Changes in Protein Characteristics during Soybean Storage under Adverse Conditions As Related to Tofu Making

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ABSTRACT: Soybeans stored under adverse conditions decrease in protein recovery (content) in the soymilk and tofu yield. This study investigated how protein structural changes contributed to the decrease in tofu yield. Soymilks were produced from original soybeans (Proto and IA2032 cultivars) and adversely stored soybeans, respectively, and soymilk protein contents were adjusted to the same level before making into tofu. Tofu yield was compared with that made from soybeans without protein content adjustment. For understanding protein structural changes, soy proteins were extracted from Proto soybean by using different solvents, including distilled water, sodium dodecyl sulfate (SDS), and 2-mercaptoethanol. The proteins in the extracts were analyzed by using SDS-PAGE and gel filtration. Results showed that tofu yield was more significantly affected by protein structural characteristics than the protein content in soymilk. Different levels of aggregations among 7S and 11S proteins during adverse storage were responsible for decreasing protein recovery in the soymilk.

KEYWORDS: soybean, storage, soymilk, tofu, proteins, gel filtration, SDS-PAGE

■ INTRODUCTION

After harvest, soybeans are subjected to storage until they are processed into soy foods. Soybean seeds are living tissues, which undergo physicochemical and biological changes during storage. The major factors affecting the storability of soybeans include ambient relative humidity (RH), seed moisture content (MC), temperature (T), and duration time of storage (t). Soymilk and tofu are important commercial products of soybeans. Storage at adverse conditions can cause significant decrease in the protein content in soymilk as well as tofu yield.[1] A primary factor responsible for the loss of tofu yield during storage is the decrease in protein extractability and the changes in the protein structures. Denaturation of soybean protein during storage was reported. Soy proteins tend to denature when subjected to adverse conditions such as extreme pH and temperatures.[2−5,13] Structural characteristics of the two major globular proteins, glycinin (11S) and β-conglycinin (7S), can change significantly during storage as a result of various reactions including glycosylation, formation of intramolecular disulfide bonds, and the decrease in the surface hydrophobicity.[2−5] Aggregation of proteins is among the major reasons for the decrease of extractability, and the forces may vary between the two globular proteins.[17] Hoshi and others (1982) reported that the hydrogen, hydrophobic, and disulfide bonds participate in the polymerization of 7S globulin and that the disulfide bond is strongly related to the polymerization of 11S globulins.[4] Hou and Chang[5] also reported changes in the structural properties of glycinin and β-conglycinin during adverse storage of high humidity and high temperature. The molecular changes include a decrease in secondary β-pleated sheet structure, a decrease in hydrophobicity, and an increase in disulfide bonds with the increase in storage time.[3,5] Some differences in the behavior of glycinin and β-conglycinin during storage also exist and may lead to different extraction ability

that will subsequently alter the composition and other properties of soymilk and tofu.[16,18,19]

It has been known that soybean storage at adverse conditions leads to decreases in protein content in soymilks which subsequently resulted in decrease in tofu yield,[1] and the changes in protein structures and functionality may also contribute to the decrease in tofu yield and quality.[3,5] However, scientific evidence to support this viewpoint has not been reported in the literature. Therefore, the objectives of this study were to (1) investigate how changes in protein quality affect tofu yield and (2) study the structural features and associated forces of protein and subunits in stored soybeans and the soymilk extracts that may cause the loss of tofu yield. Protein content were adjusted to the same value in the soymilk extracted from both normal and adversely stored soybeans before making tofu with an aim to eliminate the effect of protein content variability. The tofu yield was then compared with the soymilk extracts without protein adjustment to assess the role of protein quality in tofu making. Different solvents were used to extract proteins in order to reveal molecular association forces involved in protein structural changes. Gel filtration and SDS-PAGE were used to examine the formation/disassociation of aggregates and subunits. This study is expected to enhance the understanding of the biochemistry of tofu making as affected by the changes of protein characteristics during storage.

■ MATERIALS AND METHODS

Soybeans and Chemicals. The soybeans used in storage study included Proto and IA2032 harvested in 2001 and 2002. Proto is the most popular cultivar in North Dakota; it was used in this study for tofu making and protein analysis . IA2032 is a genetically modified variety that is lipoxygenase (LOX) free, which was only used for tofu making as a comparison variety. Storage conditions include RH 60%, 65%, 70%, 75%, and 80%; temperatures include room temperature (22 °C), 30, and 40 °C. The storage duration was up to one year depending on storage conditions. Soybeans stored under freezing conditions (−18 to −20 °C) were used as control. Our test indicated that no significant change in tofu yield had occurred for this condition. Detailed methods for storage experiments, including the selection of saturated salts for achieving particular RHs, were described in previously published paper.[1] Chemicals used in this study were reagent grade from Fisher Scientific (Pittsburgh, PA), Sigma Chemical Co. (St. Louis, MO), and VWR Scientific Products (Minneapolis, MN).

