JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 10893–10900 **10893**

Protein Recovery in Soymilk and Various Soluble Fractions as a Function of Genotype Differences, Changes during Heating, and Homogenization

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Harovinton, a variety of tofu type soybean, and 11 derived null soybean genotypes lacking specific glycinin (11S) and β-conglycinin (7S) protein subunits were investigated to determine whether changes in protein composition affected the protein recovery in soymilk and its soluble fractions after various centrifugation steps. As both heating and homogenization have a marked effect on the increase in protein solubility, the changes occurring during these processing steps were studied for each soybean genotype. Harovinton and 11S-null genotypes showed significantly higher protein yields than the other genotypes evaluated. Subunits of group I (A_1, A_2) of glycinin had a negative impact on protein solubility in all treatments, but this effect was the greatest in unheated soymilk samples. Samples containing a high β-conglycinin to glycinin ratio showed an effect of heating on the solubility of the protein, as β-conglycinin subunits aggregate with heating. The presence of the α' subunit of β-conglycinin aids in the recovery of protein in the supernatant prepared from lines containing group I ($A_{1,4}$ A_2) glycinin. The results of this study will help determine which specific protein composition will confer an increased stability in soymilk and soymilk-derived products.

KEYWORDS: Soymilk; homogenization; Harovinton

INTRODUCTION

Because of their nutritional value, the role of soybean-based products is increasing in importance in the western diet. Soymilk is a colloidal dispersion extracted from ground soybeans; therefore, most components that are present in the seed are present in soymilk. Soymilk composition, including its total solids content, varies depending on the processing method and soybean varieties (*1*−*3*). In general, soymilk contains 8−10% total solids, comprised of 3.6% protein, 2% fat, 2.9% carbohydrates, and 0.5% ash, depending on the water to soybean ratio employed during processing (*4*).

The protein content of soybeans is approximately 40−45% on a dry matter basis (*4*). Glycinin (11S) and β-conglycinin (7S) are the two major soy storage proteins, for approximately 40 and 30% of the total seed protein, respectively (*5*). These two proteins differ in their structure, amino acid composition, functionality, and processing properties. Glycinin is a hexamer with a molecular mass of 320−380 kDa. Constituent subunits of glycinin are classified into two groups: Group I consists of three subunits $A_{1a}B_{1b}$, $A_{1b}B_2$, and A_2B_{1a} (*6, 7*), group IIa consists of $A_5A_4B_3$, and group IIb consists of A_3B_4 (*8, 9*). Each monomer subunit consists of one basic and one acidic polypeptide, linked together by a single disulfide bond, except for the acidic polypeptide A_4 (*10*). β-Conglycinin is a trimeric glycoprotein with a molecular mass of 180 kDa consisting of three subunit types, α' (57−72 kDa), α (57−68 kDa), and β (45−52 kDa) (*11*), in seven different combinations ($\beta\beta\beta$, $\beta\beta\alpha'$, $\beta\beta\alpha$, $\beta\alpha\alpha'$, $\beta\alpha\alpha$, $\alpha\alpha\alpha'$, and $\alpha\alpha\alpha$) (*12, 13*). The subunits are associated via hydrophobic and hydrogen bonding without any disulfide bonds (*14*).

It has been reported that the variety of soybeans used to prepare soymilk has a significant effect on soymilk yield and quality (*3, 15, 16*). As soybean protein content increases, the protein recovery and the total solids in soymilk also increase (*17*). In the past few years, specific soybean lines with different protein composition have become available and have been studied to improve soybean quality. Only recently, more focus has been given to the effect of varieties on the processing performance of soybean. For example, it has been demonstrated that breeding of

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10.1021/jf800897s CCC: $40.75
Published on Web 10/23/2008

special varieties (such as Harovinton) high in both protein quality and quantity can improve tofu quality (*15, 16, 18*).

Heat treatment is a necessary operation for making soymilk: It not only increases shelf life and microbial quality but also increases protein digestibility and reduces beany flavor (*4*). Heat treatment causes dissociation, denaturation, and aggregation of both glycinin and β-conglycinin (*2*). While β-conglycinin denatures at 65−75 °C, glycinin denatures at between 85−95 °C depending on soybean variety and environmental conditions (*19, 20*). Soymilk can be further stabilized with subsequent homogenization; however, very little has been reported on the physicochemical properties of homogenized soymilk.

Research also has been carried out on high pressure treatment, as it has a significant effect on soy protein conformation in soymilk without causing any change in taste and flavor of the final products (*21*). High pressure denatures soy proteins, exposes hydrophobic regions, and increases soymilk viscosity at 500 MPa (*20*). Denaturation occurs at 300 and 400 MPa at the room temperature for β-conglycinin (7S) and glycinin (11S) proteins in soymilk, respectively (*21*). High pressure can dissociate soy proteins into subunits, some of which aggregate and become insoluble (*21, 22*).

The effects of heat and high pressure treatment on soy protein conformation and functionality have been studied but in most cases dealt with pure protein solutions; only a few studies employed soymilk, which contains not only proteins but also triglycerides, phospholipids, carbohydrates, and other constituents. In addition, in soymilk, the protein structures are in native form and have to be dissociated from the protein bodies. Although there is some understanding of the effect of different subunits on the processing behavior of soy proteins, most work has been carried out on purified materials with various processing histories.

The present research focused on evaluating the effect of soy protein composition on the protein profiles recovered in soymilk, using a set of soybean lines developed from the cultivar Harovinton and a series of protein variants. The changes occurring during heating and homogenization were determined. As the processing history of the proteins is fully comparable, this research will evaluate the effect of subunit composition on the recovery of soluble protein in various soymilk fractions.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Mississauga, ON, Canada). Ultrapure water was used for the preparation of soymilk and buffer solutions.

