J Am Oil Chem Soc (2009) 86:409–417
DOI 10.1007/s11746-009-1367-6

ORIGINAL PAPER

Fractionation of Soybean Globulins Using Ca^{2+} and Mg^{2+}: A Comparative Analysis

Zi Teng · Chong Liu · Xiaoquan Yang · Lin Li · Chuanhe Tang · Yueming Jiang

Received: 4 August 2008 / Revised: 25 February 2009 / Accepted: 2 March 2009 / Published online: 24 March 2009

Abstract The substitution of $CaCl_2$ by $MgCl_2$ was undertaken in Deak's two-step process of separating the soybean 11S and 7S globulins, aiming at higher purities and lower phytic acid (PA) contents of recovered protein fractions. The effects of pH and the addition of NaCl were also evaluated. Compared with $CaCl_2$, $MgCl_2$ reduced the PA content of the 11S-rich fraction by 63–71% but increased that of the 7S-rich fraction by 14–28%, depending on pH. Correspondingly, more Ca^{2+} was recovered in the 11S-rich fraction, while more Mg^{2+} co-precipitated with the 7S-rich fraction. NaCl increased the purity of the 11S-rich fraction and reduced its PA content, but the purity of the 7S-rich fraction was reduced by using 50–100 mM NaCl. Lowering pHs from 6.4 and 4.8 to 5.6 and 4.0 in the two precipitation steps increased the yield of both fractions. The optimized fractionating procedure was as follows: the 11S-rich fraction was precipitated at pH 5.8 by using 5 mM $MgCl_2$, 10 mM $NaHSO_3$ and 20 mM NaCl, followed by the precipitation of the 7S-rich fraction at pH 4.5. The new method provided both fractions with satisfactory protein yields (22% for 11S and 16% for 7S), purities (88% for 11S and 80% for 7S) and PA contents (0.356% for 11S and 0.882% for 7S).

Z. Teng · C. Liu · X. Yang (✉) · L. Li · C. Tang
Department of Food Science and Technology,
South China University of Technology, Guangzhou 510640,
People's Republic of China
e-mail: fexqyang@163.com; fexqyang@scut.edu.cn

Y. Jiang
South China Botanical Garden, Chinese Academy of Sciences,
Guangzhou 510650, People's Republic of China

Keywords Soybean protein · 11S globulin · 7S globulin · Fractionation · $CaCl_2$ · $MgCl_2$ · Phytic acid

Introduction

Soy globulins have become an attractive source of vegetable proteins in recent years due to their desirable digestibility and functionalities. Glycinin and β-conglycinin, often inexactly referred to as 11S and 7S globulins respectively according to their sedimentation coefficients, are the major two storage protein components in soybean seed. Glycinin is a hexametric molecule, and each subunit is composed of an acidic polypeptide chain (40 kDa) and a basic (20 kDa) one linked by a single disulfide bond [1]. β-conglycinin is a trimer which comprises of α′, α and β-subunits with MW of approximately 57, 57 and 42 kDa, respectively [2]. Because of their distinctive structures, individual soy globulin components exhibit significant differences in nutritional and functional properties. For instance, 11S globulin presents superior gel-forming ability while 7S globulin shows better solubility in aqueous system [3, 4]. Furthermore, increasing attention has been paid to the unique nutritional benefits of 7S globulin including triglyceride-lowering and anticarcinogenic functions [5–7].

One of the classical methods for separating soy globulins [8–11] is Nagano's three-step method, in which soy protein is extracted under mildly alkaline condition and its individual components are sequentially fractionated by precisely adjusting temperature, pH and ionic strength [8]. Accurate but complicated, this method has been extensively modified and optimized to meet the requirement of industrial production since the late 1990s [12–14]. For instance, Wu et al. [14] proposed a pilot-scale separating procedure based on Nagano's method and obtained from

soy flakes both 11S-rich and 7S-rich products with 90% purity. The solid yields were 12.9% for glycinin and 9.8% for β-conglycinin, respectively, suggesting that their protein recoveries were less than 50%. Wu's team [15] also proposed a modified method using ultrafiltration that claimed to increase the recoveries of the two soy protein fractions but at the expense of their purities.

Deak et al. [16] simplified the fractionating procedure to two steps by using $CaCl_2$ instead of NaCl as precipitating agent. The principle of this method is that divalent cations associate with the 11S soy protein prior to the 7S globulin at pH 6.4, probably because of the difference in their surface charge densities [16, 17]. This method noticeably increased the protein yields of both the 11S and 7S fractions (15.1 and 23.6%, respectively), but the purities were lower than those of Wu's and Nagano's methods. Furthermore, the addition of $CaCl_2$ produced significantly higher content of phytic acid (PA) [18], a bioactive chemical that functions as a major phosphorous store and an energy store in soy seeds [19]. The reason for the increase of PA content is that Ca^{2+} formed insoluble phytates with the negatively charged PA at pH 6.4 [20]. Additionally, Ca^{2+} also participates in the form of the PA–Ca^{2+}–protein ternary complex when pH is higher than 4.5, resulting in the co-precipitation of PA with soy protein [18, 21]. Since PA impairs the nutritional value of foods by impeding the absorption of mineral elements in the human body [19], it is important to reduce its content in soy protein products. Current methods of removing PA include phytase treatment and ion exchange chromatography [22, 23], but they are either complicated or costly.

Compared with calcium phytate, magnesium phytate is more soluble at pH 5.0–6.5 [20, 24]. This behavior implied that the substitution of Ca^{2+} by Mg^{2+} in the fractionating procedure might weaken the co-precipitation of PA, thus lowering the PA content in the product. Another effective method for improving the fractionation of soy globulins is by pH adjustment, as the solubilities of soy protein and PA are influenced greatly by pH [25]. In all of the current isolation methods [8, 14–16], glycinin and β-conglycinin were obtained at pH 6.4 and pH 4.8, respectively. However, it is still uncertain if the fractionation of 7S and 11S globulins is optimal at these pH values in the presence of $MgCl_2$.