The columns and resins used for gel filtration (Sephacryl S-300 high-resolution, column dimensions: 2.6 cm diameter × 95 cm height) chromatography were from Amersham Pharmacia Biotech (Piscataway, NJ). Gel electrophoresis was performed in a Bio-Rad Protean II chamber (Bio-Rad Laboratory, Hercules, CA). The MW-GF-1000 molecular mass marker kit, consisting of carbonic anhydrase (29000), bovine serum albumin (66000), alcohol dehydrogenase (150000), β-amylase (200000), apoferritin (443000), thyroglobulin (669000), and blue dextran (2000000), was from the Sigma Chemical Co.

Proximate Analysis. Soybeans were ground with an analytical mill (model A-10, Tekmar Company, Cincinnati, OH) in which the resultant flour was passed through a 60 mesh screen. Moisture contents were measured by using a vacuum oven method (AOAC 925.09, 1995). Solids content was calculated by subtracting moisture from sample weight. The same method was used to determine the moisture contents of the freeze-dried soymilk and tofu.

Water activity of stored soybeans was measured with an Aqualab instrument (Decagon Devices, Inc., Pullman, Washington, 99163). The pH of soymilk was measured with a digital pH/millivolt meter 611 (Thermo Orion Corporation, Beverly, MA, 01915). The crude protein contents in soybeans and freeze-dried soymilk/tofu were determined using Kjeldahl method (AOAC 955.04, 1995).

Tofu Processing with Protein Adjustment. Soybeans under various adverse storage conditions were used for tofu making. Table 1

Table 1. Soybean Varieties and Storage Conditions Used for Protein Adjustment Experiments

RH (%)	T (°C)	variety[a]
60	30	Proto
60	40	Proto
65	30	IA2032
65	40	Proto, IA2032
70	30	Proto, IA2032
75	30	Proto
80	40	Proto

[a]Initial moisture contents are 13.34% for Proto and 12.02% for IA2032.

shows the storage conditions and varieties used for this study. Soymilk and soft tofu were prepared using the method of Cai and Chang with slight modifications.[6] Soybeans (431 g) were soaked in room temperature overnight (10−11 h) and then ground in water (water/bean = 7:1) using a soybean grinding machine (Chang-Seng Mechanical Company, Taoyuan, Taiwan) equipped with a centrifugal separator (120 mesh screen) that separated solid residue (okara) and soymilk. Total volume of soymilk was measured, and a small portion of the soymilk sample was freeze-dried for chemical analysis. The protein content in soymilk was determined using the Dumas combustion method (AOAC 992.23, 1995) in which a Leco system (model FP-

2000, Leco Corporation, St. Joseph, MI) was employed to determine nitrogen content in soymilk. The protein content was calculated by using a conversion factor of 6.25. The protein content in the soymilk was adjusted to 4.25% (0.68% in nitrogen) (w/v) by adding water. Some samples stored at adverse conditions had very low extractability in proteins such as the samples stored at 40 °C for one year. In this case, the water:bean ratio was reduced to obtain a required protein level in soymilk. For samples stored at 40 °C, the water/bean ratio could be reduced to 6:1. We assume that the small range of variation in water:bean ratio had no significant impact on soymilk property. A portion of 910 mL of such soymilk was used to make tofu. Soymilk was boiled for 5 min before being quickly cooled to 87 °C by stirring. A coagulant suspension, containing 2.6 g (approximately 2% of the soybean weight) of calcium sulfate dispersed in 20 mL of water, was poured into soymilk rapidly; meanwhile, the soymilk was stirred at 150 rpm using a stirrer (model RZR1, Caframo LTD, Wiarton, Ontario, Canada), equipped with a rectangle paddle (14 cm length × 1.5 cm width). After the addition of coagulant, stirring continued for several seconds equal to the optimum stirring time (t_m) to produce the highest tofu yield. The t_m for each storage condition was developed by trial and error.[1] Then the mixture was poured immediately into a muslin cloth lined wooden mold (13 cm × 13 cm × 7.5 cm), which was lined with a plastic film. The curd was left to incubate for 8 min. The plastic film was then removed. The tofu curd was packed tightly with the muslin cloth, followed by pressing with iron blocks to remove water. The iron blocks were used to first produce a pressure of 6300 N/m² on tofu gel for 15 min, followed by 9500 N/m² for another 15 min. After that, the tofu was removed from the cloth, stored in water at 4−5 °C overnight, and used for color and textural analysis. The tofu weight was recorded as g/910 mL soymilk with 4.25% protein content.

Soy Protein Extraction and 7S/11S Isolation. Soybeans were ground in an analytical mill (model A-10, Tekmar Company, Cincinnati, Ohio) and the powder passed through a 60 mesh sieve. Soybean powder was defatted by *n*-hexane (soy flour/hexane = 1:5, v/v) for 1 h at 4 °C, followed by centrifugation at 8000g for 15 min. The supernatant solvent, containing soybean oil was removed and solids were extracted two more times. After defatting, the solvent was evaporated under a fume hood. The defatted flours were used for soy protein extraction.

