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Effects of Surfactant and Salt Species in Reverse Micellar Forward Extraction Efficiency of Isoflavones with Enriched Protein from Soy Flour

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Abstract Suitability of reverse micelles of anionic surfactant sodium bis(2-ethyl hexyl) sulfosuccinate (AOT) and sodium dodecyl sulfate (SDS), cationic surfactant hexadecyl trimethyl ammonium bromide (CTAB) and nonionic surfactant polyoxyethylene *p*-t-octylphenol (TritonX-100) in organic solvent isooctane for extraction of soy isoflavone-enriching proteins was investigated. The results showed that the order of combined isoflavone contents was SDS>CTAB>Triton X-100>AOT, while the order of protein recovery was SDS>AOT>TritonX-100>CTAB. As compared with ACN-HCl extraction, the total amount of isoflavones was lower than reverse micellar extraction. Ion strength was one of the important conditions to control extraction of isoflavone-enriching proteins with AOT reversed micelles. For the six salt systems, KNO_3, KCl, $MgCl_2$, $CaCl_2$, NaCl, and Na_2SO_4, extracted fraction of isoflavone-enriching proteins was measured. Salt solutions greatly influenced the extraction efficiency of isoflavones in an order of $KNO_3>MgCl_2>CaCl_2>KCl>NaCl>Na_2SO_4$, while protein in an order of $MgCl_2>CaCl_2>NaCl>KNO_3>Na_2SO_4>KCl$.

Keywords Reverse micelle · Extraction · Soybean flour · Protein · Isoflavone

Introduction

Study showed that soy protein-enriched isoflavones can result in positive vascular effects in healthy postmenopausal women [1]. The isoflavone profiles of soy-based products are known to be highly dependent on various chemical and physical treatments subjected to

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them [2]. There are many methods for the extraction of the isoflavones from soybean and soy products including organic solvent extraction with methanol [3], ethanol [4], acetonitrile [5] using simple mixing, soxhlet, or ultrasonification techniques for a few to several hours or supercritical fluid [6]. These procedures have some important drawbacks such as long extraction time, consuming large quantities of solvents, and making proteins denatured.

Reverse micelles are nanometer sized droplets of a polar solvent surrounded by layer of nonpolar or weakly polar solvent. In reverse micelles, the main driving forces responsible for the solute distribution between the organized assembly and the organic medium are considered to be mainly hydrophobic effects, hydrogen bonding interactions, and electrostatic interactions, all of which may affect the extraction efficiency of soybean isoflavones. Since reverse micelles are water-in-oil microemulsion droplets stabilized by surfactants in apolar system [7], they are able to uptake both hydrophobic and hydrophilic isoflavone conjugates from soy flour during forward extraction. Hydrophillic isoflavones tend to be solubilized within the water core of the reversed micelles, while lipophilic isoflavones can either stay in the interface or even partially exposed to the organic phase. A few reports were about the investigation of the factors affecting the extraction efficiency of proteins by reverse micelles [8–10] and only few reports are available on the application of reverse micelles in soybean isoflavone extraction [11]. The surfactant aggregates in reverse micelles are thus closely packed globules where the polar head group of the surfactant molecules occupies the interior of the aggregates whereas the hydrophobic tails extend into the bulk apolar solvent, with water encapsulated in compartments [7]. The surfactants in reverse micelles can change the interfacial tension and increase the solubility, mobility, bioavailability, and subsequent biodegradation of hydrophobic or insoluble organic compounds [12]. The results may be attributed to the hydrophobic or hydrogen bonding interaction between the surfactant molecules and the isoflavone or protein, and the electrostatic interaction between the isoflavone or protein and a surfactant layer at the interface. The salts added to the aqueous solution to adjust its ionic strength have a large influence on the isoflavone and protein extraction. For some salt systems, the order of the dependency for each salt corresponds to Hofmeister's series [13, 14]. In reverse micellar systems, this phenomenon may be attributed to the interaction between the direct isoflavones or proteins and ions. In water-poor media of reverse micelles with salt ions added, the binding to isoflavones followed the Hofmeister series of the ions present while an ion-exchange mechanism is seen in reverse micelles [15, 16]. Li et al. [17] found that the salting-out ability of different salts might also be related to the Gibbs energy of hydration of the ions, and the order of salting-out ability of the anions follows the Hofmerister series for the strength of the kosmotropic salts. These facts imply the importance of hydration of ions to discuss extraction behaviors in reversed micellar systems depending on the salt type. But, not much work has been reported about the effect of the surfactant and salt species in reverse micelle on the extraction efficiency of soybean isoflavones. So, it is important to study the effect of the surfactant and salt species on the soybean isoflavones.