The addition of NaCl is also a potential approach to lowering the PA content, because Na^+ disrupts the association of Ca^{2+} (and possibly Mg^{2+}) with soy protein and PA anion [17]. In addition, appropriate application of the salting-in/out effect of NaCl on soybean proteins may also benefit the process of separation [26].

The main objectives of the present study were to evaluate the effect of pH and Ca^{2+} and Mg^{2+} concentrations on the fractionation of soy globulins and to investigate the influence of NaCl on the separation procedure. The contents of PA and divalent cations in the isolated protein fractions were also evaluated.

Experimental Procedures

Materials and Reagents

Defatted white flakes were provided by Yuwang Industrial & Commercial Co., Ltd., Shandong, China. The flakes were ground and passed through a 60-mesh sieve to yield defatted soy flour (DSF). Reagents required for atomic absorption spectrometric (AAS) analysis and electrophoresis were of the corresponding grades. Other reagents were of analytical grade.

Isolation of Soybean Globulins

The control fractionating procedure was Deak's method [16] with minor modification (Fig. 1). About 240 g of DSF was extracted with deionized water at 15:1 (v/w) water-to-flour ratio, then the pH was adjusted to 8.0 with 2 N NaOH, and the resulting slurry was mechanically stirred for 1 h at 25 °C. After centrifugation at 3,000*g* for 15 min, the insoluble residue was discarded. Sodium bisulfite (SBS) and $CaCl_2$ was added to the supernatant to achieve the concentrations, respectively of 10 and 5 mM. The resulting slurry was adjusted to pH 6.4 and stirred for 1 h. The 11S-rich fraction was obtained as the precipitated curd after a centrifugation at 3,000*g* for 15 min, while the supernatant was then adjusted to pH 4.8 with 2 N HCl and stirred for 1 h. After the third centrifugation at 3,000*g* for

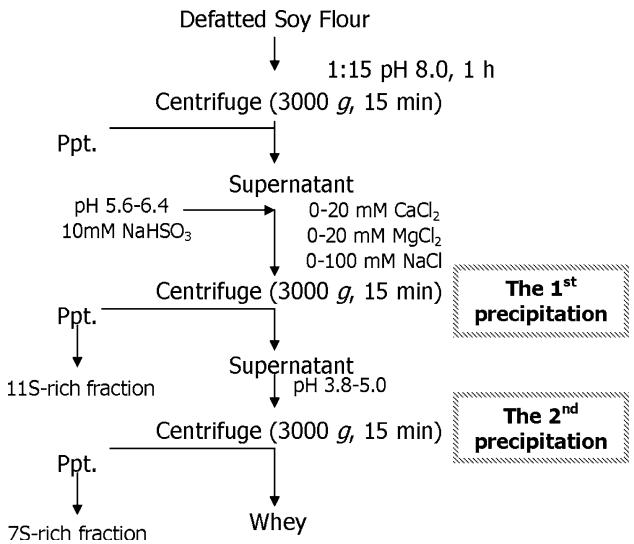

Fig. 1 Flow diagram for the procedure of fractionating soybean 7S/11S globulins

15 min, the precipitated phase was collected as the 7S-rich fraction. Both fractions were redispersed in deionized water and lyophilized. The protein contents of the samples were determined by Kjeldahl method ($N \times 6.25$).

The Effect of pH and NaCl in the Presence of Mg^{2+} or Ca^{2+}

Four experiments were conducted to examine the fractionation processes. Experiment 1 studied the influence of 5 mM $CaCl_2$ or $MgCl_2$ at various pH values (from 5.6 to 6.4) in the first precipitation step. Experiment 2 evaluated the effect of concentration of $CaCl_2$ or $MgCl_2$ (from 0 to 20 mM) on the fractionation. Experiment 3 assessed the efficiency of separation as influenced by 0 to 100 mM NaCl in the presence of $CaCl_2$ or $MgCl_2$. Experiment 4 examined the yields and purities of the 7S-rich fraction obtained at pH 3.8–5.0 in the second precipitation step. Each experiment was at least duplicated.

Analysis of Protein Constitution by SDS-PAGE

SDS-PAGE was performed with 12% separating gel and 4% stacking gel in a 1-mm vertical slab gel, according to the method of Laemmli [27]. Two milligram of each sample (protein basis) was dissolved in 500 μL reducing sample buffer (pH 6.8) containing 60 mM Tris–HCl, 2% SDS, 14 mM β-mercaptoethanol, 25% glycerol and 1% bromphenol blue. The solution was then heated in boiling water for 5 min. Five microliter of solution was loaded in each lane. Electrophoresis was performed at 40 mA at first, and then the current was increased to 80 mA when samples entered the separating gel. The gel was stained with Coomassie brilliant blue R-250 and destained in 7% acetic acid [methanol: acetic: water = 1:1:8 (v/v/v)]. The band patterns were then photographed and analyzed with Quantity One software version 4.4 (Bio-Rad Laboratories Inc., USA).

The purity of a single fraction was calculated according to Deak et al. [16]. Fraction purity (%) = (summed light intensity of bands corresponding to a designated fraction)/(summed light intensity of all bands) × 100. Glycinin and β-conglycinin subunit bands were confirmed by using purified standards produced according to the methods of O'Keefe et al. [28]. All measurements were at least duplicated.

Determination of Calcium and Magnesium

Each sample (0.500 g) was weighed and digested with mixed acid (75% HNO_3 and 25% $HClO_4$) at the boiling point of the mixture for 5 h. The residue of digestion was cooled down and then deionized water was added to a final volume of 25 mL. The resulting solution was then analyzed by AAS in Polarized Zieman AAS Hitachi Z-5000 Equipment (Hitachi Ltd., Japan) according to AOAC method 985.01 [29] to determine the contents of Ca or Mg.