The fractionation method reported by Bogracheva et al. with slight modifications by Hou and Chang[3,5] was used to fractionate glycinin (11S) and β-conglycinin (7S) from defatted soybean flour.[3,7] Briefly, defatted soy flour was added with 10-fold (w/v) distilled water and mixed for 1 h. The mixture was centrifuged at 12000g at 4 °C for 30 min. The supernatant is referred to as the total protein extract. The supernatant was then adjusted pH to 6.5 with 0.1 N HCl and stood overnight at 4 °C before centrifugation at 12000g at 4 °C for 20 min. The pellet was the crude 11S. The supernatant was added with 0.1 N HCl to adjust pH to 5.5 before centrifugation at 12000g at 4 °C for 20 min. The supernatant pH was then adjusted to 4.8, and another centrifugation was conducted at the same condition as described. The pellet was collected as crude 7S. The crude proteins were washed twice with ice-cold water and then dispersed in proper volumes of a standard buffer (2.6 mM KH₂PO₄, 32.5 mM K₂HPO₄, 0.4 M NaCl, and 0.2% NaN₃; pH 7.6). Traces of precipitate were removed by centrifugation at 1000g for 30 min. The isolated proteins were kept in the standard buffer containing sodium azide (NaN₃) (5 mM) for future analysis. The Biuret method was used to determine the protein content of isolated glycinin and β-conglycinin. Bovine serum albumin (BSA) was used for establishing the standard curve. Protein extractability was expressed as the extracted protein weight divided by the weight of defatted soybean powder and multiplied by 100%.

To investigate the molecular forces involved in the protein aggregation, 1% SDS and 5 mM 2-mercaptoethonal (2-ME) were used as extraction solvents and their effect on the isolation yields for the total protein, 7S, and 11S were compared with the extraction result with pH 7.8 distilled water.

Gel Filtration Chromatography. A Sephacryl S-300 superfine gel filtration (2.6 cm × 95 cm) column with a molecular mass fractionation range of 10000−1500000 Da was used to analyze
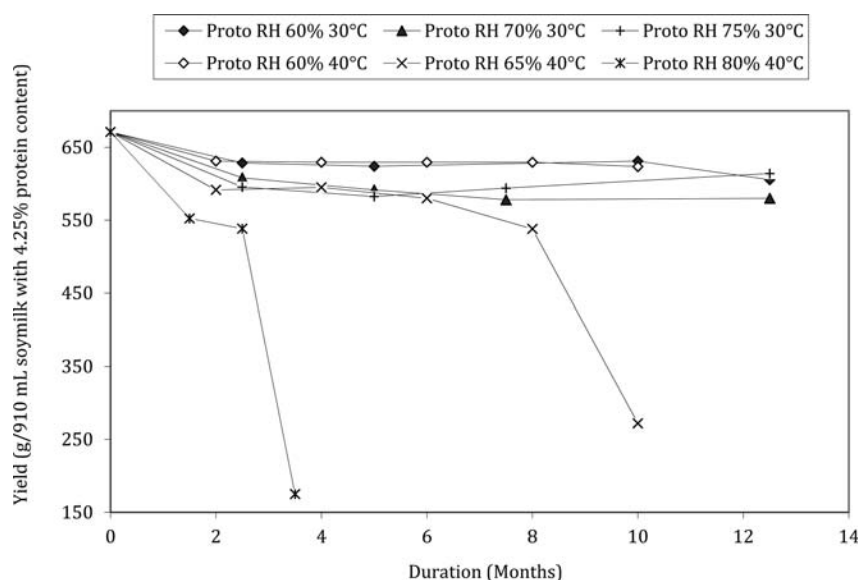
Figure 1. Yields of tofu made from Proto soybeans stored under various conditions. Protein content was adjusted to 4.25% for all the conditions. Each data point is an average of two replicates.

soybean extract. The Sephacryl S-300 column was calibrated with the MW-GF-1000 molecular weight marker kit from Sigma Chemical Co. The soybean protein extract was eluted with the standard phosphate buffer (0.05 M) at pH 7.0 at 4 °C to retain its structure as close as possible to its native structure. Approximately 5 mL of the soy extract was applied onto the column. The column was eluted with one column volume of buffer at a flow rate of 20 mL/h. Fractions of 5 mL/tube were collected, and the absorbance at 280 nm was measured. Fractions of the major peak were analyzed by electrophoresis (SDS-PAGE) as described in the next section.

SDS Electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Hou and Chang.[5] A Bio-Rad Protein II vertical slab gel apparatus (Bio-Rad Laboratory, Hercules, CA) was employed to produce a discontinuous acrylamide gel. The stacking gel was a nonrestrictive gel with 4.0% acrylamide-bisacrylamide (40% T, 4.5% C), 0.1% SDS, 0.05% ammonium persulfate, and 0.1% TEMED at 0.125 M Tris, pH 6.8. The separating gel was a linear gradient acrylamide gel with concentrations varying from 8 to 16% and contained 0.1% SDS, 0.05% ammonium persulfate, 0.05% TEMED at 0.375 M Tris, pH 8.8. A 16 cm ×16 cm × 0.15 cm (length × width × thickness) slab gel was prepared, with 12 cm being separating gel and 4 cm being stacking gel. A 1.5 mm thickness comb was used to create 15 wells on the gel top. The protein concentration was diluted to 1 mg/mL with a buffer, which consisted of 12.5% Tris-HCl stock solution (0.5 M, pH 6.8), 10% glycerol, 20% sodium dodecyl sulfate (SDS) stock solution (10% w/v), 5% 2-mercaptoe-thanol (ME), and 5% bromophenol blue stock solution (1% w/v) and heated in a water bath at 95 °C for 5 min. The protein sample (∼10 uL) was applied to the gel and run at a constant current of 35 mA per gel until the tracking dye reached the bottom of the gel.