Leser & Luisi [18] explored the possibility of utilizing AOT reverse micelle for the extraction of proteins from oil-rich vegetable meal, such as the sunflower and soybean. The procedure of protein and oil extraction from vegetables by reverse micelles consisted of two steps: a forward extraction and a backward extraction. Forward extraction (proteins from vegetables and oil to micellar solutions) is achieved by adjusting experimental conditions to let the partition favor the transfer of proteins into the organic micellar phase, while the backward extraction was a process that could recover the solubilized protein and oil from the reverse micellar solution. The use of reverse micelles to simultaneous extract oil and protein from soybean is attractive since soybeans represent one of the major oilseeds for producing edible oils [18]. Soy proteins using reverse micelle extraction have been considered in detail about their structures, stability, and textural properties [19, 20]. Very limited information is available on investigation of the extraction of isoflavones from soybean flour using reverse micelles.

In this article, the dry-addition method was used to dissolve isoflavone-enriching proteins in different reverse micellar solutions, namely, anionic surfactant AOT and SDS, non-ionic surfactant TritonX-100, and cationic surfactant CTAB. In present study, we aimed to study the effect of surfactant and salt species on the amount of soy proteins and isoflavones forward-extracted from reverse micellar systems. This could allow the development of functional food product with a higher content of biologically active substances. We have compared the calculated results of the isoflavone and protein contents in the reverse micellar solution extraction with acetonitrile–HCl (ACN–HCl) extraction.

Materials and Methods

Materials

Daidzein, glycitein, genistein, daidzin, glycitin, genistin, 6″-*O*-malonyl-daidzin, 6″-*O*-malonyl-glycitin, 6″-*O*-acetyl-glycitin, and 6″ -*O*-malonyl-genistin, acetyl daidzin, and acetyl genistin were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). AOT, SDS, TritonX-100, and CTAB were obtained Sigma Chemical Company. BCA protein assay kit was purchased from Pierce Biotechnology, Inc., IL, USA. All reagents and water used in this study were of HPLC grade. All other chemicals were of analytical grade. Soybean (No.8) was from local market, Beijing. The soy flour consisted of 37.53% protein, 7.99% moisture, 20.75% oil. All values are given in weight percent (wt.%) of the total weight of the flour.

Preparation of Reverse Micellar Systems and Isoflavone Extraction

AOT reverse micelle was used to isolate protein from soy flour [21]. The reverse micellar systems were formed by AOT, isooctane, and salt solutions. Stock solution of 0.05 M AOT was obtained first by AOT mixing with isooctane and stirring at room temperature. When AOT dissolved completely, KCl, $MgCl_2$, $CaCl_2$, NaCl, Na_2SO_4, and KNO_3 solutions were added with certain concentrations (0.05 mol/L) and pH (7.0), respectively. The water content in reverse micelles was usually expressed as W_0, the molar ratio of water to surfactant, i.e., $W_0 = [H_2O]/[AOT]$. By fixing AOT concentration (0.05 M) and adding different amounts of salt solutions (the desired value of W_0=18, 21, 21, 20, 22, and 20, respectively), W_0 was adjusted. The water content was determined by the Karl–Fischer method. Then, the forward extraction procedure was prepared by adding soybean flour to the AOT reverse micellar system (1:20, *w*/*w*). Solubilization was conducted in a magnetically agitated Erlenmeyer flask for 30 min at 45 °C. The resulting mixture was centrifuged at 6,000 rpm for 10 min; the clear supernatant was named the forward-extraction solution. In the end, the volume of supernatant was measured. The protein content in the reverse micellar system was determined with BCA method (microplate procedure, microplate reader 550: Japan Bio-RAD Company) [22]. For measuring the isoflavone content, the supernatants were filtered through a 0.45-μm syringe filter prior to HPLC analysis.

In the case of SDS, Triton X-100, and CTAB, microemulsions can only be obtained with the addition of a cosurfactant such as a short-chain octanol, while the choice of the compositions was based on the phase water-1-octanol-SDS–isooctane, water-1-octanol-Triton X-100-isooctane, and water-1-octanol-CTAB-isooctane. The concentrations of SDS, Triton X-100, and CTAB were 0.05 M, 0.05 M, and 0.06 M, respectively, and the molar ratios of cosurfactant to SDS, Triton X-100, and CTAB were kept constant (isooctane: cosurfactant, 4:1, *v/v*). The total amount of water was adjusted to give the desired value of the ratio W_0=25, 22, and 21, respectively.