The recoveries of divalent cations were calculated as follows:

$$\text{Recovery}(\%) = (m_S \times c_S)/(m_F \times c_F + m_A) \times 100$$

where

- c_S the mass proportion of Ca or Mg in the samples, g/g sample
- c_F the mass proportion in the starting flour, g/g flour
- m_A the mass of additional Mg^{2+} or Ca^{2+}, calculated as the additional molar concentration multiplied by 24 and 40 for Mg and Ca, respectively

Each determination was performed in triplicate.

Phytic Acid Determination

Phytic acid determination was carried out according to the method of Thompson et al. [30] with minor modifications. The sample (0.5 g) was extracted with 25 mL 1.2% (w/w) HCl, followed by centrifugation at 17,200*g* for 15 min. Two milliliters of the supernatant was digested as described in the preceding section and analyzed spectrometrically for the total phosphorus content [31]. An excess amount of $FeCl_3$ was added to the remaining supernatant. The mixture was then incubated at 97 °C for 75 min and then centrifuged at 17,200*g* for 15 min. The remaining supernatant was digested for the analysis of the inorganic P content. The conversion factor of the content of organic P (calculated as total P minus inorganic P) to that of PA content was 1/0.282. Analysis of each sample was performed in triplicate.

Statistics

All the data were averaged using Microsoft Excel 2007. Analysis of variance (ANOVA) was performed using Origin 7.5 (OriginLab Corp., USA).

Results and Discussion

Analysis of Starting Flour

The total protein content in the starting flour was 52.7% (dry basis). Glycinin and β-conglycinin represented 50.2 and 32.1% of the total protein, respectively. The PA content was 1.52%, which was in accordance with De Boland's report [32] but was 40% lower than that in Deak's study [18]. This result explained why the PA contents of our products obtained by the control method (1.24 and 0.75% for 11S and 7S, respectively, as shown in Table 1) were 40% lower than those of Deak's samples (1.89 and

Table 1 Distribution of divalent cations and phytic acid as influenced by pH

pH	$CaCl_2$ used				$MgCl_2$ used			
	PA (%)	Δc (PA) (%)[a]	Ca (%)	Ca rec (%)[a]	PA (%)	Δc (PA) (%)[a]	Mg (%)	Mg rec (%)[a]
11S-rich fraction								
5.6	0.657c	−47.0	0.156a,b	12.829a	0.457a	−63.1	0.135c	10.182a
5.8	1.071b	−13.6	0.171a	11.969b	0.356c	−71.3	0.146c	10.066a,b
6.0	1.039b	−16.2	0.141b	7.404c	0.398c	−67.9	0.160b	9.266b
6.4	1.240a	0.0[b]	0.170a	6.548d	0.409b	−67.0	0.182a	7.246c
7S-rich fraction								
5.6	1.162a	56.0	0.139a	2.673d	0.856b	14.9	0.157a	3.445d
5.8	0.763b	2.4	0.097c	3.308c	0.882b	18.4	0.148a	4.892c
6.0	0.765b	2.7	0.106b	4.565a	0.957a	28.5	0.132b	6.348b
6.4	0.745b	0.0[b]	0.121a	4.464b	0.847b	13.7	0.120c	7.797a

Different letters in the same column following each figure indicate significant differences at $P < 0.05$, $n = 3$

[a] Positive (or negative) values indicates that the PA content in the product is higher (or lower) than that of the sample obtained by the control method

[b] PA content of the samples produced by the control method

1.20% for 11S and 7S, respectively) [18]. The initial contents of Ca^{2+} and Mg^{2+} in the flour were 2.42 and 2.43 mg/g, respectively. These contents were within the range of concentrations reported by Porter [33].

Effect of $CaCl_2$ or $MgCl_2$ and pH in the First Precipitation Stage

The effect of $CaCl_2$ or $MgCl_2$ on the fractionation process largely depended on the pH applied in the first precipitating step. When the pH in this stage was adjusted from 6.4 to 5.6, the yield of the 11S-rich fraction doubled (Fig. 2). This was attributed to the sharp decrease in protein solubility when pH decreased [17, 25]. In contrast, the yields of the 7S-rich fraction decreased by 70 and 50% for $MgCl_2$ and $CaCl_2$, respectively. The purity of 11S declined but that of 7S increased when pH was adjusted to 5.6. This behavior appears to be consistent with different pH-solubility profiles of the two globulins [17, 34].

$MgCl_2$ had a similar yield of the 11S-rich fraction as $CaCl_2$ did at pH 6.4 (Fig. 2), while the recovery of Mg^{2+} was significantly higher than that of Ca^{2+} (Table 1). This was also true when the pH was adjusted to 6.0. These results suggest that more Mg^{2+} than Ca^{2+} co-participated with equivalent glycinin in the pH range of 6.0–6.4. A reasonable deduction was that the ionic radius of Mg^{2+} is less than that of Ca^{2+}, allowing Mg^{2+} to occupy more binding sites on the protein molecules. However, further studies are needed to confirm this assumption since there has been little information on the association of different cations with soy protein.