Upon the completion of electrophoresis, the gel was stained in a staining solution which consisted of 0.1% Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid on an orbital shaker (LabLine Instruments Inc., Melrose Park, IL). After 8 h of staining, the gel was destained in a quick destaining solution, which consisted of 40% methanol and 10% acetic acid, for 3−4 h to remove background stain. Subsequently, the gel was gently shaken in a regular destaining solution, which consisted of 5% methanol and 7.5% acetic acid, with at least two to three changes to enhance the destaining process until the gel was almost visibly cleared of the dye background. A broad range of molecular mass standard proteins including α-actinin (100000), bovine serum albumin (66000), actin (42000), carbonic anhydrase (29000), and cytochrome C (12400) were used to calculate molecular weight.

RESULTS AND DISCUSSION

Tofu Production at the Same Soymilk Protein Concentration. In our previous paper, we reported that storage under adverse conditions caused significant decreases in tofu yield.[1] One reason for the yield decreases was the reduced protein extractability in stored soybeans, and the degree of reduction increased with higher temperature and RH. In addition, the deterioration in protein functionality such as weakened hydrophobic interactions may also significantly contribute to the yield loss. To assess this effect, we adjusted the protein content in the soybean extracts (soymilk). The soymilk protein contents were adjusted to the same level (4.25% w/v) by adding water or changing water-to-bean ratio before making into tofu, which was analyzed for yield. Figure 1 shows the tofu yield changes for Proto soybeans stored at selected storage conditions, as expressed by g tofu/910 mL soymilk. The tofu yield decreased with increase in temperature and RH. The yield decreased slightly for the samples stored at 30 °C and all RHs and significantly for those stored at 40 °C and RH > 65%. These results indicated that deterioration in the quality of soymilk proteins significantly contributed to tofu yield decrease. These figures also showed some critical conditions, e.g., 80% RH and 40 °C and 3.5 months, and 65% RH and 40 °C and 10 months. Similarly, IA2032 soybeans showed dramatic quality deterioration at storage conditions (critical condition) of 65% RH and 40 °C and 8 months. We had previously defined "critical conditions" as the conditions that caused a drastic decrease in tofu yield in our previous publication.[1] When stored at these critical conditions, tofu yield losses were drastic even if the soymilk protein content was adjusted to the same 4.25% level. Large granules formed in the soymilk after addition of coagulant. The gel matrix was very loose and easy to collapse.

To illustrate the effect of protein quality on tofu yield, the tofu yields for the soybeans with/without soymilk protein adjustment for Proto soybean stored under 65% RH and 40 °C were plotted for a comparison (Figure 2). The protein content before adjustment was labeled for each data point on the curve. The results showed that these two curves were close, and both
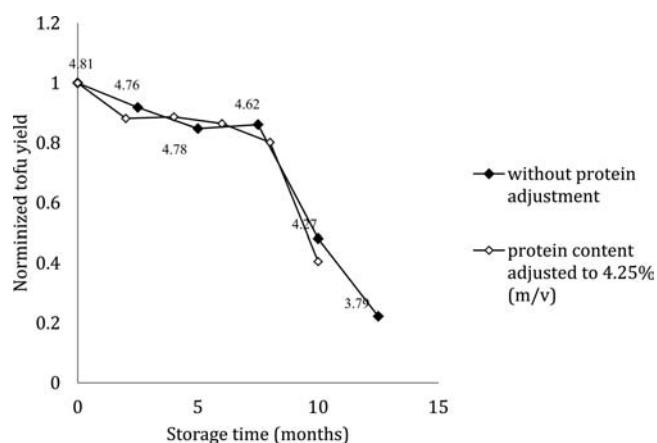
Figure 2. Comparison between yields of tofu made from soymilk with or without protein adjustment. The soybeans were Proto stored under 65% 40 °C. The values denoted for each point on the curve without protein adjustment are protein content in soymilk. Normalized tofu yield is the ratio of the yield of stored soybeans to that of original soybeans.

showed the appearance of critical conditions at approximately 10 months, even though the protein contents were significantly different. Similar results were observed in other storage conditions and IA2032 soybeans. This result indicated that the protein functionality was a more important factor than protein content in affecting the tofu yield. Storage under adverse conditions caused deterioration in the functionality of proteins to subsequently result in poor water holding capacity

and less ability to form a gel network during tofu making. The resultant tofu had high solids content, with a hard and rough texture similar to the ones without protein adjustment as described in our previous paper.[1]

It had been reported that the formation of tofu gel network was partly a result of hydrophobic interactions.[8] Hou and Chang found that glycinin and β-conglycinin after storage at adverse conditions (84% RH and 30 °C) exhibited a significant decrease in surface hydrophobicity.[3,5,9] These changes could have significantly impact the extent of protein gelation and consequently tofu yield. More detailed study, such as examination of tofu gel structure and distribution of protein particles in soymilk, is needed to fully understand how storage caused the weakened protein interactions.