Determination of Isoflavone Contents in Soy Flour by ACN–HCl Extraction

ACN–HCl extraction was a conventional extraction method for isoflavones. Isoflavones in soy flour were extracted using the ACN-HCl method of Jackson et al. [23]. Exactly 0.6000 g of samples was weighed into 125-mL flat-bottom flasks and 20 mL extraction solution (ACN, 0.1 mol/L; HCl in 5:1 ratio) was added to each flask. The solution was mixed using a rotary shaker (120 rpm) 4 h at room temperature. Extracts were suction-filtered into 250-mL round bottom flasks through Whatman no. 42 filter paper and washed twice with 20 mL extraction solution. Samples were condensed to approximately 1 mL using a vacuum rotary evaporator at 30°C, redissolved in 80% methanol (*v/v*) to a final volume of 10 mL and filtered through a 0.45-μm syringe filter prior to HPLC analysis.

High Performance Liquid Chromatography

Analysis of isoflavones was based by a method described by Wang & Murphy [5] with some slight modification. A LC-10ATvp liquid chromatography (Shimadzu) equipped with a Diamonsil HPLC column (5 μm, 250×4.6 mm id, Dikma Inc., Beijing, China,) and an ultraviolet spectrophotometer were used. The column temperature was 40°C, and the absorption was measured at 254 nm. The mobile phases for HPLC consisted of solvent (A) 0.1% (*v/v*) acetic acid in filtered Milli Q water, and (B) 0.1% (*v/v*) acetic acid in ACN. The solvent B gradient was as follows: solvent B original concentration was 15%, then increased from 15% to 25% over 35 min, then increased to 26.5% within the next 12 min, and finally to 50% within 30 s prior to being held for 14.50 min. The flow rate was 1mL/min up to 48 min and was increased to 1.3 mL/min within 30 s and held until 63 min, namely, the analytic time was 63 min for sample. The sensitivity was 0.02 AUFS, and the volumn of injection sample was 20 μL. Isoflavones were identified based on the retention times of all 12 standard compounds. Quantitative data for each isoflavone was obtained by comparison to known standards. Calibration curves were constructed from the peak height counts. The correlation coefficients were above 0.999, and relative standard deviations were 0.3–7.8% (*n*=3). All measurements were carried out in triplicate.

Statistical Analysis

Analyses of variance using the general linear models were conducted and were averaged and reported along with the standard deviation (±SD). The differences in mean were calculated using the Duncan's multiple-range tests for means with 95% confidence limit ($p \leq 0.05$). Statistical analysis of the data was done using the SAS software (SAS Institute Inc.; Cary, NC, USA).

Results and Discussion

Changes in the Combined Contents of Isoflavone-Enriching Proteins and the Mass Distribution Profile of Isoflavone Isomers in Forward-Extraction

Forward-extraction experiments were performed by contacting a solid feed with a fresh organic solution containing water at the conditions. Percentages of soy proteins in different forward-extraction solutions were shown in Fig. 1. In forward-extraction, approximately 67.6%, 72.9%, 46.2%, and 50.6% of soy proteins was transferred into the AOT, SDS, Triton X-100, and CTAB reverse micelles, respectively. The extraction ratio of proteins was greatly influenced by the surfactant species, and the order was SDS>AOT>CTAB>Triton X-100. The forward-extraction was governed primarily by electrostatic interactions between the charged proteins and polar head of surfactants [7] and was also affected by hydrophobic interactions between the non-polar region of the proteins and surfactant tails [24]. The result indicated that the interaction between soybean proteins and surfactants was a dominant factor influencing the protein recovery in reverse micelles. The reason might be attributed to the environment changes of soy protein [24].

The contents of isoflavones in four reverse micellar solutions were analyzed using HPLC method. The sampling amount was adjusted so as not to exceed the calibration range.

