Trp) was strongly fluorescent in green excitation Raman (causing a rising baseline), but unlike fluorescence spectroscopy, the micro-Raman spectra were not affected to the point where they could not be measured easily. By comparing with the Trp fingerprint regions of 7S and 11S globulins using two extraction methods, in 7S and 11S globulins by AOT reverse micelle extraction, new peaks appeared in the Raman spectrum at 755, 744 and 756 cm^{-1} (Fig. 2C and D), respectively, which were assigned to tryptophan. The reason was attributed that H-bonds of 7S and 11S globulins might be changed in microenvironment of AOT reverse micelle.

Another clear characteristic Raman band in the amino acid family derived from the S–S stretching deformation in the (oxidised) dimerised cystine (Cys), was formed by bridging the thiol groups of two cysteine molecules (Carey, 1982). This generated an intense Raman signal peak at around 500 cm^{-1}. The exact position of the S–S band was clearly indicated to some extent by the conformation of the residues contributing the disulphide bridge and hence the three-dimensional structure of proteins, as shown in Fig. 2A–D. The S–S bands of 7S and 11S globulins using two extraction methods were 509, 504, 509 and 509 cm^{-1}, respec-

tively, which indicated that the AOT reverse micelle had not affect the C–C–S–S–C–C structure of S–S. The C–S stretching mode in the reduced monomer cystine, as well as reduced cystine-containing proteins, was found over a much wider wave number range about 580–740 cm^{-1}, depending on the backbone (primary) molecular structure as well as microenvironment (Monti et al., 2007). The vibration peaks of C–S in 7S and 11S globulins were 643, 644, 646 and 644 cm^{-1} (Fig. 2A–D), respectively. The results indicated that the structure of C–S using AOT reverse micelle extraction was unchanged.

4. Conclusions

By Raman spectroscopy, experimental results showed that the main chain conformation of 7S and 11S globulins by AOT reverse micelle extraction was different from those by aqueous buffer extraction. The relative amount of different secondary structures of 7S and 11S globulins by aqueous buffer extraction, which determined from amide I and amide III second-derivative based areas, agreed closely with the results determined by other method or reported in the literature. In comparison with the structures of 7S and 11S globulins using aqueous buffer extraction, the percentages of α-helix, β-sheet, turn and unordered structures from 7S and 11S globulins with using AOT reverse micelle extraction showed different changes. In view of widening the functional food utility of soybean proteins as food material, it is of interest to explore various chemical modification approaches able to complement the intrinsic outstanding properties of soybean proteins and to enhance its end-use performance. The results obtained in this study will form the framework for further functional property investigations aimed at demonstrating the functional utility of soybean proteins with reverse micelle extraction.

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