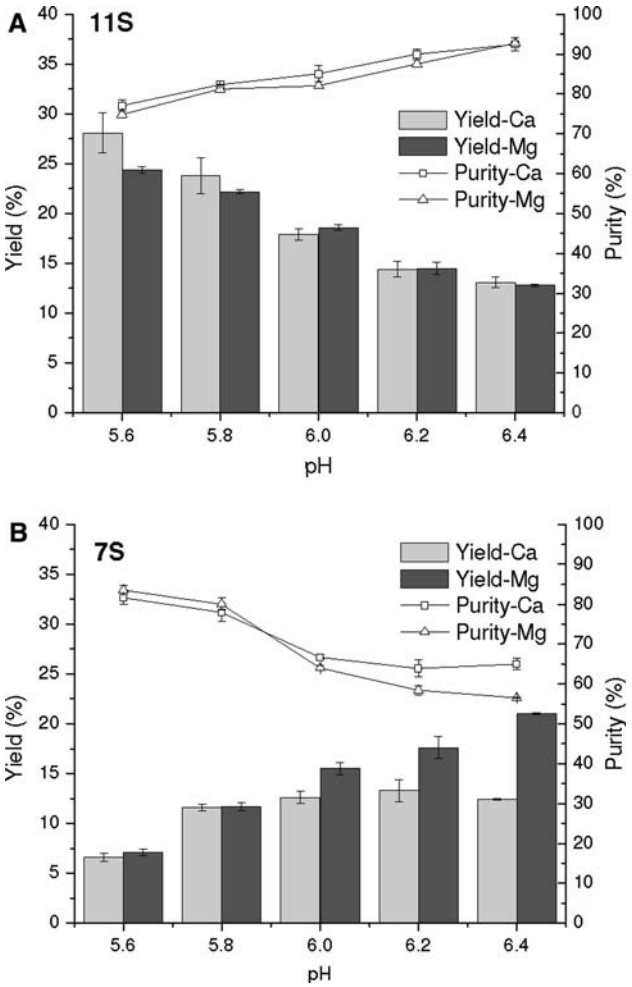

Fig. 2 Effects of 5 mM $CaCl_2$ or $MgCl_2$ on the fractionation of soybean globulins at different pHs in the first precipitating step

When pH was adjusted to 5.8 or 5.6, Mg^{2+} yielded lower amount of glycinin than Ca^{2+} (Fig. 2). Correspondingly, the recovery of Mg^{2+} was less than that of Ca^{2+} in the 11S-rich fraction (Table 1). This was probably because more free hydrogen ions competed with Ca^{2+} and Mg^{2+} for the same binding sites on the protein molecule [16, 35]. Compared with Ca^{2+}, such influence might be more significant on Mg^{2+} because more Mg^{2+} was needed to precipitate equivalent amount of protein than Ca^{2+} as discussed previously. We deduced that Mg^{2+} apparently has lower associating ability with soy protein in pH 5.6–5.8.

Whatever pH was used in the first step, a 7S-rich fraction with higher yield but lower purity was obtained in the second step when $MgCl_2$ replaced $CaCl_2$. The recovery of Mg^{2+} was also higher than that of Ca^{2+} (Fig. 2b), which implied that Mg^{2+} was more effective than Ca^{2+} in precipitating the remaining glycinin (and β-conglycinin in some observations) at pH 4.8.

The PA content in the 11S-rich fraction was reduced when pH was adjusted from 6.4 to 5.8 (Table 1). This is because more calcium phytate or magnesium phytate becomes soluble when pH decreases within this range [20, 24]. However, there was an unexpected increase in PA content by 28% at pH 5.6 when $MgCl_2$ was used. Grynspan et al. [21] observed an increase in the precipitation of calcium phytate when pH decreased from 5.5 to 4.5. Based on our experimental results, we deduce that a similar increase in the precipitation of magnesium phytate started at pH 5.8. When $MgCl_2$ was used instead of $CaCl_2$, the PA content of the 11S-rich fraction (precipitated at pH 6.4) was decreased by 63–71% (Table 1). This observation is supported by Grynspan's study, which noted that more calcium phytate than magnesium phytate precipitated at pH 5.6–6.4 [20, 24].

The PA content of the 7S-rich fraction was observed to be insensitive to pH adjustment in the first step (Table 1). At pH 4.8, the solubility of PA was nearly zero in the presence of divalent cations and soy protein perhaps because of the precipitation of the protein–cation–PA complex [21]. Thus, the PA content in the 7S-rich fraction mainly depended on its residual concentration in the second step. Compared with $CaCl_2$, $MgCl_2$ co-precipitated with less PA in the first step and produced higher residual PA concentration in the second one. Therefore, the substitution of $CaCl_2$ by $MgCl_2$ induced an increase of 14–28% in the PA content of the 7S-rich fraction, depending on pH.

Based on these results, both fractions were obtained with desirable yields and purities when the first precipitation took place at pH 5.8; thus, this pH was adopted in the subsequent studies.

Effect of $CaCl_2$ or $MgCl_2$ Concentration on the First Precipitation Step

The effect of calcium or magnesium concentration on the fractionation process at pH 5.8 is presented in Fig. 3. Ten millimolar $CaCl_2$ or 15 mM $MgCl_2$ precipitated the maximum amount of glycinin. When the concentration of $CaCl_2$ or $MgCl_2$ was increased to 20 mM, excess divalent cations might precipitate β-conglycinin, resulting in a decreased purity of the 11S-rich fraction. Meanwhile, the yield of the 11S-rich fraction decreased slightly in the presence of 20 mM $CaCl_2$ or $MgCl_2$, indicating that some minor salting-in effects existed. For the 7S-rich fraction, the yield kept decreasing and its purity increased as the concentration of either precipitating agent increased.

Compared with $CaCl_2$, $MgCl_2$ precipitated less amount of 11S-rich fraction, but its purity was higher. At the concentration of 10–20 mM, $MgCl_2$ yielded more amount of 7S-rich fraction with lower purity than $CaCl_2$ (Fig. 3).