The content of isoflavones was calculated to construct calibration curves. The distributions of various isoflavone conjugates extracted in the different forward-extraction were shown in Table 1. The results showed that isoflavones could be solubilized in reverse micelles by the hydrophobic, hydrogen bonding, and electrostatic interactions between reverse micellar solution and isoflavones [25]. The order of combined isoflavone contents in forward-extraction of four reverse micelles was SDS (1147.66 μg/g)>CTAB(859.65 μg/g)>Triton X-100(669.92 μg/g)>AOT (489.33 μg/g). It could be concluded from experiment that the total content of proteins or isoflavones in SDS reverse micelle were significantly higher ($P<0.05$) than in AOT, Triton X-100, and CTAB reverse micelles. While the combined content of isoflavones in AOT reverse micelle was significantly lower ($P<0.05$) than in the SDS, CTAB, and Triton X-100 reverse micelles. In the forward-extraction of AOT reverse micelle, the content of daidzein was 76.53 μg/g, which was significantly higher ($P<0.05$) than in SDS, Triton X-100, and CTAB reverse micelles. While the contents of daidzin, glycitin, *6″-O*–malonyl-genistin, *6″-O*–malonyl-glycitin, *6″-O*-acetyl-daidzin, *6″-*

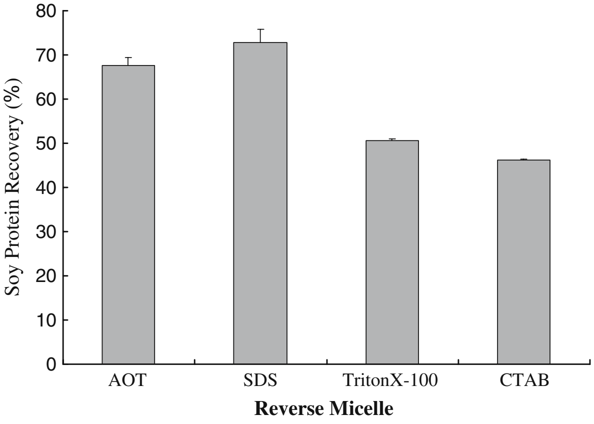

Fig. 1 Effect of reverse micelle composition on soybean protein recovery

Table 1 Effects of different extraction methods on isoflavones from soybean flour (μg/g; mean±SD, *n*=3).

Isoflavones		Reverse micelle type				ACN-HCl extraction
		AOT	SDS	Triton X-100	CTAB	
Glucoside	Din	74.55 ± 2.25^{a}	187.39 ± 4.01^{a}	167.93 ± 4.89^{a}	183.37 ± 5.12^{a}	120.94 ± 5.12^{a}
	Gin	69.94 ± 2.67^{a}	86.08 ± 2.45^{b}	118.24 ± 4.98^{a}	161.68 ± 3.96^{a}	54.53 ± 2.17^{b}
	Glin	66.71 ± 2.35^{b}	162.40 ± 4.69^{a}	154.21 ± 5.78^{a}	160.31 ± 4.73^{a}	2.76 ± 1.01^{c}
Malonyl	Din	Nd	Nd	83.36 ± 0.55^{b}	97.30 ± 0.87^{a}	59.33 ± 2.31^{b}
	Gin	20.65 ± 2.23^{c}	72.05 ± 4.25^{b}	22.21 ± 1.25^{c}	Nd	Nd
	Glin	59.32 ± 3.47^{b}	289.94 ± 7.28^{a}	Nd	Nd	37.98 ± 3.15^{c}
Acetyl	Din	Nd	60.86 ± 3.67^{b}	44.94 ± 4.31^{c}	49.56 ± 2.19^{b}	35.55 ± 3.11^{c}
	Gin	59.68 ± 2.27^{b}	80.82 ± 3.43^{b}	Nd	Nd	65.78 ± 5.05^{b}
	Glin	Nd	33.22 ± 1.16^{c}	Nd	Nd	Nd
Aglycone	Dein	76.53 ± 6.33^{a}	60.86 ± 3.67^{b}	Nd	Nd	8.05 ± 2.25^{c}
	Gein	51.95 ± 2.31^{b}	80.82 ± 3.43^{b}	79.03 ± 4.31^{c}	207.43 ± 8.09^{a}	90.68 ± 6.23^{a}
	Glein	Nd	33.22 ± 1.16^{c}	Nd	Nd	Nd
Combined		489.33	1,147.66	669.92	859.65	474.66

Values in the same line with different letters were significantly different ($p<0.05$). Abbreviations: *Din* daidzin, *Gin* genistin, *Glin* glycitin; *Dein* daidzein, Gein, genistein, and *Glein*, glycitein, *Nd* not detected.