Soybean Genotypes. The lines with different glycinin and β-conglycinin compositions used in this study were developed and grown at the Harrow Research Centre (Harrow, ON, Canada) of Agriculture and Agri-Food Canada (*16*). The protein subunit composition of soybean genotypes is listed in **Table 1** and shown in **Figure 1**. The subunit null soybean genotypes were developed from crosses and backcrosses between Harovinton (*23*) and "Iwate-1", which lacks all glycinin subunits, and "Iwate-3", which lacks the α' subunit of β-conglycinin and all glycinin subunits (*16*). The composition of soybean genotypes, percentage of glycinin (11S) and β-conglycinin (7S), and their ratio are summarized in **Table 2**. The amount of oil varied from 17.7% in V12 to 20.5% in V2. The protein content and subunit composition were derived from near infrared (NIR) and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) analysis (*16*). Among all genotypes, V12 contained the highest (45.5%) and V2 the lowest (41.5%) protein content. The genotypes were also different in the ratio between 11S and 7S. V4, V1, and Harovinton (V1) showed a higher 11S/7S ratio followed by V2, V7, V8, and V12, and genotypes V5, V6, V11, V10, and V9 had the lowest ratio. The soy protein composition of Harovinton and 11 null soybean genotypes was identified by SDS-PAGE electrophoresis (**Figure 1**; see below for the methods).

Table 1. Glycinin and β-Conglycinin Subunit Compositions of Harovinton and Null Soybean Genotypes

soybean genotypes		subunits absent		
			glycinin	
line	designation	β-conglycinin	acidic polypeptide[a]	grouping
V1	Harovinton			
V2	SQ98-0110-3-1		A_3	IIb
V3	SQ97-0263-54-1-5	α'	A_4, A_5	α', IIa
V4	SQ98-0105-6-1	α'	A_3	α', IIb
V5	SQ97-0263-71-1-3		A_1, A_2, A_4, A_5	I, IIa
V6	SQ98-0105-1-1b		A_3, A_4, A_5	IIa, IIb
V7	SQ97-0263-21-7-2	α'	A_3, A_4, A_5	α', IIa, IIb
V8	SQ97-0263-3-10-1	α'	A_1, A_2, A_3	α', I, IIb
V9	SQ97-0252-S17-2-1		A_1, A_2, A_3, A_4, A_5	11S
V10	SQ97-0252-S17-2-3		A_1, A_2, A_3, A_4, A_5	11S
V11	SQ97-0263-3-1a	α'	A_1, A_2, A_3, A_4, A_5	α', 11S
V12	SQ98-0112-S7-1		A_1, A_2	I

[a] Corresponding basic polypeptides are absent in the genotypes as well as acidic polypeptides.

The results confirmed that the parent variety Harovinton (lane 1) contains all glycinin and β-conglycinin subunits. The five null genotypes in lanes 3, 4, 7, 8, and 11 lack the α' subunit of β-conglycinin. The null genotypes in lanes 5, 8, and 12 do not contain the polypeptide chains of group I (A_1, A_2), while the genotypes in lanes 3 and 5−7 are missing the group IIa (A_4, A_5). The lines in lanes 2, 4, and 6−8 lack group IIb (A_3); lanes 9−11 are free of glycinin (11S) subunits. Although genotypes in lanes 9−11 are considered as null in 11S in this study, the amount of glycinin subunits is very low but not completely absent (**Table 2**).

Soymilk Preparation. Soymilk was prepared as described by Mullin et al. (*3*) with slight modifications (*24*). Soybeans were weighed (100 g) and soaked in 1 L of ultrapure water, overnight at room temperature, drained and rinsed with cold water, and drained again. The soaked beans were weighed again to determine the water uptake, calculated by dividing the weight of the soaked beans by the initial weight of the dry beans. The amount of additional water needed to obtain a ratio of 18:1 water to protein was then calculated by subtracting the amount of absorbed water. The ratio was calculated on a protein basis, so the soymilk samples would be equal in protein.

Approximately half of the additional water needed was then added to the beans at 20 °C and blended (commercial blender, Waring, New Hartford, CT) at high speed for 3.5 min. The remaining water was heated to 60 °C and added to the slurry for better protein extraction, and the whole mixture was blended at high speed for another 30 s. A two-step filtration was then carried out to remove the coarse material (okara, which is mainly composed of fiber material): The slurry was filtered through a juice extractor (Juiceman, professional series 211, Korea), and the okara was collected and extracted again. The soymilk obtained from the juice extractor was filtered through cheesecloth to remove fines (*3*). The soymilk was then collected for analysis (unheated soymilk, SM-UH).

For this study, the soymilk was given three treatments: unheated, heated, and heated-homogenized. A portion of soymilk was divided into test tubes (10 mL each tube) and heated in boiling water (95−100 °C) for 5 min (with additional 2 min to reach temperature), following a previously published procedure (*24*), and cooled to room temperature in an ice waterbath (SM-H, heated soymilk). A portion of the heated soymilk was passed four times through a valve homogenizer (Emulsiflex C5, Avestin, ON, Canada) at 69 MPa at room temperature (SM-H-H, heated-homogenized soymilk).

To better characterize the colloidal particles present in the soymilk, a stepwise centrifugation procedure was applied to obtain four different supernatant phases by centrifuging the soy milk at 8000*g* (SN1), 15000*g* (SN2), 40000*g* (SN3), and 122000*g* (SN4) following a previously published procedure (*25*) at 20 °C for 30 min using a refrigerated ultracentrifuge (Optima LE-80K Beckman Coulter, CA). This selective

