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Effects of Long-Term Frozen Storage on Electrophoretic Patterns, Immunoreactivity, and Pepsin *in Vitro* Digestibility of Soybean (*Glycine max* L.) Proteins

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Soybean flours stored for 20 years at −20 °C retained protein polypeptide profile integrity. Proteins in stored soybean flours retained their immunoreactivity. Long-term frozen storage of seed flours at −20 °C did not adversely affect seed protein *in vitro* pepsin digestibility.

KEYWORDS: Soybean; long-term storage; protein; immunoreactivity; antibody

INTRODUCTION

In 2007, the U.S. soybean (*Glycine max* L.) production of 70 364 916 tons [1 ton of soybeans = 36.74 bushels, http://www.ussec.org/resources/conversions.html (accessed Nov 30, 2008)] was valued at $26 752 197 000 (*1*). In 2007, the U.S. accounted for 32.71% of global soybean production (*2*). By some estimates (*3*), global soybean production is projected to be about 277 million metric tons by 2015−2016. Soybeans are an important source of proteins, oil, fiber, and certain minerals in human and animal nutrition. Soybean proteins are considered to be of good nutritional quality because they are easily digested (*4*) and, with the exception of methionine, contain all of the essential amino acids required by humans (*5, 6*). Typically, mature soybean seeds contain 40−45% protein on a dry weight basis (*7*), with two storage globulins glycinin (11S) and β-conglycinin (7S) accounting for ∼70% of the total seed proteins (*8*). A significant variation in 11S, 31.4−38.3% of total seed proteins, as a function of the seed variety has been demonstrated by Hughes and Murphy (*9*). Dependent upon the seed type and cultivar, the ratio of 11S and 7S (w/w) may range from 1:3 to 3:1 (*8, 10*). Protein polypeptide variation between wild and cultivated soybeans has also been reported (*11*). A recent study (*12*) found 7S and 11S contents to be higher in seeds with higher total protein content compared to the soybeans containing lower protein amounts. Additionally, the study also revealed the individual subunit polypeptide concentrations to be significantly different between different seed cultivars.

Soybeans are widely used to produce soy-based foods, as an ingredient in the manufacture of several foods, and as an animal feed. Soybean proteins are important in the manufacture of several food products, such as tofu, protein concentrates/isolates, and protein hydrolyzates used as flavoring agents/flavor enhancers. For these and several additional reasons, molecular (*13−17*), functional (*18−23*), and nutritional (*24−27*) properties of soybean proteins have been investigated. Although dry soybean seeds may be stored for long periods, only a limited number of studies on the storage stability of soybean seeds/flours have been reported. Often, such studies are limited to several months to less than 2 years of seed storage and focused on off-flavor developments as a result of lipid oxidation (*28−30*), protein solubility and functionality (*31−33*), and nutritional quality (*34*). However, the effects of long-term storage on molecular properties of soy proteins remain largely unexplored.

Soybeans are one of the "big 8" allergens (*35*) in human food supply. Dependent upon the patient sensitivity and the soybean cultivar, several soybean proteins may be potentially allergenic (*36−40*). Accidental exposure of sensitive individuals to soybeans and/or soybean products is therefore of concern. As soybeans and soybean products find increased use in human food and animal feed supply, the potential for unintended exposure of sensitive humans and animals to soybeans and soybean-derived ingredients also increases. It is therefore important to assess whether or not immunoreactivity of soybean seed proteins is altered during the long-term storage. At least one study (*41*) has reported the generation of neoallergens during storage, where fresh soybeans were reported to be less allergenic than stored soybeans. The increase in allergenicity was attributed to the increase in temperature upon storage/transportation. We were therefore interested in assessing polypeptide stability of proteins extracted from stored soybean flours.

The current investigation was therefore undertaken to assess polypeptide profile stability, immunoreactivity, and *in vitro* protein pepsin digestibility of soluble proteins from the selected soybean seeds stored frozen for 20 years at −20 °C.