O-acetyl-genistin, *6″-O*-acetyl-glycitin, daidzein, and glycitein in forward-extraction of SDS reverse micelle were a large yield of 187.39, 162.40, 72.05, 289.94, 60.86, 80.82, 33.32, 60.86, and 33.22 μg/g significantly higher ($P< 0.05$) than in other reverse micelles, respectively, but some higher contents of daidzin (183.37 μg/g), genistin (161.68 μg/g), *6″-O*–malonyl-daidzin (97.30 μg/g), and *6″-O*-aglucon-genitin (207.43 μg/g) were in the forward-extraction of CTAB reverse micelle (Table 1). The results indicated that the surfactant species and cosurfactant (octanol) in reverse micellar systems could affect the content of soybean isoflavone conjugates.

According to the above results, the contents of proteins and isoflavones in SDS reverse micelle were significantly higher than in other reverse micelles. Detailed information could be obtained by analyzing the contents of isoflavone conjugates using reverse micelle and ACN-HCl extraction.

Comparison on Amount of Isoflavones Extracted Using ACN-HCl and Reverse Micelles

To determine the amount of isoflavones in soy flour, isoflavones was first extracted using the conventional ACN-HCl extraction method of Jackson et al. [23] prior to measurement. Amount of various isoflavone conjugates from soy flour recovered in ACN-HCl extraction and forward-extraction solutions were shown in Table 1. It was interesting to note that the amounts of genistin, glycitin, *6″-O*-malonyl-genistin, *6″-O*-malonyl-glycitin, *6″-O*-acetyl-genistin, daidzein, and aglucon-daidzein extracted using reverse micellar solutions were significantly many-fold higher ($P< 0.05$) than those in ACN-HCl solution. The ACN-HCl extraction method failed to recover *6″-O*-malonyl-genistin, *6″-O*- acetyl-glycitin, and aglucon-glycitein from soy flour, but the three isoflavone conjugates were detected in the SDS reverse micellar solution.

The choice of solvents for extracting isoflavones was crucial in determining the types and amounts of isoflavones. Isoflavones were a group of compounds with diverse chemical and physical properties such as water solubility, hydrophobicity, and connectivity index [26]. For instance, hydrophobicity of isoflavone forms was aglycone>acetyl-β-glucoside>malonyl-β-glucoside>β-glucoside [27]. At least four factors contributed to the changes of soybean isoflavones in reverse micelles. Firstly, the unusual properties of water localized in the interior of reverse micelles could bring about stronger interaction with the charged groups of isoflavones [28]. Secondly, the interaction of surfactants in reverse micelles and isoflavones could affect the content of isoflavones. The third factor, the contact of isoflavone molecule with solvent in the process of solubilization could also affect the isoflavone contents [25]. Last but not the least, the state of water and water–head group interaction in reverse micelle microemulsion could also change the isoflavone contents from soybean flour. Rostagno et al. [4] found that certain amount of water was necessary in the extracting solvent in order to improve the extraction ratio of isoflavones from soy flour due to the relative polarity of these compounds. The co-effect of these factors had changed the extraction environment, which either made the content of the isoflavones and proteins from soybean flour changed. Moreover, addition of acid to ACN lower the amounts of malonyl-β-genistin, daidzein, genistein, and total isoflavones extracted from various soy products [29]. Furthermore, the instability for malonyl form of isoflavones and tendency to decarboxylate with time after extraction could be a result for the failure of ACN-HCl extraction method to detect *6″-O*-malonyl-genistin and *6″-O*-malonyl-glycitin in soy flour [23]. Hence, reverse micellar solutions might be better than ACN-HCl to extract isoflavones with higher diversity and quantity.

Effect of Salt Species on Isoflavones and Proteins in Reverse Micelle

Ronnie et al. [30] reported that different ions in reverse micelles could lead to different distribution of proteins. Figure 2 showed the protein recovery of forward-extraction under various salts; ionic strengths were adjusted by adding different salt solutions in the aqueous phase, such as $MgCl_2$, Na_2SO_4, KNO_3, $CaCl_2$, NaCl, and KCl, respectively. It was found that the forward-extraction efficiency was different for different salt solutions. The contents of protein in the forward-extraction of reverse micelle with adding $MgCl_2$, Na_2SO_4, KNO_3, $CaCl_2$, NaCl, and KCl solutions were 69.3%, 56.2%, 57.8%, 67.6%, 61.8%, and 54.9%, respectively. The forward-extraction efficiency was greatly influenced by the cation species and the order of extraction efficiency was NaCl>KNO_3>Na_2SO_4>KCl for monovalent