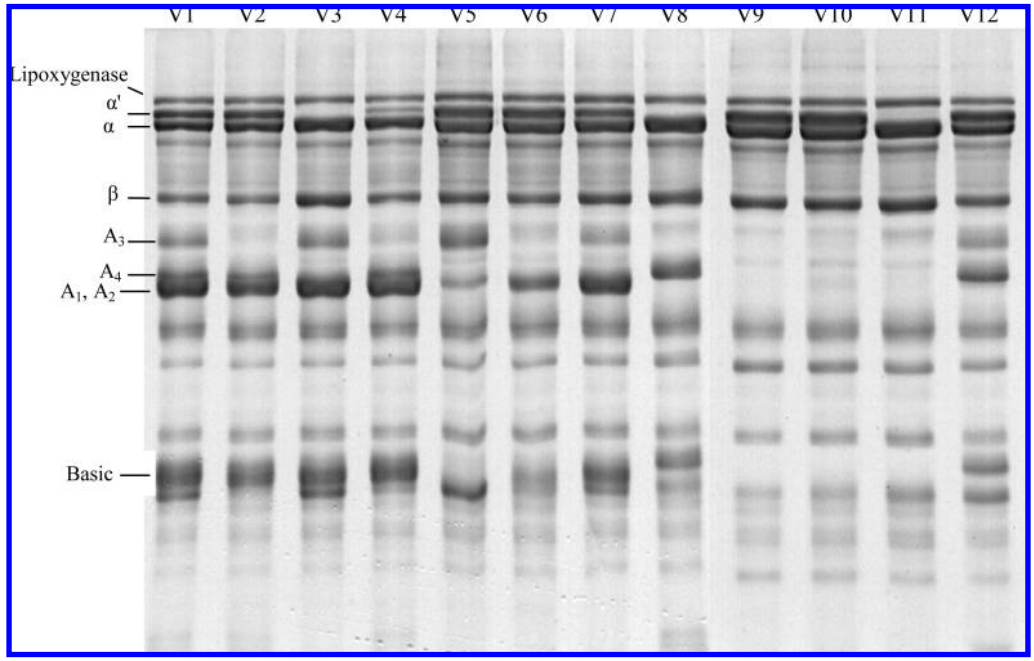

Figure 1. SDS-PAGE electrophoresis gel of Harovinton and 11 null soybean genotypes differing in their protein subunit composition. The lanes are marked at the top as follows: V1 (Harovinton), V2 (null A_3), V3 (null α′, A_4, A_5), V4 (null α′, A_3), V5 (null A_1, A_2, A_4, A_5), V6 (null A_3, A_4, A_5), V7 (null α′, A_3, A_4, A_5), V8 (null α′, A_1, A_2, A_3), V9 (null 11S), V10 (null 11S), V11 (null α′, 11S), and V12 (null A_1, A_2). A_5 polypeptide is not indicated.

Table 2. Soybean and Soy Protein Composition of Harovinton and 11 Null Soybean Genotypes

cultivar names							β-conglycinin		glycinin			
line	genotype	% oil	% protein	% 11S	% 7S	11S/7S	% (α′ + α)	% (β)	% (A_3)	% (A_1, A_2, A_4)	% A_5	% (basic)
V1	Harovinton	20.40	43.50	40.40	31.13	1.30	24.02	7.11	5.21	15.73	1.53	17.93
V2	SQ98-0110-3-1	20.50	41.50	32.64	37.78	0.87	29.62	8.17	0.86	13.81	1.62	16.35
V3	SQ97-0263-54-1-5	19.20	44.60	38.85	33.04	1.18	20.10	12.94	5.12	14.02	1.34	18.38
V4	SQ98-0105-6-1	20.40	44.40	42.36	28.35	1.50	19.61	8.73	2.44	18.85	2.74	18.32
V5	SQ97-0263-71-1-3	19.50	41.90	22.81	47.15	0.49	38.84	8.30	6.80	2.59	1.06	12.36
V6	SQ98-0105-1-1b	19.60	43.30	20.52	44.38	0.47	36.57	7.81	1.41	6.54	1.56	11.01
V7	SQ97-0263-21-7-2	19.70	43.00	30.53	40.21	0.76	29.35	10.86	2.71	11.73	0.72	15.37
V8	SQ97-0263-3-10-1	18.50	45.40	28.37	38.97	0.73	27.65	11.31	1.67	12.83	2.88	10.99
V9	SQ97-0252-S17-2-1	18.90	42.90	11.70	56.76	0.21	43.88	12.88	1.31	2.50	1.81	6.08
V10	SQ97-0252-S17-2-3	18.90	42.00	14.92	55.44	0.27	43.59	11.85	2.49	3.39	1.96	7.08
V11	SQ97-0263-3-1a	19.20	42.70	12.87	48.83	0.27	34.00	14.83	0.97	1.64	0.96	9.31
V12	SQ98-0112-S7-1	17.70	45.50	28.20	43.28	0.65	33.73	9.55	4.67	10.97	3.60	8.96

centrifugation method was reported to distinguish between soluble fractions in soymilk, with a clear difference between samples containing high levels of glycinin (with higher losses) as compared to high levels of β-conglycinin soymilk (*25*). The protein content in soymilk and the various supernatant fractions was measured following the Dumas method using a Nitrogen Analyzer (LECO, FP-528, Mississauga, ON, Canada) with EDTA as the standard for calibration of the instrument. The protein concentration was calculated using a conversion factor of 6.25. The term protein solubility in this paper refers to the amount of protein recovered after each centrifugation step. The protein recovered could be in the form of soluble aggregates, small soluble complexes, or oligomeric forms in solution.

SDS-PAGE Electrophoresis. SDS-PAGE was carried out in a vertical slab gel of 1.5 mm thickness with 12.5% acrylamide running gel and 4% stacking gel in a Bio-Rad mini-protein electrophoresis system (Bio-Rad Laboratories, Hercules, CA) at a constant voltage of 200 V.

The proteins were extracted from defatted soybean samples (6 mg) in extraction buffer (420 μL) containing 50 mM Tris-HCl, 5 M urea, 1% SDS, and 4% 2-mercaptoethanol. After 1 h of incubation at room temperature, 420 μL of electrophoresis sample buffer containing 125 mM Tris-HCl, 5 M urea, 1% SDS, 20% glycerol, and 1% bromophenol blue were added. The solution was heated at 95 °C for 5 min and centrifuged at 5000*g* for 4 min using an Eppendorf centrifuge (Brinkmann Instruments, Westbury, NY). Aliquots (6 μL) were then loaded onto the gels. Samples of soymilk or supernatant fractions (100 μL) were mixed with 250 μL of extraction buffer as described above. Aliquots (6 μL) were then loaded to the gel.