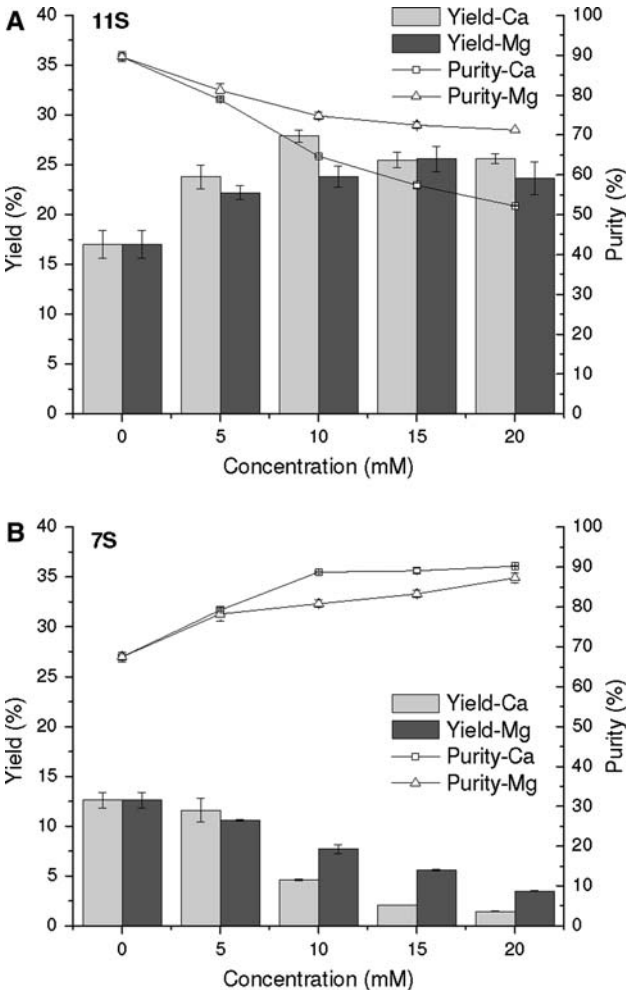

Fig. 3 Influence of $CaCl_2$ or $MgCl_2$ concentration on the fractionation of soybean globulins at pH 5.8

Higher recovery of Ca^{2+} than Mg^{2+} was observed in the 11S-rich fraction, whereas larger proportion of Mg^{2+} precipitated with the 7S-rich fraction than Ca^{2+} (Table 2). These results further confirmed our previous theory that Mg^{2+} presented lower associating ability with soy protein than Ca^{2+} at pH 5.8 but showed stronger capacity to bind remaining glycinin at pH 4.8.

Even when 20 mM $CaCl_2$ or $MgCl_2$ was used, the contents of Ca and Mg in the final products were below those in the starting flour. This was in accordance with Kroll's [35] finding that less than 30% of the Ca^{2+} (and possibly Mg^{2+}) precipitated at pH 5.8. Increased concentration of added $CaCl_2$ promoted the precipitation of PA much more rapidly than that of $MgCl_2$. Compared with 0 mM salt, 20 mM $CaCl_2$ increased the PA content in the 11S-rich fraction by fivefold, whereas 20 mM $MgCl_2$ increased that content by only 28% (Table 2). This may be because magnesium phytates was more soluble than calcium phytates at pH 5.8 [20, 24]. The PA contents in the 7S-rich fraction were generally low while the contents in the 11S-rich fraction were high.

Effect of NaCl on Fractionation in the Presence of Ca^{2+} or Mg^{2+}

Figure 4 shows the effect of NaCl on the fractionation of soybean globulins in the presence of 5 mM $CaCl_2$ or $MgCl_2$ at pH 5.8. The purity of the 11S-rich fraction progressively increased with increasing concentration of NaCl, but the protein yield declined by 3.5% when 100 mM NaCl was added. These results suggest that NaCl inhibited the precipitation of soy protein and that β-conglycinin was easier to 'salt-in' than glycinin. This is in agreement with Nagano's and Deak's [8, 26] studies where NaCl was employed to precipitate the remaining glycinin while keeping β-conglycinin soluble. Besides salting-in soy proteins, NaCl also disrupted the Ca^{2+} (or Mg^{2+})–protein association [36]. According to Yuan's study [17], the number of Ca^{2+} ions needed to bind all the glycinin and β-conglycinin were 79 and 164 (ions/g protein), respectively, and those values increased to 435 and 1,000 (ions/g protein) when 100 mM NaCl was added. According to their results, NaCl led to a more negative effect on the precipitation of β-conglycinin than glycinin, and the purity of the 11S-rich fraction increased as a consequence.

The purity of the 7S-rich fraction decreased with increasing NaCl concentration (Fig. 4), which could be also attributed to the negative effect of NaCl on the precipitation of β-conglycinin. With or without NaCl, $MgCl_2$ gave lower yields and higher purities of the 11S-rich fraction than did $CaCl_2$. The purity of the 7S-rich fraction obtained with $MgCl_2$ was lower than that precipitated with $CaCl_2$.

Table 3 presents the effect of NaCl on the precipitation of divalent cations and PA. The PA content in the 11S-rich fraction increased when 10 mM NaCl was added, but it decreased in the presence of 50 or 100 mM NaCl. A possible reason was that 10 mM of Na^+ weakened the ionic shield of soy protein and allowed it to associate with more PA while 50–100 mM Na^+ disrupted the association between Ca^{2+} (or Mg^{2+}) and PA through forming soluble sodium phytate [37]. The profile of divalent cations' recoveries paralleled that of PA contents, suggesting that NaCl influenced the solubilities of Ca^{2+} or Mg^{2+} and PA by disrupting the association between them.

Effect of pH Adjustment on the Second Precipitation Step

The protein yield increased remarkably when pH decreased from 5.0 to 4.0, but it declined slightly at pH 3.8 (Fig. 5).

Table 2 Distribution of divalent cations and phytic acid in the presence of Ca^{2+} or Mg^{2+}

Ca or Mg (mM)	$CaCl_2$ used				$MgCl_2$ used			
	PA (%)	Δc (PA) (%)[a]	Ca (%)	Ca rec (%)[a]	PA (%)	Δc (PA) (%)[a]	Mg (%)	Mg rec (%)[a]
11S-rich fraction								
0	0.333d	−73.1	0.041d	14.754a	0.333b	−73.1	0.107d	11.230a
5	1.071c	−13.6	0.171c	11.969b	0.356b	−71.3	0.146c	10.066b
10	1.761b	42.0	0.263a	10.473c	0.424a	−65.8	0.179a	7.772c
20	2.156a	73.9	0.233b	5.857d	0.425a	−65.7	0.156b	4.305d
7S-rich fraction								
0	0.856a	14.9	0.041d	12.478a	0.856b	14.9	0.142b	12.586a
5	0.763b	2.4	0.097c	3.308b	0.882b	18.4	0.148b	4.892b
10	0.740b	−0.7	0.046b	1.097c	1.079a	44.8	0.089c	1.355c
20	0.646c	−13.3	0.052a	0.175d	1.063a	42.7	0.184a	0.737d