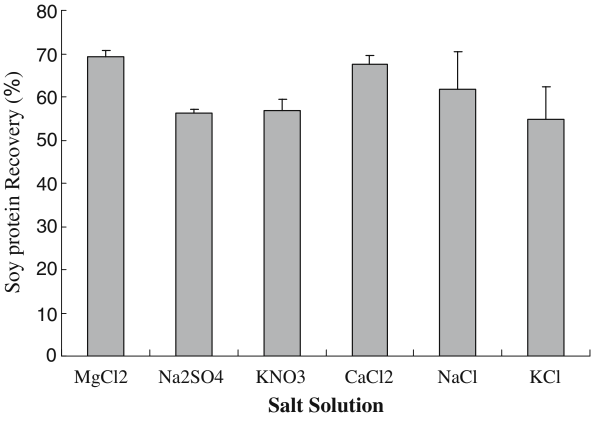

Fig. 2 Effect of salt solution in AOT reverse micelle on soybean protein recovery

cations and $MgCl_2$>$CaCl_2$ for divalent cations. The extraction efficiency was totally higher for divalent cations than for monovalent ones. The results were supported the investigation of Kinugasa et al. [31]. The reasons might be attributed to electrostatic effect of different ions. The ionic strength of the aqueous phase was closely related to the degree of shielding of electrostatic potential imposed by a charged surface and subsequently affected the protein separation process [24].

The interactive effect of salt species on the isoflavone contents in the forward-extraction of AOT reverse micelle was shown Table 2. With the addition of salts, the order of the amount of isoflavones was KNO_3(862.91 μg/g)>$MgCl_2$(742.39 μg/g)>$CaCl_2$ (594.54 μg/g)>KCl (489.33 μg/g)>NaCl(473.70 μg/g)>Na_2SO_4(439.89 μg/g). It was noted that the total amounts of isoflavones were higher for monovalent ones than for divalent cations except for using KNO_3 (Table 2). The fact that its order was consistent with that of extraction efficiency indicated the possibility of the influence of the size exclusion effect on isoflavone extraction provided that the size of reverse micelles did not change before and after extraction. In reverse micellar systems using KNO_3, the content of combined isoflavones was significantly higher ($P<0.05$) than using other salts. Some isoflavone conjugates were significantly different ($P<0.05$), some were similar. A different trend occurred in reverse micelle containing different salt solutions, with a large yield of 81.88, 210.37, 44.18, 64.30, and 107.77 μg/g in reverse micelle containing KNO_3 for *6″-O*-malonyl-daidzin, *6″-O*-malonyl-glycitin, *6″- O*-acetyl-genitin, *6″-O*–acetyl-dadzein, and aglycone-genistein, respectively 141.70, 97.33, 84.80, and 47.37 μg/g in reverse micelle containing $CaCl_2$ for glucoside-daidzin, gucoside-glycitin, *6″-O*-acetyl-daidzin, and glycitein, respectively, but a high content of *6″-O*-malonyl-genitin (54.52 μg/g) in reverse micelle containing NaCl. The results indicated that the isoflavone conjugates were sensitive to salts in reverse micelle. The change of isoflavone solubilization in the presence of salts might be due to two possible factors. First, electrostatic screening reduced the electrostatic interaction between polar part of the

Table 2 Effects of different salt solutions in AOT reverse micelle on soybean flour isoflavones (μg/g; mean±SD, $n=3$).