After the run, gels were immediately stained using Coomassie blue R-250 for 30 min and destained with a solution composed of 45% ultrapure water, 45% methanol, and 10% acetic acid for 2 h with two changes and then destained overnight with a destaining solution containing 22.5% methanol and 5% acetic acid. Gels were scanned using a SHARP JX-330 scanner (Amersham Biosciences, Quebec), and the bands were analyzed using image analysis software (ImageMaster 1D, Version 2.0, Amersham Biosciences).

Statistical Analysis. All results presented are the average of at least three independent replicates. Significant differences were determined by the general linear model (GLM) and Duncan multiple range test (SAS version 9.1). The statistical significance of genotypes, treatments, centrifugation steps, and their two way interactions was tested using three way interaction (genotypes × treatments × centrifugation) as the error term. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Protein concentrations present in soymilk and corresponding soymilk supernatants obtained from Harovinton and 11 null soybean lines are summarized in **Table 3**. It is important to

Table 3. Amount of Protein (%) in Soymilk (SM) and Recovered after Subsequent Centrifugations (SN1−SN4) for Harovinton and 11 Null Soybean Genotypes[a]

	V1: Harovinton	genotypes lacking various glycinin and β-conglycinin subunits										
		V2: (A_3)	V3: (α', A_4, A_5)	V4: (α', A_3)	V5: (A_1, A_2, A_4, A_5)	V6: (A_3, A_4, A_5)	V7: (α', A_3, A_4, A_5)	V8: (α', A_1, A_2, A_3)	V9: (11S)	V10: (11S)	V11: (α', 11S)	V12: (A_1, A_2)
Unheated												
SM	4.90 ± 0.51 a	4.64 ± 0.23 a,b	4.20 ± 0.13 c,b	4.23 ± 0.25 c,b	3.97 ± 0.18 c	4.08 ± 0.06 b,c	4.41 ± 0.26 b,c	4.16 ± 0.41 b,c	4.18 ± 0.16 b,c	4.49 ± 0.44 a,b,c	4.55 ± 0.39 a,b,c	4.59 ± 0.18 a,b
SN1	3.79 ± 0.18 a,b,c*	3.87 ± 0.28 a,b,c*	2.36 ± 0.38 d*	2.53 ± 0.39 d*	3.75 ± 0.55 a,b,c	3.52 ± 0.33 b,c	3.38 ± 0.28 c*	4.02 ± 0.46 a,b,c	3.91 ± 0.24 a,b,c	4.21 ± 0.50 a,b	4.35 ± 0.41 a	4.40 ± 0.12 a
SN2	3.52 ± 0.30 a,b,c	3.53 ± 0.11 a,b,c	2.27 ± 0.37 d	2.46 ± 0.46 d	3.43 ± 0.11 a,b,c	3.09 ± 0.14 c	3.18 ± 0.23 b,c	3.81 ± 0.46 a,b	3.63 ± 0.12 a,b,c	3.88 ± 0.57 a	4.03 ± 0.28 a	3.86 ± 0.40 a
SN3	3.46 ± 0.34 a,b,c	3.20 ± 0.15 b,c,d	2.24 ± 0.37 e	2.21 ± 0.44 e	3.33 ± 0.19 a,b,c,d	2.93 ± 0.11 c,d	2.76 ± 0.25 e	3.68 ± 0.38 a,b	3.57 ± 0.21 a,b	3.68 ± 0.6 a,b	3.86 ± 0.18 a	3.77 ± 0.34 a,b
SN4	3.11 ± 0.11 b,c	2.75 ± 0.15 c	2.18 ± 0.58 d,e	2.02 ± 0.49 e	2.96 ± 0.06 c,b	2.66 ± 0.11 c,d	2.16 ± 0.39 d,e	3.36 ± 0.3 a,b	3.21 ± 0.07 a,b,c,d	3.07 ± 0.15 b,c	3.70 ± 0.34 a	3.12 ± 0.20 b,c
Heated												
SM	4.94 ± 0.41 a	4.32 ± 0.28 b,c,d	4.28 ± 0.26 b,c,d	4.00 ± 0.13 c,d	3.97 ± 0.24 d	4.04 ± 0.17 c,d	4.18 ± 0.23 b,c,d	4.21 ± 0.38 b,c,d	4.19 ± 0.09 b,c,d	4.53 ± 0.53 a,b,c,d	4.73 ± 0.32 a,b	4.57 ± 0.24 a,b,c
SN1	4.85 ± 0.41 a	3.76 ± 0.25 b	3.87 ± 0.44 b	3.36 ± 0.14 b	3.62 ± 0.36 b	3.50 ± 0.17 b	3.58 ± 0.75 b	3.61 ± 0.59 b	3.94 ± 0.26 b	3.96 ± 0.43 b	4.11 ± 0.54 b*	4.03 ± 0.21 b
SN2	4.59 ± 0.26 a	3.42 ± 0.39 b,c,d	3.72 ± 0.27 b	3.30 ± 0.14 b,c,d	3.58 ± 0.28 b,c	2.94 ± 0.11 d	2.99 ± 0.50 c,d*	3.42 ± 0.28 b,c,d	3.58 ± 0.50 b,c*	3.46 ± 0.42 b,c,d	3.45 ± 0.25 b,c,d	3.80 ± 013 b
SN3	3.64 ± 0.25 a*	2.65 ± 0.19 c,d*	3.19 ± 0.61 a,b,c	2.40 ± 0.32 d*	2.98 ± 0.06 b,c,d*	2.63 ± 0.29 c,d	2.63 ± 0.56 c,d	2.82 ± 0.35 c,d*	3.23 ± 0.64 a,b,c	3.07 ± 0.52 a,b,c,d	2.87 ± 0.12 b,c,d	3.54 ± 0.07 a,b
SN4	2.62 ± 0.14 a,b*	2.05 ± 0.25 b,c,d,e	1.82 ± 0.22 d,e*	1.81 ± 0.47 d,e	2.30 ± 0.15 a,b,c,d*	2.40 ± 0.21 a,b,c	1.61 ± 0.21 e*	2.14 ± 0.35 a,b,c,d,e	2.54 ± 0.05 a,b,c*	2.49 ± 0.5 a,b,c	1.98 ± 0.16 c,d,e*	2.65 ± 0.35 a*
Heated−Homogenized												
SM	5.07 ± 0.38 a	4.18 ± 0.14 b,c,d	4.19 ± 0.27 b,c,d	4.13 ± 0.08 c,d	3.95 ± 0.03 d	4.15 ± 0.20 c,d	4.37 ± 0.37 b,c,d	4.29 ± 0.23 b,c,d	4.16 ± 0.06 c,d	4.53 ± 0.42 b,c	4.69 ± 0.35 a,b	4.47 ± 0.28 b,c
SN1	4.99 ± 0.3a	4.01 ± 0.14 b,c	3.81 ± 0.14 b,c	3.67 ± 0.09 b,c	3.83 ± 0.17 b,c	3.61 ± 0.08 c	3.81 ± 0.44 b,c	3.87 ± 0.32 b,c	3.63 ± 0.09 b,c	4.18 ± 0.36 b	3.94 ± 0.37 b,c	4.19 ± 0.31 b
SN2	4.72 ± 0.24 a	3.61 ± 0.21 b,c	3.51 ± 0.15 b,c	3.31 ± 0.37 c	3.69 ± 0.06 b,c	3.37 ± 0.36 c	3.57 ± 0.36 b,c	3.45 ± 0.26 b,c	3.65 ± 0.35 b,c*	3.91 ± 0.25 b	3.63 ± 0.22 b,c	3.82 ± 0.18 b,c
SN3	3.90 ± 0.23 a	2.59 ± 0.32 d*	3.13 ± 0.42 b,c,d	2.69 ± 0.31 d	3.62 ± 0.09 a,b	2.76 ± 0.46 c,d*	2.58 ± 0.36 d*	2.68 ± 0.21 d*	3.12 ± 0.39 b,c,d*	3.36 ± 0.26 a,b,c	3.07 ± 0.13 b,c,d	3.36 ± 0.55 a,b,c
SN4	3.15 ± 0.11 a	2.45 ± 0.33 b,c	2.41 ± 0.23 b,c*	1.81 ± 0.18 d*	2.46 ± 0.40 b,c*	2.40 ± 0.15 b,c	2.16 ± 0.04 c,d	2.05 ± 0.38 c,d	2.57 ± 0.28 b,c*	2.84 ± 0.41 a,b	2.36 ± 0.14 b,c	2.42 ± 0.44 b,c*