Progressive Changes in the Elution Pattern of Protein Extracts of Stored Soybeans. To understand how storage induced the decrease in tofu yield, the composition of the soybean extract was examined using gel filtration. The protein extracts (in distilled water) of four Proto samples were tested, including the original samples, and samples were stored for 12 months at 65% RH and 22 °C, 65% RH and 30 °C, and 65% RH and 40 °C. Figure 3a−d shows the progressive changes in elution patterns. It was noted that each profile had 8−9 peaks. SDS-PAGE electrophoresis was conducted to identify these peaks, and the patterns as shown in Figure 4 showed that these peaks contained different components: peak 1, 7S + 11S complex I (larger aggregate, molecular weight >1500 kDa); peak 2, 7S trimers and/or 11S hexamers (molecular weight ~296 kDa); peak 3, presumably mixture of many smaller proteins, with molecular weight 65 kDa; peak 4, presumably β-
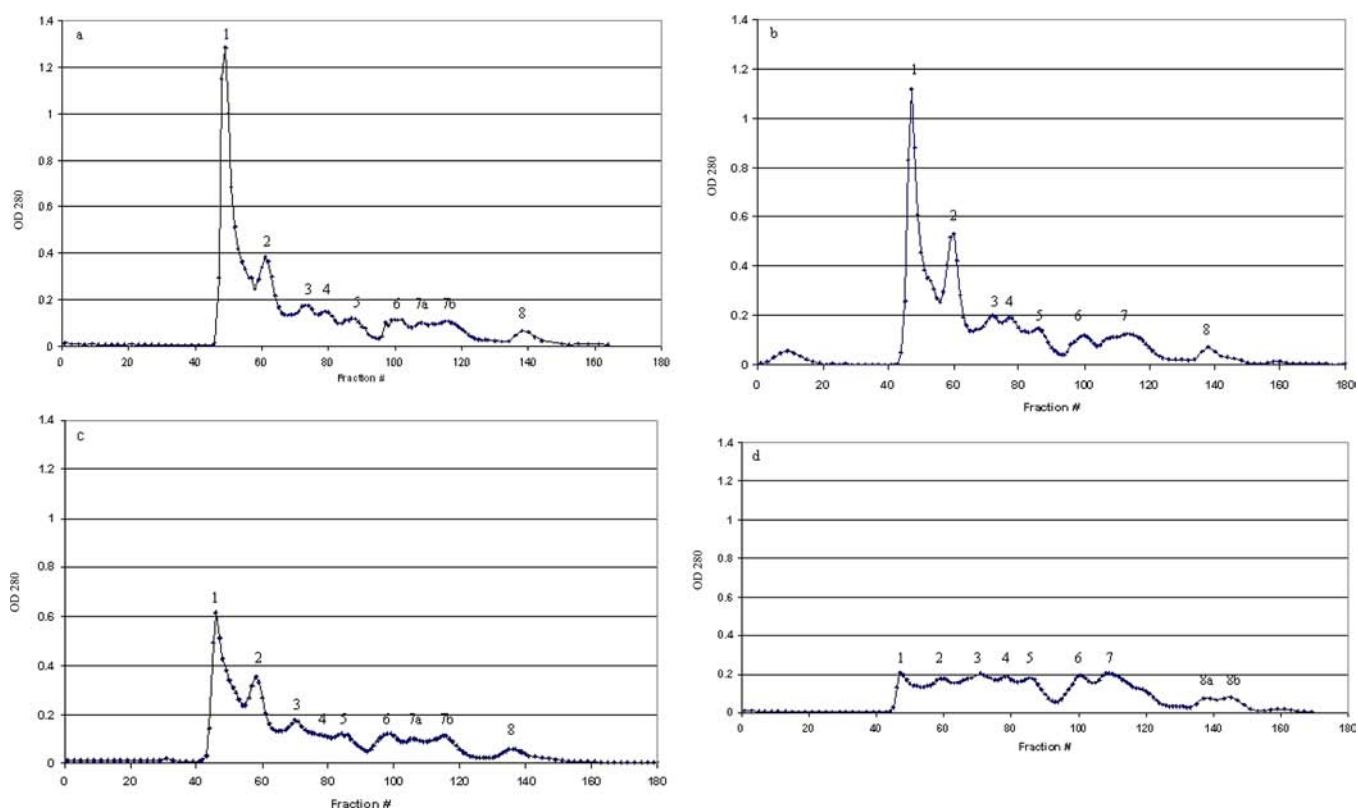