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MATERIALS AND METHODS

Materials. Sources of soybean seeds have been reported earlier (*42*, *48*). Williams 82 and North Dakota (ND) soybean seeds were kindly provided by Dr. W. J. Wolf [United States Department of Agriculture (USDA), NRRL, Peoria, IL] and Dr. S. Chang [North Dakota State University (NDSU), Fargo, ND] and served as controls. Electrophoresis and immunoassay chemicals and reagents were purchased from Fisher Scientific (Orlando, FL) and Sigma Chemical Co. (St. Louis, MO).

Methods. *Preparation and Storage of Flour.* Seed flour was prepared as described earlier (*42*). Briefly, whole soybean seeds were ground in an Osterizer blender (Galaxy model 869-18R, Jaden Consumer Solutions, Boca Raton, FL; speed setting "grind") and defatted for 6−8 h in a Soxhlet apparatus using petroleum ether (boiling point range of 38.2−54.3 °C, Fisher Scientific, Fair Lawn, NJ). Defatted flour was spread in a thin layer on an aluminum foil and dried overnight (12−14 h) under a fume hood at room temperature (RT, ∼25 °C). Dried, defatted flour was further ground in an Osterizer blender to pass through 40 mesh sieve and stored in capped airtight plastic containers in a −20 °C freezer (Model 326F, Fisher Scientific Co.) for 20 years. Williams 82 and ND soybean defatted flours were not subjected to 20 years of storage at −20 °C and were used as controls.

Protein Extraction and Determination. Proteins subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) in the presence and absence of urea were solubilized and extracted as described earlier (*42*). For immunoassays, proteins were solubilized using borate saline buffer (BSB; 0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, and 0.075 M NaCl at pH 8.45). Briefly, soybean flours were extracted with 10 vol of BSB with constant vortex shaking (Vortex Genie 2, setting 8; American Scientific Products, McGaw, IL) at RT for 1 h and centrifuged (13600*g*, 20 min, RT). The supernatants were collected and used for subsequent analyses. Soluble protein content of the supernatants was determined using Bradford (*43*) and Lowry et al. (*44*) assay procedures.

Sulfur-Rich Protein (SRP) Purification. SRP was purified as described earlier (*45*).

Electrophoresis. SDS−PAGE in the presence and absence of urea was performed as described earlier (*42*). Protein extracts were mixed with an equal volume of the corresponding sample buffer, heat-denatured (100 °C boiling water bath for 10 min), cooled to RT, and 20 μL (equivalent to 10 μL of the original extract) each was used for gels.

Production of Polyclonal Antibodies (pAbs). Purified SRP from Century soybean (1 mg) in 1 mL of Freund's complete adjuvant was administered intradermally (id) to a female New Zealand white rabbit (*Oryctulagus cuniculus*). Approximately 4 weeks after the initial immunization, one booster dose (1 mg of SRP in 1 mL of incomplete Freund's adjuvant, id) was administered. Pre- and post-immunization blood was collected from the marginal ear vein. Blood was allowed to clot for 30 min, followed by centrifuging (5000*g*, 20 min, RT) and removal of the supernatant (serum). Serum aliquots were kept at 4 °C for immediate use and at −20 °C for long-term storage. BSB-extracted Century soybean proteins (660 μg) mixed with SRP (340 μg) dissolved in BSB (final total protein concentration of 1 mg/mL) were similarly used for the production of anti-whole soybean protein + SRP rabbit pAbs.