[a] Subunits missing are indicated. Data are the average of at least three independent experiments. Duncan's multiple range test. Within a treatment and centrifugation step (within a row), means with different letters are significantly different at $p < 0.05$. Within a genotype and a treatment, means are compared to the previous centrifugation step, and means with * are significantly different at $p < 0.05$.

Figure 2. Protein content of unheated (solid bar), heated (white bar), and heated−homogenized (hatched bar) soymilk samples prepared with different soybean genotypes. Means are compared within a treatment (same type bars); means with different superscripts are significantly different at $p < 0.05$.

note that different means comparison are shown in **Table 3** than in **Figure 3**, to be able to compare the results within treatment or within genotype. Genotypes were highly significant in affecting the protein composition of the soymilks following the various treatments, as well as all of the supernatant fractions. In addition to the effect of each individual factor, the interaction effects genotype by treatment and centrifugation by treatment were much larger than genotype by centrifugation effects (data not shown).

Although the ratio of water to beans used during soymilk preparation was calculated based on protein, the amount of protein present in the resulting soymilk depended on the genotype. The soymilks prepared from Harovinton and null soybean lines had a range of protein content, averaging from 5.07 to 3.95% among all genotypes and all treatments. Soymilk samples made from Harovinton (V1) had the highest protein content (4.9%), although the protein content of soymilks made from null genotypes V2 (null A_3), V10 (null 11S), V11 (null α', 11S), and V12 [null A_1, A_2 (group I)] was not significantly different from Harovinton (**Table 3**). **Figure 2** summarizes the effect of treatment on protein recovery in soymilk made from different genotypes (data also in **Table 3**, but statistical means comparisons are depicted within treatments, i.e., UH, H, and H-H). As expected, prior to centrifugation, the effects of treatments (UH, H, and H-H) on protein content of soymilks were not significant within each genotype (**Table 4**). As the processing history was the same for all of the soybean lines, genotype was the only source of variation in the protein content of the soymilks. The various centrifugation steps significantly decreased the amount of protein recovered in the sample.

After the centrifugation steps, the protein content in the various supernatants also was significantly affected by genotype. **Figure 3** summarizes the loss of protein during the various steps of centrifugation for all soymilk samples: unheated, heated, and heated-homogenized. After centrifugation at 8000*g* (SN1), in unheated soymilk samples, the largest decrease in protein (40−45%) was observed in V3 (null α', A_4, A_5) and V4 (null α', A_3). A loss between 13 and 25% was shown in V6 (null A_3, A_4, A_5), V7 (null α', A_3, A_4, A_5), Harovinton, and V2 (null A_3). Unheated samples of V5, V8, V9, V10, V11, and V12, which are 11S-null lines or lines with little glycinin, did not show a significant decrease in protein content after the first centrifugation step (**Figure 3A** and **Table 3**). Changes occurring in the unheated samples give an indication of the solubility of