Different letters in the same column following each figure indicate significant differences at $P < 0.05$, $n = 3$

[a] Refer to Table 1 for abbreviations and definitions

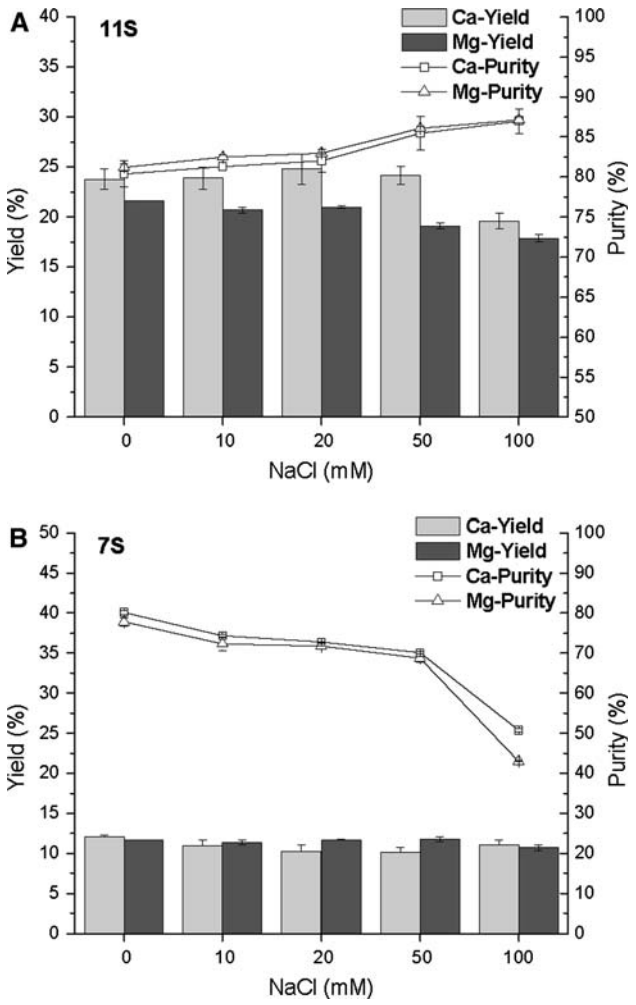

Fig. 4 Fractionation of soybean globulins using NaCl in the presence of 5 mM $MgCl_2$ or $CaCl_2$ at pH 5.8

Additionally, the purity of the 7S-rich fraction appeared independent of pH in the second step (data not shown). Interestingly, the difference in protein yields achieved by using different salts was significant at pH 4.8–5.0, but it appeared not obvious at pH 3.8–4.5. This was probably because the net charge of soy protein was almost zero at the latter pHs, thus the effect of the cation–protein electric interaction being negligible [21].

When $CaCl_2$ was used as precipitating agent, the PA content increased dramatically with lower pH and achieved the maximum value of 2.182% at pH 4.0 (Fig. 5). This finding was similar to that observed by Grynspan [21], who reported that the precipitation of PA was enhanced when pH decreased from 5.0 to 3.0 in the presence of soy protein and divalent cations. Similar results were observed when $MgCl_2$ or NaCl was employed (data not shown).

SDS-PAGE of Selected Fractions

SDS-PAGE was used to examine the purity of different fractions obtained by different methods (Fig. 6). The major contaminant of the 11S-rich fraction obtained at pH 6.4 with $CaCl_2$ or $MgCl_2$ (lanes 7 and 8) or that precipitated at pH 5.8 with no salt (lane 6) was the β subunit of β-conglycinin. At pH 5.8, the addition of $CaCl_2$ or $MgCl_2$ induced a contamination of α' and α subunit as well as LP (lipid-associated protein) [38] in the 11S-rich fraction, as shown in lanes 1–5. Divalent cations increased the purity of the 7S-rich fraction at pH 5.8 (lanes 1, 2 and 5, compared with lane 6). $MgCl_2$ (lane 2) co-precipitated with a lower amount of LP in the 7S-rich fraction at pH 5.8 than did $CaCl_2$ (lane 5). The relative quantities of the 11S globulin in lanes 3–4 (11S section) were higher than those in lanes 2

Table 3 Distribution of divalent cations and phytic acid as influenced by NaCl

NaCl (mM)	$CaCl_2$ used				$MgCl_2$ used			
	PA (%)	Δc (PA) (%)[a]	Ca (%)	Ca rec (%)[a]	PA (%)	Δc (PA) (%)[a]	Mg (%)	Mg rec (%)[a]
11S-rich fraction								
0	1.071b	−13.6	0.171a	11.969a	0.356c	−71.3	0.146b	10.066b
10	1.179a	−4.9	0.169b	11.847a,b	0.515a	−58.5	0.182a	11.727a
50	0.755c	−39.1	0.140c	9.928b	0.418b	−66.3	0.140b	8.301c
100	0.776c	−37.4	0.133d	7.645c	0.338c	−72.7	0.132c	7.331c
7S-rich fraction								
0	0.763a	2.4	0.097a	3.308a	0.882a	18.4	0.148a	4.892a
10	0.763a	2.4	0.093a	2.981b	0.882a	18.4	0.121b	4.614b
50	0.720a	−3.4	0.098a	2.894c	0.890a	19.5	0.120b	4.664a,b
100	0.598b	−19.7	0.096a	3.122b	0.802b	7.7	0.126b	4.564b

Different letters in the same column following each figure indicate significant differences at $P < 0.05$, $n = 3$