Immunoassays. (A) Western Immunoblotting. BSB-extracted protein solutions were electrophoresed following the Fling and Gregerson (*46*) method as described earlier (*42*). After extracting the whole soybean flours, each with 10 volumes of BSB as described under protein extraction, the supernatants were mixed with an equal volume of SDS−PAGE sample buffer containing 2% (v/v) β-ME and heat-denatured as described under electrophoresis and 20 μL each were used for SDS−PAGE. After electrophoresis, proteins were transferred from polyacryalmide gels onto a Protran nitrocellulose (NC) membrane (0.2 μm porosity, Whatman Plc, Middlesex, U.K.) according to the method of Towbin et al. (*47*). After transfer, membranes were stained with Ponceau S stain to visualize and document polypeptide profiles. The membranes were subsequently washed with Tris-buffered saline [TBS-T; 10 mM Tris, 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20] until Ponceau S stain was removed. The unbound sites on the NC membranes were blocked with 5% (w/v) nonfat dry milk (NFDM) in TBS-T for 1 h at RT. The blots were washed twice, 5 min each, in TBS-T and then incubated overnight at 4 °C with primary antibody. The primary antibody was either 10 000-fold (v/v) diluted rabbit pAbs raised against whole soybean protein + SRP or anti-soybean SRP in TBS-T. Excess pAb was washed off the NC membranes with TBS-T (once for 15 min followed by three 5 min washes). The membranes were then incubated for 1 h with 40 000-fold diluted (v/v) horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (secondary antibody) in TBS-T at RT. The blots were washed with TBS-T (once for 15 min followed by three 5 min washes) and developed using a luminol/*p*-coumaric acid system and Kodak BioMax XAR Film photo film (Carestream Health France, Paris, France).

(B) Dot Blotting (DB). Dot blots were performed using the Biodot apparatus (Bio-Rad, Hercules, CA) according to instructions of the manufacturer. Appropriate protein amount (150−750 ng) in 100 μL of BSB was applied on prewetted 0.2 μm NC paper. Excess BSB was removed by applying vacuum, and the NC paper was blocked and thereafter treated as described in Western blotting. A densitometer (Molecular Imager ChemiDoc XRS System, Bio-Rad Laboratories, Hercules, CA) was used to quantify SRP and to determine immunoreactivity of desired samples using ChemiDoc software (version 4.2).

(C) Competitive Inhibition Enzyme Linked Immunosorbent Assay (ELISA). Immunoreactivity of soybean was measured using ELISA as described by Monaghan et al. (*48*). SRP (dissolved in BSB) used for coating the plates was purified from Century soybeans. Briefly, microtiter plates were coated with 250 ng of SRP/well in citrate−phosphate buffer (pH 5.0) for 1 h at 37 °C, followed by washing 3 times with TBS-T and blocking with 5% NFDM in TBS-T for 1 h at 37 °C. In a separate plate, 1:10000 (v/v) diluted rabbit anti-SRP (90 μL/well) was mixed with 10 μL of suitably diluted soybean protein extract. In the top well, 10 μL of 1 mg soybean protein/mL (i.e., 100 000 ng/mL final concentration for soybean protein) was used. In the subsequent wells, 10-fold dilutions of soybean protein extract were used. The final soybean protein concentration was in the range of 100 000−0.01 ng/mL. The plate was incubated at 37 °C for 1 h. A total of 50 μL of the pAb−soybean protein mixture from each well from the plate was transferred to the respective well in the previously blocked plate followed by incubation at 37 °C for 1 h. The plate was washed 3 times with TBS-T and incubated with 1:5000 (v/v) diluted alkaline phosphatase-labeled goat anti-rabbit IgG for 1 h at 37 °C. The plate was washed 3 times with TBS-T and color-developed using *p*-nitrophenyl phosphate (PNPP) substrate (5 mg/plate). The reaction was stopped by adding 50 μL of 3 N NaOH in each well, and the plate was read at 405 nm. The four-parametric equation was plotted to determine IC_{50} to assess immunoreactivity. IC_{50} is the concentration of the inhibitor protein effecting 50% inhibition in ELISA response.

In Vitro Protein Digestibility. Solubilized proteins, undenatured and heat-denatured (30 min heating in a 100 °C boiling water bath), were subjected to pepsin (porcine stomach mucosa, Sigma Chemical Co., St. Louis, MO) hydrolysis. Final digestion conditions were substrate protein concentration, 2 mg/mL; 0.1 M HCl; substrate/enzyme ratio, 100:1 (w/w); incubation temperature, 37 °C (controlled temperature water bath); digestion time, 30 min; and final digestion volume, 500 μL. At the end of the digestion period, 10 μL of 5 N NaOH (to neutralize the HCl) followed by 490 μL of SDS−PAGE sample buffer [containing 2% (v/v) β-ME] were added to the digestion mixture. Samples were immediately heat-denatured in a 100 °C boiling water bath for 10 min to inactivate pepsin, cooled to RT, and subjected to SDS−PAGE using 8−25% linear acrylamide gradient gels.