Isoflavones		Salt solution in AOT reverse micelle					
		$MgCl_2$	Na_2SO_4	KNO_3	$CaCl_2$	NaCl	KCl
Glucoside	Din	83.94 ± 3.47^{d}	80.80 ± 5.63^{e}	132.90 ± 5.15^{b}	141.70 ± 4.29^{a}	88.76 ± 3.73^{c}	74.55 ± 2.25^{a}
	Gin	75.98 ± 2.53^{a}	72.81 ± 2.61^{c}	64.41 ± 4.58^{a}	64.68 ± 3.65^{a}	73.33 ± 2.61^{c}	69.94 ± 2.67^{a}
	Glin	104.91 ± 4.89^{b}	76.27 ± 3.77^{c}	84.86 ± 3.78	97.33 ± 5.92^{a}	107.26 ± 5.79^{b}	66.71 ± 2.35^{b}
Malonyl	Din	53.30 ± 2.11^{c}	73.04 ± 4.47^{b}	81.88 ± 6.43^{a}	Nd	Nd	Nd
	Gin	Nd	Nd	Nd	Nd	54.52 ± 0.27	20.65 ± 2.23^{c}
	Glin	206.87 ± 9.13^{b}	$72,20\pm7.68^{c}$	210.37 ± 10.27^{a}	54.75 ± 6.56^{d}	Nd	59.32 ± 3.47^{b}
Acetyl	Din	67.68 ± 5.71^{c}	21.44 ± 1.08^{e}	44.18 ± 2.26^{d}	84.60 ± 5.96^{a}	75.28 ± 3.95^{b}	Nd
	Gin	66.22 ± 5.67^{b}	28.43 ± 1.17^{f}	64.30 ± 6.48^{a}	47.37 ± 4.45^{d}	38.83 ± 4.15^{e}	59.68 ± 2.27^{b}
	Glin	Nd	Nd	Nd	Nd	Nd	Nd
Aglycone	Dein	83.49 ± 6.79^{b}	14.90 ± 0.11^{e}	72.24 ± 6.02^{a}	39.86 ± 1.78^{c}	35.72 ± 1.66^{d}	76.53 ± 6.33^{a}
	Gein	Nd	Nd	107.77 ± 5.08^{a}	Nd	Nd	51.95 ± 2.31^{b}
	Glein	Nd	Nd	Nd	64.50 ± 3.57^{a}	Nd	Nd
Combined		742.39	439.89	862.91	594.54	473.70	489.33

Values in the same line with different letters were significantly different ($p<0.05$). Abbreviations: *Din* daidzin, *Gin* genistin, *Glin* glycitin, *Dein* daidzein, *Gein* genistein, *Glein* glycitein, *Nd* not detected surfactant and charged groups. The reduced protein solubilization at higher ionic strength had been reported by other studies [32]. Second, the inverse relationship between salt concentration and micelle size was reported in reverse micelles formed by anionic surfactants such as AOT [21], and the reduced micelle size possibly resulted in change of isoflavone solubilization. It indicated that the decreased isoflavone solubilization in the presence of salts was probably due to an electrostatic screening effect rather than reduced micelle size and resulted in decreasing the content of isoflavones. Noh & Imm [33] supported this view.

Table 2 showed that the effect of anion species on the extraction of soy isoflavones. The effect of anions was less significant than that of cations. Also in this case, the isoflavone extraction was relatively sensitive to the anion species and the order of extraction easiness was $NaCl>Na_2SO_4$ and $KNO_3>KCl$. This was consistent with the lyotropic series of anions. That was, anions brought about a hydrophobic effect and the isoflavone extraction became easier in a system containing anions with high salting out effect. Because of this, the extraction of higher hydrophilic isoflavone was less influenced by the salting out effect and the anion species. Kinugasa et al. [31] also observed the effect of anion hydrophobicity on extraction ratio of protein. The results indicated that the cation and anion could affect the isoflavone contents in reverse micelle solution, as well as the protein recovery in reverse micelle solution [34].

Conclusions

This study pointed to the potential of reverse micelles as a new analytical extraction method for isoflavones from soy sample. By reversed micelles, the proteins and isoflavones from soybean flour could simultaneously be extracted. The extraction efficiency of isoflavones was greatly influenced by surfactants in the order of SDS>CTAB>Triton X-100>AOT, while the order of protein recovery was SDS>AOT>Triton X-100>CTAB. The effect of divalent cations on the extraction efficiency of isoflavones was smaller than that of monovalent cations except for KNO_3 solution and was in the order of $KNO_3>MgCl_2>CaCl_2>KCl>NaCl>Na_2SO_4$. The extraction efficiency of protein was greatly influenced by the salt species in order to $NaCl>KNO_3>Na_2SO_4>KCl$ for monovalent cations and $MgCl_2>CaCl_2$ for divalent cations. The extraction efficiency of proteins was totally higher for divalent cations than for monovalent ones. The effect of anion species on the extraction efficiency of proteins or isoflavones was smaller than that of cation species. This was consistent with the lyotropic series and indicated the important relation between the salting out effect and isoflavone or protein extraction. By modifying surfactant and ionic strength in reverse micelles, soy isoflavone-enriching proteins could be produced from soy flour.

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