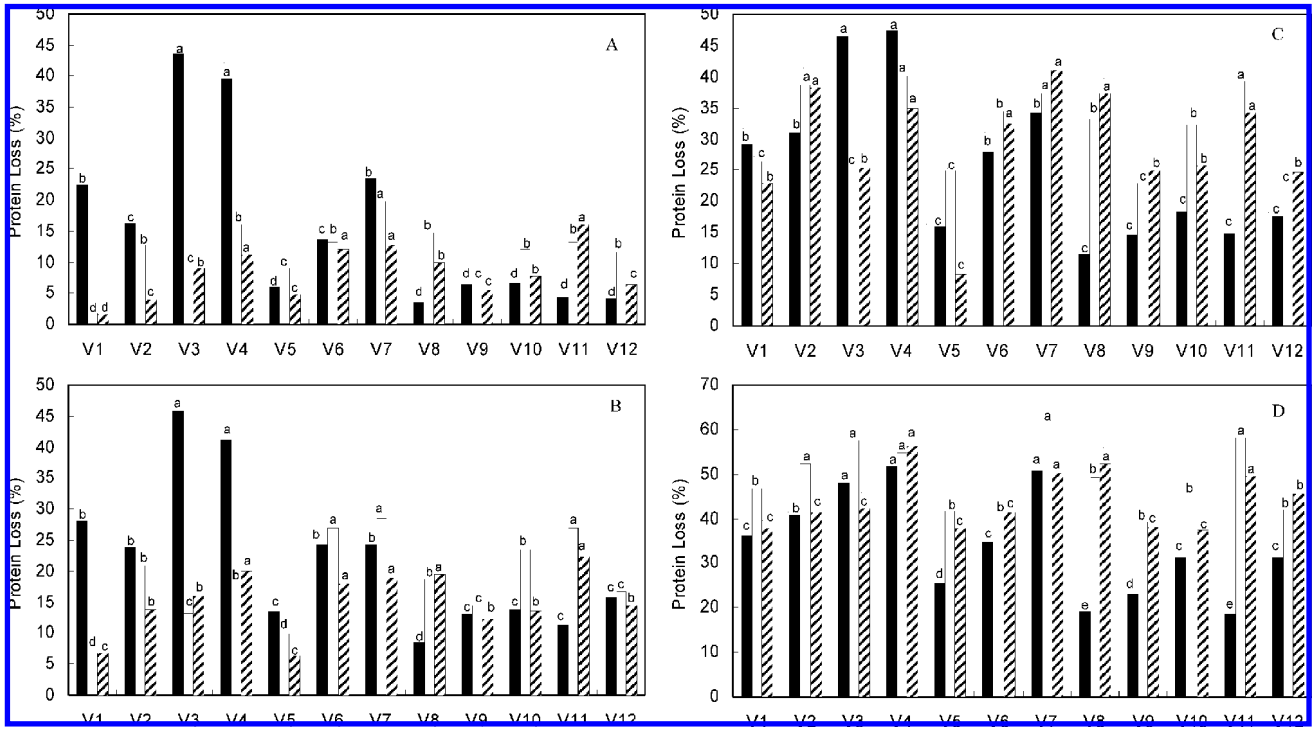

Figure 3. Protein loss (as % of the initial protein present in the soymilk) for supernatant fractions of unheated (solid bar), heated (white bar), and heated−homogenized (hatched bar) samples, after the first centrifugation step (SN1) (**A**), the second centrifugation step (SN2) (**B**), the third centrifugation step (SN3) (**C**), and the fourth centrifugation step (SN4) (**D**). Means are compared within a treatment (same type bars); means with different superscripts are significantly different at $p < 0.05$.

Table 4. Mean Square Values for Treatment Effects (Unheated, Heated, and Heated−Homogenized) on Protein Content for the 12 Genotypes[a]

	genotypes lacking various glycinin and β-conglycinin subunits											
	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
SM	0.023 NS	0.168 NS	0.008 NS	0.041 NS	0.001 NS	0.004 NS	0.03 NS	0.012 NS	0.001 NS	0.001 NS	0.026 NS	0.012 NS
SN1	1.301*	0.047 NS	2.193*	1.036*	0.035 NS	0.01 NS	0.194 NS	0.129 NS	0.001 NS	0.048 NS	0.131 NS	0.102 NS
SN2	1.292*	0.026 NS	1.833*	0.714*	0.052 NS	0.144 NS	0.241 NS	0.136 NS	0.004 NS	0.187 NS	0.263 NS	0.003 NS
SN3	0.143 NS	0.342*	0.84 NS	0.175 NS	0.308*	0.068 NS	0.026 NS	0.87*	0.169 NS	0.276 NS	0.83*	0.115 NS
SN4	0.258*	0.377*	0.271 NS	0.046 NS	0.35*	0.069 NS	0.304 NS	1.589*	0.427 NS	0.259 NS	2.456*	0.378 NS

[a] NS, nonsignificant; and *significant at $p < 0.05$, respectively.

the native protein aggregates and how these are affected by changes in the subunit composition. These results suggest that the proteins of genotype V3 and V4 were the most affected by heating and homogenization. These two genotypes had the highest ratio of 11S to 7S. It should be noted that while this is important for understanding the physicochemical properties of the proteins, the study of unheated soymilk has little industrial relevance, as all soymilk is subjected to heating during processing. Heating alone or in combination with homogenization was also evaluated.

Results shown in **Figure 3** demonstrate that there are significant genotypic differences. Harovinton (V1), V3, and V4 lose less protein with heating and heating-homogenization; therefore, these treatments favor solubilization of soy proteins of genotypes that contain almost all 11S proteins. Genotypes V5, V8, V10, V11, and V12, which do not contain 11S or group I of 11S, showed nonsignificant changes in protein solubility. Analyzing SN1, we can conclude that the presence of 11S or group I of 11S in genotype is important so as to avoid protein losses at 8000*g* (**Figure 3A**). It has been previously demonstrated (*24*) that heating significantly improves the recovery of protein in the soluble fraction of soymilk, by causing a compositional and structural rearrangement of the soluble protein macroaggregates present in the unheated soymilk. These results suggest that heating causes aggregation of the β-conglycinin subunits in genotypes null in 11S, due to higher protein loss in H and H-H samples as compared to UH (**Figure 3A**). **Figure 3** also summarizes the protein losses during subsequent centrifugations. As expected, a gradual decrease in total protein content for all genotypes and for all treatments was observed during the second (at 15000*g*, SN2), third (40000*g*, SN3), and fourth centrifugation steps (122000*g*, SN4) (**Table 3**).