Statistics. Data were expressed as mean ± standard error of the mean (SEM). Appropriate data were analyzed for significance ($p = 0.05$) using one-way analysis of variation (ANOVA; SPSS for Windows, Microsoft Corp., version 15.0, Chicago, IL) and Fisher's least significant difference (LSD).

RESULTS AND DISCUSSION

Electrophoresis. Electrophoretic profiles for the soluble proteins in the presence of urea (**Figure 1**) indicated that major polypeptides were stable with respect to their electrophoretic mobility. Mobilities of 7S subunits α′, α, and β as well as those

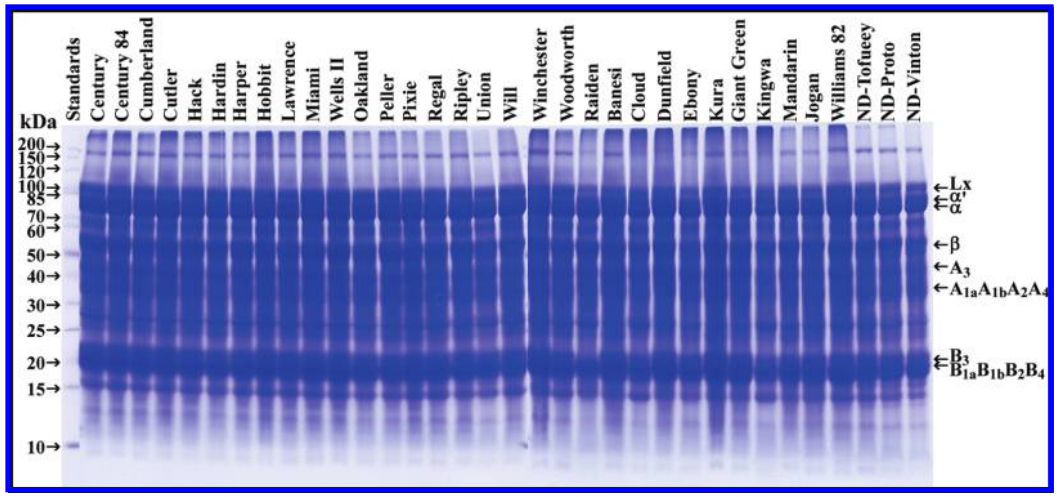

Figure 1. SDS−PAGE profiles of 28 soybean varieties stored for 20 years and 4 soybean varieties used as a control, in the presence of 6 M urea. This figure is a composite of two 8−25% monomer acrylamide linear gradient gels. The protein load for each variety was 10 μL of original extract. Molecular weights of standard proteins are shown in the left margin. Soybean protein polypeptides indicated in the right margin are identified on the basis of their known electrophoretic mobilities. Lx, lipoxygenase; ά, α, and β, 7S subunits; A_3, A_{1a}, A_{1b}, A_2, and A_4, 11S acidic subunits; and B_3, B_{1a}, B_{1b}, B_2, and B_4, 11S basic subunits.