Figure 3. Elution patterns (UV 280 absorbance) of protein extracts from gel filtration column. Samples: (a) Proto original extract, 5 mL, and 12.67 mg/mL; (b) Proto 65% RH, 22 °C, 12 months, 5 mL, and 12.87 mg/mL; (c) Proto 65% RH, 30 °C, 12 months, 5 mL, and 10.49 mg/mL; (d) Proto 65% RH, 40 °C, 12 months, 8 mL, and 4.49 mg/mL.
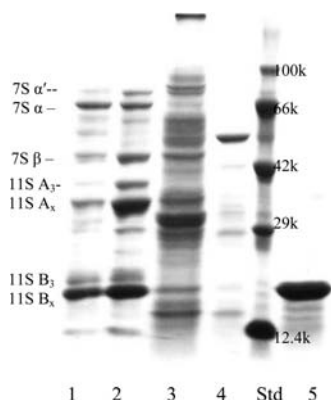
Figure 4. SDS-PAGE for the peaks of gel filtration elution patterns. Sample identified: lane 1, peak 1 (7S + 11S complex); lane 2, peak 2 (7S + 11S complex); lane 3, peak 3 (presumably a mixture of many proteins); lane 4, peak 4 (presumably β-amylase or γ-conglycinin); lane Std, molecular mass standards; lane 5, peak 5 (11S basic subunit). A_x represents acidic subunits $A_1A_2A_4$, and B_x represents basic subunits $B_1B_2B_4$.

amylase or γ-conglycinin; peak 5, 11S basic subunit; peak 6–8 did not show protein subunits and they were most probably smaller peptides or amino acids from the degraded proteins that absorbed UV 280 nm radiation but too small to be detected by the specific SDS-PAGE system, which had 8–16% gel gradient that could not retain molecules smaller than 10000 Da.

Comparison between the elution profiles as shown in Figure 3a–d gives a picture as to how the protein components evolved when storage environment changed from mild to adverse conditions. Figure 3a implies that the aggregates (complexes) of 7S and 11S, mainly found in peaks 1, were the most abundant in the soymilk made with the original soybeans (stored at freezing conditions). Therefore, the proteins in soymilk are mostly in the form of aggregates of 7S and 11S, other than monomeric subunits. Figure 3b–d implies that adverse storage had promoted protein aggregation in the seeds in which large aggregated proteins became insoluble in water and thereby resulted in a reduction of the amount of soluble complexes of 7S/11S in the soymilk extract. As a result, the height of peaks 1 and 2 gradually decreased with the increase in the adversity of the storage conditions. Insoluble aggregates formed might be also filtered out before samples were placed in gel filtration column and thus could not be shown in the elution profiles. In addition, the contents of small-molecule substances (peaks 6–8) did not change significantly in the four profiles, implying that degradation might not be a major reaction as compared to aggregation.

Protein Extractability in pH 7.8 Distilled Water. To understand how storage induced decreases in protein extractability, the Proto samples stored for one year at 65% RH and different temperatures were extracted with distilled water. Table 2 shows that the isolation yields of total protein, 7S, and 11S were all decreased with elevated temperatures. The samples stored at 40 °C showed the highest decrease in extractability: total protein recovery decreased from 44.4 to 15.2%, 7S recovery from 6.3 to 2.9%, and 11S from 6.0 to 0.1%. The greater decreases in 11S than 7S were consistent with previous findings that 7S was more resistant to adverse storage conditions.[3,10,11] The difference in the storability of 7S and 11S may be caused by the different molecular interaction forces

Table 2. Isolation Yields of Proteins Extracted in pH 7.8 Distilled H_2O^a

variety	total protein yield (%)	7S yield (%)	11S yield (%)
original	44.4 ± 3.6	6.3 ± 0.4	6.0 ± 0.3
RH 65% 22 °C 12 months	44.0 ± 2.2	5.3 ± 0.1	3.4 ± 0.1
RH 65% 30 °C 12 months	34.6 ± 0.1	3.5 ± 0.2	0.9 ± 0.0
RH 65% 40 °C 12 months	15.2 ± 0.6	2.9 ± 0.1	0.1 ± 0.0

[a]Yield is based on defatted soybean flour weight. Data are expressed as mean ± standard deviation for total protein yield and are the means of two replicates.

involved in their structures. The 11S polypeptides contained more disulfide bonds than 7S proteins, and their disulfide bonds increased during storage,[3,5] which may result in more polymerization between subunits and subsequently a more significant decrease in solubility. For 7S protein, the quaternary structure is mainly stabilized by hydrophobic interactions.[5]

Figure 5 shows the SDS-PAGE images for the extracted 7S and 11S proteins. The 7S and 11S subunits in the original
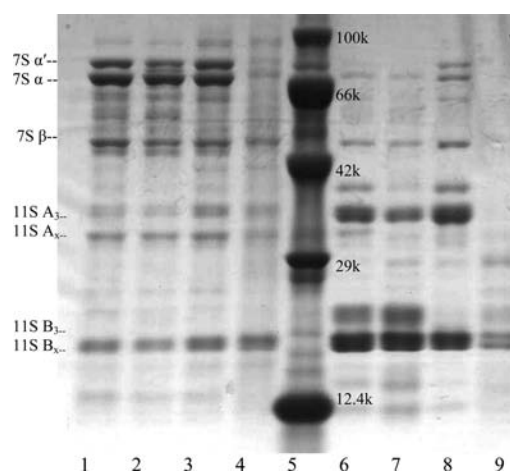