[a] Refer to Table 1 for abbreviations and definitions

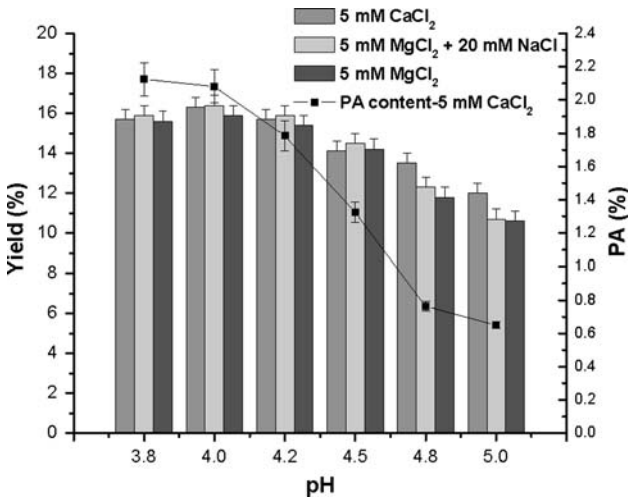

Fig. 5 Effect of pH in the second precipitating step on the yield of the 7S-rich fraction

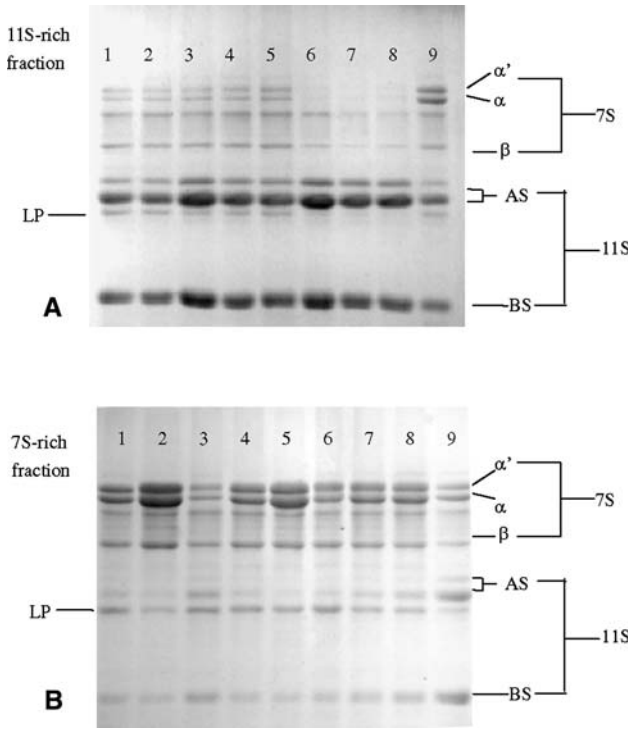

Fig. 6 SDS-PAGE band patterns of the 11S-rich and 7S-rich fractions obtained by different methods: *1* 10 mM $MgCl_2$, pH 5.8 (employed in the first step); *2* 5 mM $MgCl_2$, pH 5.8; *3* 5 mM $CaCl_2$ + 100 mM NaCl, pH 5.8; *4* 5 mM $CaCl_2$ + 50 mM NaCl, pH 5.8; *5* 5 mM $CaCl_2$, pH 5.8; *6* No salt, pH 5.8; *7* 5 mM $MgCl_2$, pH 6.4; *8* 5 mM $CaCl_2$, pH 6.4; *9* The starting extract. All the 7S-rich fractions were precipitated at pH 4.5. *AS* and *BS* denoted acidic and basic peptides of glycinin respectively, while *LP* denoted the lipid-associated protein. 25 μg of each sample (protein basis) was loaded

and 5, while the relative contents of the 7S globulin in the former lanes (7S section) were lower than those in the latter ones. These results suggest that the addition of 50 or 100 mM NaCl improved the purity of the 11S-rich fraction but reduced that of the 7S-rich fraction.

Conclusion

The substitution of $CaCl_2$ by $MgCl_2$ for fractionation of soybean 7S/11S globulins was successfully performed in our study. Compared with $CaCl_2$, $MgCl_2$ produced significantly lower PA content of the 11S-rich fraction without any obvious reduction in its yield and purity. However, the PA content of the 7S-rich fraction increased slightly after the substitution. pH values also played an important role in the isolation process by influencing the interaction between protein and cations. The addition of NaCl together with $CaCl_2$ or $MgCl_2$ promoted the yield and reduced the content of PA in the 11S-rich fraction. However, it decreased the purity of the 7S-rich fraction, especially when its concentration was above 50 mM.

An optimized procedure was proposed to acquire desirable yields and purities of both fractions of soy protein. An 11S-rich fraction containing 88.9% glycinin was obtained first from the starting extract by using 5 mM $MgCl_2$ at pH 5.8, while a 7S-rich fraction (81.4% β-conglycinin) was subsequently obtained at pH 4.5. The solid yields of the 11S-rich and 7S-rich fractions were 22.4 and 16.2% respectively, while their PA contents were 0.356 and 0.882%, respectively. The contents of Mg in the 11S-rich and 7S-rich fractions were 0.146 and 0.148%, respectively, which were lower than the amount present in commercial SPI. This new procedure was an inexpensive, simple and relatively accurate method of fractionating soybean globulins. It also reduced the PA content of the products significantly and will promote their nutritional value.

Acknowledgments This work was a part of the research project of the National Natural Science Foundation of China (No. 20776050).