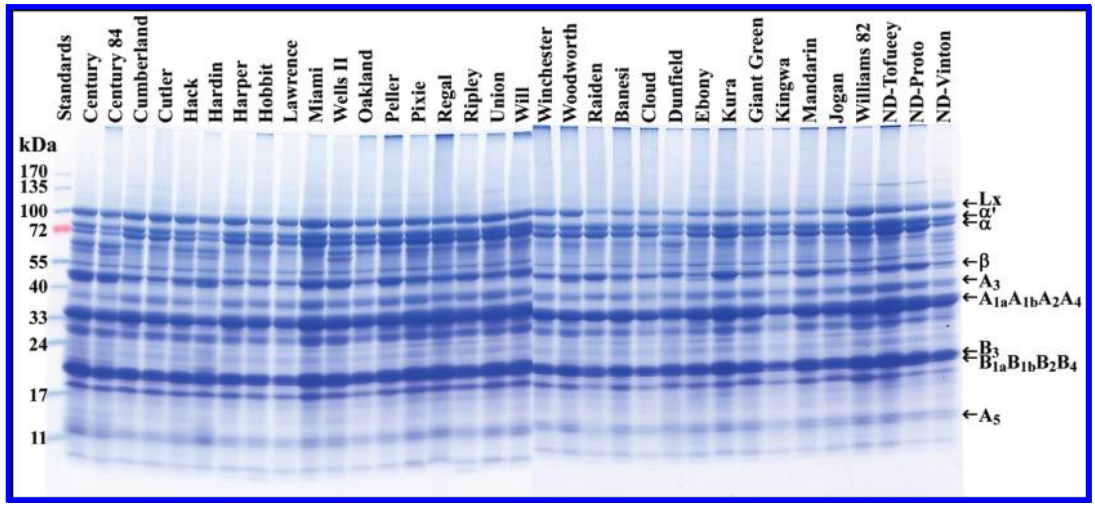

Figure 2. SDS−PAGE profiles of 28 soybean varieties stored for 20 years and 4 soybean varieties used as a control, in the absence of 6 M urea. This figure is a composite of two 8−25% monomer acrylamide linear gradient gels. The protein load for each variety was 10 μL of original extract. Molecular weights of standard proteins are shown in the left margin. Soybean protein polypeptides indicated in the right margin are identified on the basis of their known electrophoretic mobilities. Lx, lipoxygenase; ά, α, and β, 7S subunits; A_3, A_{1a}, A_{1b}, A_2, A_4, and A_5, 11S acidic subunits; and B_3, B_{1a}, B_{1b}, B_2, and B_4, 11S basic subunits.

of the acidic and basic subunits of 11S were consistent with the earlier observations (*42*). Similarly, electrophoretic mobilities of constituent polypeptides in the SDS−PAGE without urea (**Figure 2**) were stable and consistent with the earlier report (*42*). Lipoxygenase (Lx), ά, α, and β subunits of the 7S globulin, and the A and B subunits of the 11S were visible in all cultivars, indicating the storage stability of the polypeptides. Interestingly, the doublet just above the β subunit of 7S, the minor polypeptide below A_5 subunit of 11S in certain cultivars, as well as the significantly low-staining A_5 in Raiden noted in our earlier study were also noted in the current study. These data suggest that, at least qualitatively, the soybean protein polypeptide profiles remained stable during the storage period used in the current investigation.

Immunoreactivity. Immunoreactivity of soybean cultivars was compared to Century soybean by competitive inhibition ELISA and dot blots using rabbit anti-SRP pAbs. Typical standard curves obtained in ELISA and dot blot are shown in parts **A** and **B** of **Figure 3**, respectively. Dot blots did not reveal significant difference between the immunoreactivity of different cultivars. Immunoreactivity assessed by ELISA exhibited significant differences compared to the immunoreactivity of proteins from Century cultivar used as a reference (**Figure 3C**). Differences in immunoreactivity may arise because of (a) differences in the epitope profile of the soluble proteins in a given cultivar in comparison to that of the Century proteins used as the immunogen for rabbit pAb production, (b) differential expression of specific proteins that may be inherently more immunoreactive, (c) differential modification of immunoreactivity as a result of long-term storage, or (d) a combination of a−c. Only Hobbit soybean showed significantly higher immunoreactivity than Century, while many cultivars were observed having lower immunoreactivity, when assessed by ELISA. Interestingly, although Williams 82 and ND soybean seeds were not subjected to long-term storage, their immunoreactivity was lower than Century proteins. These data suggest that regardless of the cultivar, long-term storage did not adversely affect the