Figure 5. SDS-PAGE electrophoresis for 7S and 11S extracts of samples stored at 65% RH and different temperatures for 12 months. Lanes identified: lane 1, original 7S; lane 2, 7S of RH 65%, 22 °C sample; lane 3, 7S of RH65%, 30 °C sample; lane 4, 7S of RH 65%, 40 °C sample; lane 5, protein molecular mass markers; lane 6, original 11S; lane 7, 11S of RH 65%, 22 °C sample; lane 8, 11S of RH 65%, 30 °C sample; lane 9, 11S of RH 65%, 40 °C sample. All samples were Proto and stored for 12 months.

soybeans could be clearly observed in lanes 1 and 6, respectively, and those stored at room temperature were also clearly shown in lanes 2 and 7, respectively, without significant changes after storage. There were some changes that occurred on the soybeans stored at 30 °C (lanes 3 and 8). The band intensity of some peptide subunits was reduced, such as B3 (lane 8). Significant changes occurred for the samples stored at 40 °C (lanes 4 and 9), with only very little 7S and 11S subunits remaining on the lanes 4 and 9, respectively. The two lanes also showed a gray-colored path, indicating the presence of polymers of 7S and 11S subunits. Deep-colored spots were also observed on the top of these lanes, implying the formation of very large protein aggregates which were not able to penetrate into the acrylamide gel. It is thus proved that extensive aggregation occurred in soy proteins during storage that led to reduced solubility and extractability.

Protein Extraction Using Various Reagents. To understand the forces involved in storage-induced protein aggregation, SDS and 2-ME were combined into the solvent for protein extraction. When both 1% SDS and 5 mM 2-ME were combined into the solvent, the protein yields in the extract for all the four samples used in Table 2 were in the narrow range of 46 ± 4% despite the different storage conditions. This result indicated that the additions of SDS and 2-ME could extract all the proteins in the defatted soybeans, implying that the use of SDS and 2-ME was effective for solubilizing the protein aggregates.

A further experiment was conducted to compare the effects of SDS and 2-ME separately. Table 3 shows the results of

Table 3. Isolation Yield of Total Proteins Extracted with Different Chemical Reagents for Proto Stored under RH 65% and 40°C for 12 Months[a]

extraction solvent	total protein yield (%)
1%SDS + 5 mM 2-ME	46.5 ± 3.5
1%SDS	43.6 ± 3.7
pH 7.8 D·H$_2$O + 5 mM 2-ME	24.6 ± 0.9
pH 7.8 D·H$_2$O	15.2 ± 0.6

[a]Yield is based on defatted soybean flour weight. Data are expressed as mean ± standard deviation, and are the means of two replicates.

isolation yields for the soybeans stored at 65% RH and 40 °C, using different solvents. A solution of 1% SDS in distilled water extracted 43.6% of the proteins, which was close to that of the original soybeans (44.4%). However, when a solution of only 2-ME was added, the yield was only 24.6%, although it is higher than that recovered by using distilled water alone (15.2%). Therefore, the primary force involved in the formation of protein aggregates in the seeds during storage was most likely to be hydrophobic interactions, which could be reduced by SDS. Disulfide bonds also played an important role because the addition of 2-ME could improve protein recovery by 9% (from 15.2% to 24.6%). Similar molecular interactions were reported for the formation of protein particles in soymilk, where hydrophobic interaction was more important than disulfide bonds.[12]

Figure 6 shows SDS-PAGE electrophoresis for extracts with different solvents for the soybean samples listed in Table 2. The sample concentrations were adjusted to 1 mg/mL. The results indicated that for soybeans stored at RH 65% and 22 °C and at RH 65% and 30 °C had no obvious changes in the peptide subunits, whereas samples stored at 40 °C (lanes 4, 5) had significant changes, with most of 7S and 11S subunits reduced. Lanes 4 and 5 showed very similar pattern to lane 11, with large amount of aggregates spread on the path as indicated by the gray color, although lane 11 was water-extracted while lanes 4 and 5 were extracted with SDS and 2-ME. These results indicated that a large amount of indefinite molecular size aggregates existed in the proteins extracted by SDS and 2-ME. Although SDS and 2-ME could convert water-insoluble aggregates into soluble aggregates (as indicated by the increased total protein yield), they are unable to completely disassemble these soluble aggregates into monomeric 7S and 11S subunits. Therefore, complex interaction forces other than hydrophobic interactions and disulfide bonds or possible interactions with other nonprotein components might have been involved in the formation of the polymers, which could not be completely disassociated by SDS and 2-ME.
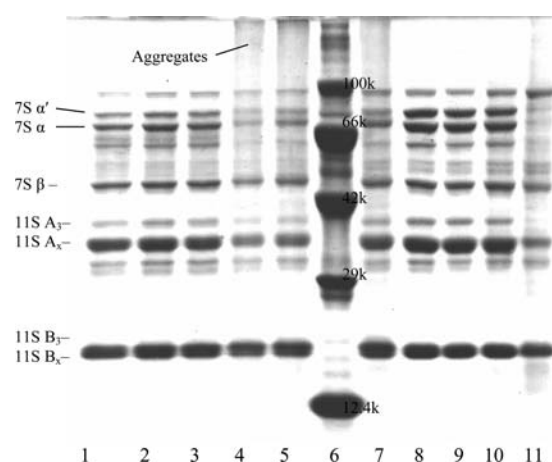