In SN2 (15000*g*), as in SN1, the effects of heat treatment and heating-homogenization were significant only for samples made from Harovinton, V3 (null α′, A_4, A_5), and V4 (null α′, A_3) (**Table 4**). Of interest are also the results from the last two centrifugation steps, as they may indicate the stability of soluble aggregates and soluble proteins. After the third centrifugation step (SN3), unheated samples continue to show large losses of proteins in the supernatant (**Figure 3C**). For unheated soymilks, the largest protein losses were observed for samples containing glycinin, except in the cases when the subunits of group IIb (A_3) were present. Line 5 (V5), for example, containing a low ratio of 11S/7S and only group IIb of glycinin, had one of the highest retentions of protein after centrifugation at 40000*g* and was also unique in the very low losses shown after heating and homogenization (<10%). It may be hypothesized that this line has an optimal ratio of subunits that cause the formation of soluble complexes during the process of making soymilk. Lines containing no glycinin (V9, V10, and V11) presented lower

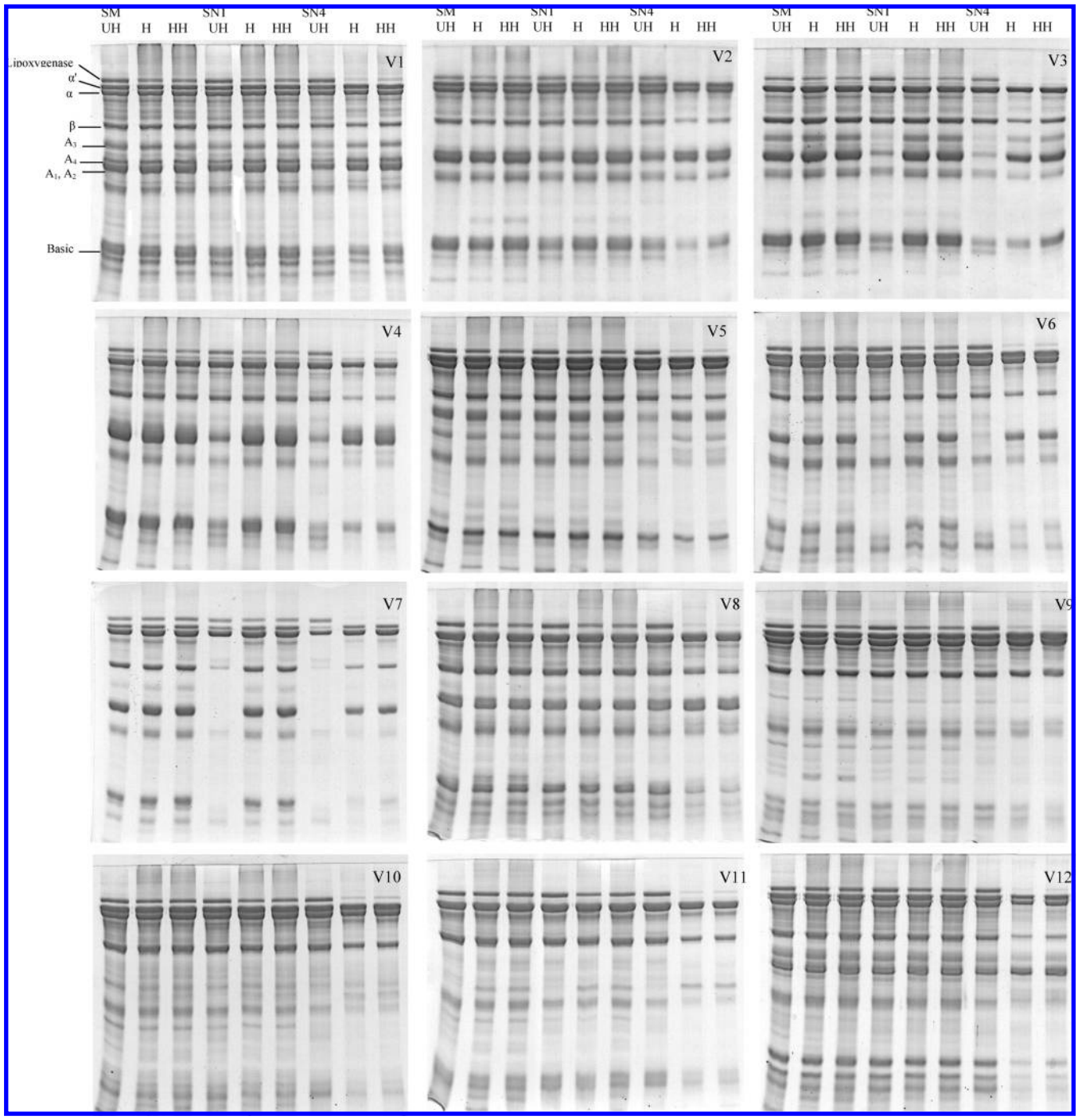

Figure 4. SDS-PAGE electrophoresis of unheated (UH), heated (H), heated−homogenized (H-H) soymilk and corresponding supernatants: SN1-UH, SN1-H, SN1-H-H, SN4-UH, SN4-H, and SN4-H-H. The soybean genotypes are marked as follows: V1 (Harovinton), V2 (null A_3), V3 (null α′, A_4, A_5), V4 (null α′, A_3), V5 (null A_1, A_2, A_4, A_5), V6 (null A_3, A_4, A_5), V7 (null α′, A_3, A_4, A_5), V8 (null α′, A_1, A_2, A_3), V9 (null 11S), V10 (null 11S), V11 (null α′, 11S), and V12 (null A_1, A_2).

protein losses than lines containing the subunits of group I (A_1,A_2) of glycinin (V1, V2, V3, V4, V6, and V7). The data also suggest that the absence of the α′ subunit of β-conglycinin may negatively affect protein solubility. Line 12 (V12) also seems to have overall lower protein losses as compared to the other samples containing 11S. This genotype contains groups IIa and IIb and has a lower 11S/7S ratio than V1, V2, V3, V4, V7, or V8. Statistical analysis on SN3 samples demonstrated that the effect of treatment on SN3 was significant only for null genotypes V2, V5, V8, and V11 (**Table 4** and **Figure 3C**).