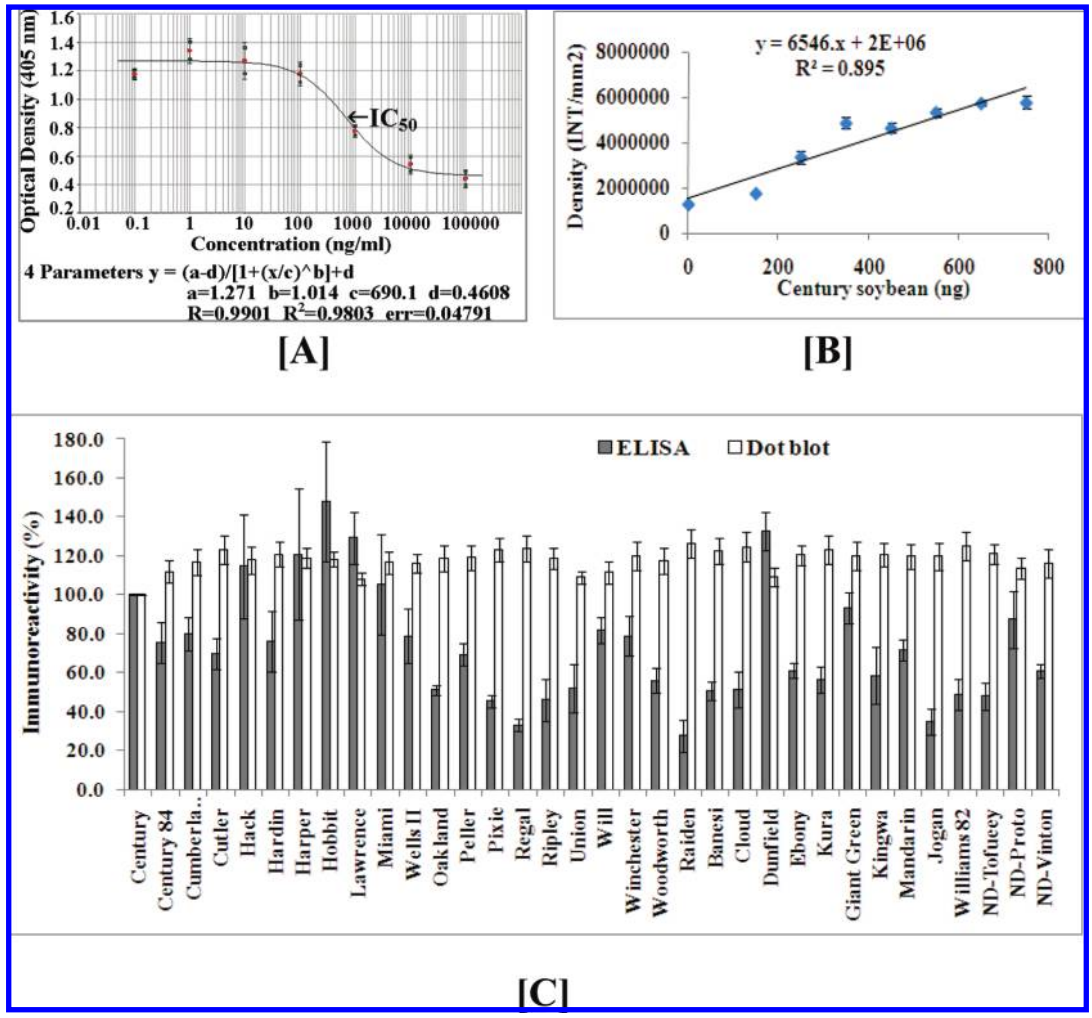

Figure 3. Typical standard curve obtained from (A) ELISA ($n = 2$) and (B) dot blot ($n = 3$) using Century proteins as the reference standard. (C) Immunoreactivity expressed as percent reactivity (mean ± SEM) compared to that of the Century soybean (arbitrarily assigned a value of 100%) using ELISA ($n = 4$, $p = 0.05$, LSD = 33) and dot blot ($n = 12$, $p = 0.05$, LSD = 26).