Figure 6. SDS-PAGE electrophoresis for protein extracts of samples stored at 65% RH and different temperatures for 12 months. Lanes identified: lane 1, original with 1% SDS + 5 mM 2-ME; lane 2, 65% RH, 22 °C with 1% SDS + 5 mM 2-ME; lane 3, 65% RH, 30 °C with 1% SDS + 5 mM 2-ME; lane 4, 65% RH, 40 °C with 1% SDS + 5 mM 2-ME; lane 5, 65% RH, 40 °C with 1% SDS; lane 6, protein markers; lane 7, 65% RH, 40 °C with pH 7.8 H$_2$O + 5 mM 2-ME; lane 8, original with pH 7.8 H$_2$O; lane 9, 65% RH, 22 °C with pH 7.8 H$_2$O; lane 10, 65% RH, 30 °C with pH 7.8 H$_2$O; lane 11, 65% RH, 40 °C with pH 7.8 H$_2$O. All samples were from Proto soybean stored for 12 months.

Similar findings were observed in the Proto soybeans stored at 70% RH and 30 or 40 °C for 10 months. The protein yield, when extracted by using only distilled water, are 44.4%, 37.2%, and 13.6%, respectively, for original soybeans, soybeans stored at 70% RH 30 °C for 10 months, and 70% 40 °C for 10 months. A solvent containing 1% SDS + 50 mM 2-ME extracted the same amount of proteins from the adversely stored samples as that from the original soybeans. However, when using SDS-PAGE to analyze extracts from distilled water and the solvent, it was observed that the electrophoresis patterns were similar for all storage conditions, which confirmed that 1% SDS + 50 mM 2-ME were unable to completely disassociate the aggregates formed during storage.

Multilevel Aggregation: A Speculation on Soy Protein Polymerization during Storage. Figures 6 implied that although applications of SDS and 2-ME could dissolve almost all of the water-insoluble proteins in the adversely stored seeds, a substantial amount of these proteins were still in the form of soluble aggregates, which were either retained at the top of the SDS-PAGE gel or spread on the electrophoresis path as indicated by a gray color. No obvious differences were observed in the SDS-PAGE patterns between the proteins extracted by distilled water and SDS + 2-ME, except that 7S subunits and 11S acidic subunits intensities were decreased somewhat. Aside from disulfide bonds and hydrophobic interactions, other forces such as hydrogen bonds, van der Waals forces, electrostatic interactions, and perhaps some other protein cross-linking through other types of covalent bonds with nonprotein components such as sugar might be involved in the polymerization of protein molecules. Further studies are needed to detect theses forces as well as structure of the aggregates.

Figure 3 implies that soybean proteins mainly exist in different forms of polymer complexes and storage-promoted aggregation leading to a reduction in water-soluble proteins. The polymerization of soy proteins during storage at adverse

conditions has been reported by a number of researchers.[2,4,15] We speculate the polymerization occurs at different levels with different forces involved. We hypothesize the terms "primary aggregates" and "secondary aggregates" to differentiate the different levels of aggregates, mainly based on the extractability of the aggregates in different solvents. Primary aggregates are water-soluble 7S and 11S polymers, which are able to be disassociated by using SDS and 2-ME. During storage, primary aggregates are interconnected into water-insoluble secondary aggregates through hydrophobic interactions and some disulfide linkages, which could not be disassociated by 1% SDS and 2-ME. Storage under adverse conditions promotes the generation of secondary aggregates, leading to a reduced extractability, as indicated by the reduced peaks 1 and 2 in gel elution profiles as shown in Figure 3. The extensive polymerization may reduce hydrophobic interactions of the protein molecules towards the outer layers of the aggregates, therefore restricting the hydrophobic interactions, which are needed for making good tofu gel, among these protein aggregates when they are suspended in water during soymilk and tofu making. As a result, the tofu gel produced a three-dimensional network, which was loosely connected among large granules (aggregates) after adding coagulant and therefore tended to collapse when they were subjected to the force during pressing to lead to a coarse tofu texture and low product yield. More studies are needed to validate this proposed mechanism such as qualitative and quantitative determination of protein interactions as well as the structural characteristics of the aggregates by using various techniques including size distribution using centrifugation[14] and other fractionation methods.

In conclusion, the major finding in this study is that the decrease in tofu yield after storage under adverse conditions is more affected by the deterioration in protein functionality than the decrease in soymilk protein content. Gel elution profiles of soybean protein extracts indicated that the soybean extract (i.e., soymilk) was mainly composed of soluble complexes of 7S and 11S proteins. Storage promoted aggregation reactions among the subunits of 7S and 11S protein. Both 7S and 11S protein yields decreased during storage, and the 11S protein decreased more than 7S. During adverse storage, complex reactions were involved in the formation of the water-insoluble secondary aggregates, making them difficult to be completely dissociated using 2-ME and SDS. It is recognized that only two varieties were included in this study. Studies with other soybean varieties are important to know whether or not the findings in this study are valid for all different genotypes of soybeans.

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Notes

The authors declare no competing financial interest.

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