Figure 3D shows the percentage of protein loss in SN4. In this case, it is expected that most colloidal particles would precipitate and only soluble proteins would remain in the supernatant. Even after this step, the amount of protein remaining was significantly affected by genotype. There were significant treatment effects on protein retention for Harovinton and null genotypes V2, V5, V8, and V11 (**Table 4**). For these genotypes, heated and heated-homogenized soymilks had significantly lower protein solubility as compared to unheated samples, except for heated-homogenized soymilk made from

Harovinton (**Table 3**). In these samples, heating caused a larger extent of aggregation of the soluble protein. It should be noted also that within genotype, the treatment effects (UH, H, and H-H) were not significantly different in the amount of protein at any centrifugation stage for V6, V7, V9, V10, and V12 (**Table 4**).

These results revealed the significant effect of protein composition and treatment applied during soymilk processing on the solubility of soy protein in soymilk. Unheated samples containing glycinin subunits of group I [Harovinton (V1), V2, V3, V4, and V7] showed the largest protein losses after the first step centrifugation (SN1) as compared to all of the genotypes not containing group I. However, it is important to note that all samples after high speed centrifugation (122000*g*) showed large protein losses after heating, indicating that soluble proteins aggregate with heating. When comparing unheated samples, the ratio of 11S/7S affects the protein solubility after 122000*g*, as heating causes aggregation of β-conglycinin subunits.

The results from the stepwise centrifugation suggest that in the absence of 11S or group I of glycinin, β-conglycinin subunits are more stable in unheated samples, and heat treatment caused aggregation in soymilk prepared from genotypes null for glycinin or null for group I (A_1, A_2) of glycinin. Homogenization showed improvement in total protein yields for heated samples, but still, the percentage of protein loss at the last centrifugation stage for heated-homogenized soymilks was higher than unheated ones in most cases.

To better identify whether the protein losses and the losses in the large particles were linked to a particular subunit composition, SDS-PAGE electrophoresis was carried out on the soymilk samples and the various supernatant fractions after centrifugation. **Figure 4** illustrates the differences in the subunit composition between the initial unheated, heated, and heated−homogenized soymilk and the corresponding supernatants following the first and fourth centrifugation steps for each genotype. These two centrifugation levels were shown for comparison, as they represent the protein population stable to low centrifugation (8000*g*) as well as the soluble protein after high centrifugation (122000*g*).

With SDS-PAGE, it is possible to separate all of the major subunits of β-conglycinin (α', α, β) and glycinin (acidic, basic) and determine whether a particular fraction is preferentially lost after centrifugation (**Figure 4**). As expected, within each genotype, no differences are shown in the soy protein composition of unheated, heated, and heated-homogenized soymilk before centrifugation. Changes can be noted after the first step centrifugation at 8000*g*, as SN1 of unheated soymilks made from V3, V4, V6, and V7 showed a decrease in the glycinin subunits (both acidic and basic), confirming that the glycinins of soy protein in unheated samples precipitate after the first centrifugation step. These glycinins form large protein particles. In samples null in glycinin, the protein patterns of the first supernatant fractions (SN1) were similar to those of soymilks.

After the fourth centrifugation step at 122000*g*, for all genotypes, the heat-induced precipitation of lipoxygenase (an iron-containing enzyme that catalyzes the oxidation of unsaturated fatty acids) was observed. Surprisingly, in unheated samples, no significant differences could be noted in the subunit composition recovered in SN1 and SN4, suggesting that the protein particles in soymilk are very poly disperse but with a similar subunit composition. This was in disagreement with what was previously reported by other authors (*25*) who noted a clear effect of subunit type on the protein retention after centrifugation. One exception was noted for SN4 of unheated samples made from V5, which showed losses in the glycinin fractions, mainly acidic polypeptides.

Unlike unheated samples, which showed the presence in the SN samples of both the acidic and the basic subunits of glycinin, heated and heated-homogenized samples showed the loss of the basic subunits. These results are in agreement with previous reports (*26*) on the formation of a precipitate during heat treatment, a precipitate composed mainly of the basic polypeptides of glycinin. The acidic polypetides are mostly recovered in the soluble phase after heating.

The electrophoresis results (**Figure 4**) show that, among the β-conglycinin protein subunits, α' and α remained unchanged for heated and heated-homogenized samples even after high-speed centrifugation (SN4, at 122000*g*). However, the β subunit of β-conglycinin decreased in soymilk supernatants from soymilks prepared from lines containing glycinin. It has been suggested previously that heat treatment causes protein dissociation and that the dissociated subunits interact with one another and form a soluble macrocomplex (*26*). It has also been reported that at least three macrocomplexes form (α'-basic, α-basic, and β-basic), but two of them undergo dissociation during SDS treatment. The interaction of the β subunit with the basic polypeptide of glycinin is stronger than that of α' and α subunits, and this β-basic macrocomplex remains intact during electrophoresis (*26, 27*). The existence of such interactions can be confirmed by the electrophoresis results for supernatants from null genotypes V9 (null 11S), V10 (null 11S), and V11 (null α', 11S). In the absence of glycinin, the β subunit of β-conglycinin remains unchanged.

The use of various genotypes to determine if subunit composition of soy protein affects the processing behavior of soybean is important, as processing history determines protein functionality. This study shows that genotype differences in 7S and 11S ratio not only affect the solubility and protein recovery in soymilk, but more importantly, by controlling the changes in subunit composition, it is possible to modulate protein−protein interactions in soy milk. Moreover, the differences between heating and heating and homogenization are also highlighted for the first time.

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Received for review March 22, 2008. Revised manuscript received June 12, 2008. Accepted July 22, 2008. This project was financially supported by the Ontario Soybean Growers.

JF800897S