References

1. Mujoo R, Trinh DT, Ng PKW (2003) Characterization of storage proteins in different soybean varieties and their relationship to tofu yield and texture. Food Chem 82:265–273
2. Thanh VH, Shibasaki K (1978) Major proteins of soybean seeds. Subunit structure of β-conglycinin. J Agric Food Chem 26: 692–695
3. Prak K, Nakatani K, Katsube-Tanaka T, Adachi M, Maruyama N, Utsumi S (2005) Structure–function relationships of soybean proglycinins at subunit levels. J Agric Food Chem 53:3650–3657
4. Utsumi S, Kinsella JE (1985) Structure–function relationships in food proteins: subunit interactions in heat-induced gelation of 7S, 11S, and soy isolate proteins. J Agric Food Chem 33:297–303
5. Fukui K, Kojima M, Tachibana N, Kohno M, Takamatsu K, Hirotsuka M, Kito M (2004) Effects of soybean beta-conglycinin

on hepatic lipid metabolism and fecal lipid excretion in normal adult rats. Biosci Biotechnol Biochem 68:1153–1155
6. Adams MR, Golden DL, Franke AA, Potter SM, Smith HS, Anthony MS (2004) Dietary soy beta-conglycinin (7S globulin) inhibits atherosclerosis in mice. J Nutr 134:511–516
7. Duranti M, Lovati MR, Dani V, Barbiroli A, Scarafoni A, Castiglioni S, Ponzone C, Morazzoni P (2004) The alpha′ subunit from soybean 7S globulin lowers plasma lipids and upregulates liver beta-VLDL receptors in rat feed a hypercholesterolemic diet. J Nutr 134:1334–1339
8. Nagano T, Hirotsuka M, Mori H, Kohyama K, Nishinari K (1992) Dynamic viscoelastic study on the gelation of 7S globulins from soybeans. J Agric Food Chem 40:941–944
9. Rickert DA, Johnson LA, Murphy PA (2004) Improved fractionation of glycinin and β-conglycinin and partitioning of phytochemicals. J Agric Food Chem 52:1726–1734
10. Saio K (1978) Differences in functional properties of 7S and 11S soybean proteins. J Texture Stud 9:1335–1347
11. Thanh VH, Shibasaki K (1977) Beta-conglycinin from soybean proteins—isolation and immunological and physicochemical properties of monomeric forms. Biochim Biophys Acta 490: 370–384
12. Golubovic M, van Hateren SH, Ottens M, Witkamp GJ, van der Wielen LAM (2005) Novel method for the production of pure glycinin from soybeans. J Agric Food Chem 53:5265–5269
13. Saito T, Kohno M, Tsumura K, Kugimiya W, Kito M (2001) Novel method using phytase for separating soybean beta-conglycinin and glycinin. Biosci Biotechnol Biochem 65:884–887
14. Wu SW, Murphy PA, Johnson LA, Fratzke AR, Reuber MA (1999) Pilot-plant fractionation of soybean glycinin and beta-conglycinin. J Am Oil Chem Soc 76:285–293
15. Wu SW, Murphy PA, Johnson LA, Reuber MA, Fratzke AR (2000) Simplified process for soybean glycinin and beta-conglycinin fractionation. J Agric Food Chem 48:2702–2708
16. Deak NA, Murphy PA, Johnson LA (2006) Fractionating soybean storage proteins using Ca^{2+} and $NaHSO_3$. J Food Sci 71:C413–C424
17. Yuan YJ, Velev OD, Chen K, Campbell BE, Kaler EW, Lenhoff AM (2002) Effect of pH and Ca^{2+} induced associations of soybean proteins. J Agric Food Chem 50:4953–4958
18. Deak NA, Johnson LA (2007) Fate of phytic acid in producing soy protein ingredients. J Am Oil Chem Soc 84:369–376
19. Jaffe G (1981) Phytic acid in soybeans. J Am Oil Chem Soc 58:493–495
20. Grynspan F, Cheryan M (1983) Calcium phytate: effect of pH and molar ratio on in vitro solubility. J Am Oil Chem Soc 60:1761–1764
21. Grynspan F, Cheryan M (1989) Phytate–calcium interactions with soy protein. J Am Oil Chem Soc 66:93–97
22. Kumagai H, Shizawa Y, Sakurai H (1998) Influence of phytate removal and structural modification on the calcium-binding properties of soybean globulins. Biosci Biotechnol Biochem 62:341–346
23. Lei XG, Porres JM (2003) Phytase enzymology, applications, and biotechnology. Biotechnol Lett 25:1787–1794
24. Cheryan M, Grynspan F (1983) Magnesium–phytate complexes: effect of pH and molar ratio on solubility characteristics. J Am Oil Chem Soc 60:235–237
25. Lui DYM, White ET, Litster JD (2007) Dissolution behavior of soy proteins and effect of initial concentration. J Agric Food Chem 55:2467–2473
26. Deak NA, Murphy PA, Johnson LA (2006) Effects of NaCl concentration on salting-in and dilution during salting-out on soy protein fractionation. J Food Sci 71:C247–C254
27. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
28. O'Keefe SF, Wilson LA, Resurreccion AP, Murphy PA (1991) Determination of the binding of hexanal to soy glycinin and beta-conglycinin in an aqueous model system using a headspace technique. J Agric Food Chem 39:1022–1028
29. AOAC (1995) Official methods of analysis, 16th edn. Gaithersburg, MD
30. Thompson DB Jr, Erdman JW (1982) Phytic acid determination in soybeans. J Food Sci 47:513–517
31. Chen PS (1956) Microdetermination of phosphorus. Anal Chem 28:1756–1758
32. De Boland AR, Garner GB, O'Dell BL (1975) Identification and properties of phytate in cereal grains and oilseed products. J Agric Food Chem 23:1186–1189
33. Porter M, Jones A (2003) Variability in soy flour composition. J Am Oil Chem Soc 80:557–562
34. Lakemond CMM, de Jongh HHJ, Hessing M, Gruppen H, Voragen AGJ (2000) Soy glycinin: influence of pH and ionic strength on solubility and molecular structure at ambient temperatures. J Agric Food Chem 48:1985–1990
35. Kroll RD (1984) Effect of pH on the binding of calcium ions by soybean proteins. Cereal Chem 64:490–495
36. Kumosinski TF, Farrell HM (1991) Calcium-induced associations of the caseins: thermodynamic linkage of calcium binding to colloidal stability of casein micelles. J Protein Chem 10:3–16
37. Rham O, Jost T (1979) Phytate–protein interactions in soybean extracts and low-phytate soy protein products. J Food Sci 44: 596–600
38. Samoto M, Maebuchi M, Miyazaki C, Kugitani H, Kohno M, Hirotsuka M, Kito M (2007) Abundant proteins associated with lecithin in soy protein isolate. Food Chem 102:317–322