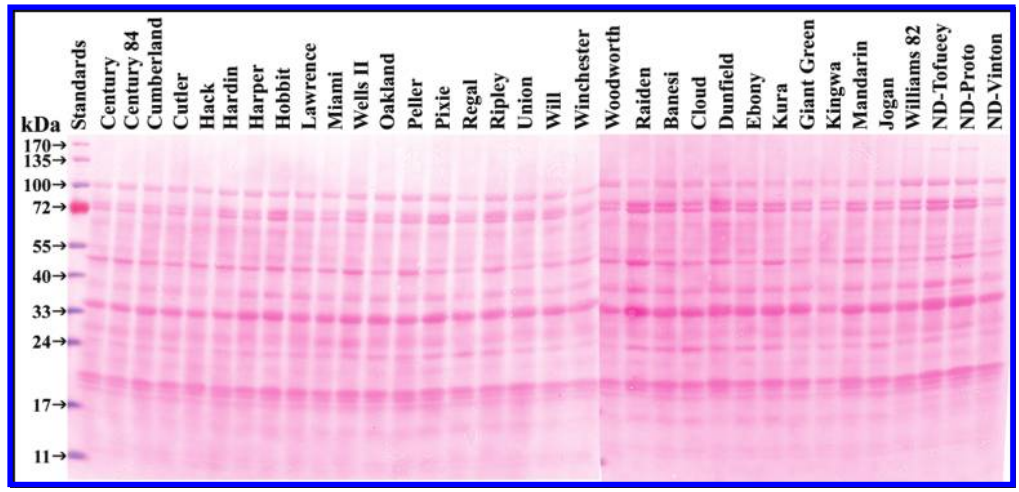

Figure 4. Ponceau S stain of BSB-extracted proteins from 28 soybean varieties stored for 20 years and 4 soybean varieties used as a control. The protein load in each lane was 30 μg. This figure is a composite of transfers from two 8−25% monomer acrylamide linear gradient gels.

immunoreactivity of soluble proteins. Although dot blots provide a useful assessment of the immunoreactivity of total soluble proteins, they do not permit the assessment of the immunoreactivity of individual proteins and protein polypeptides when present in a mixture. Lalles et al. (*49*) have shown that *in vivo* partially digested acidic subunits of 11S were detected after incubation for up to 8−10 h in the *ileal digesta* in Holstein heifer cows, while intact 7S (β-conglycinin) was detectable over the 8 h sampling period (Table 3 in their paper), thus indicating differential stability for individual proteins. Although 11S (glycinin) and 7S (β-conglycinin) are the major seed storage proteins in soybeans, SRP a storage

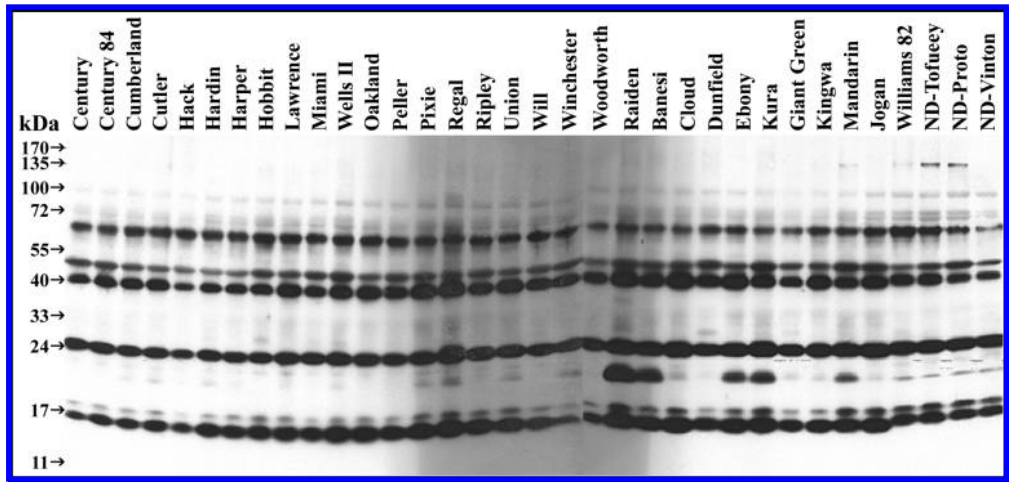

Figure 5. Western immunoblots for soybean varieties probed with rabbit anti- (whole soybean seed proteins + SRP) pAbs. The protein load in each lane was 30 μg. This figure is a composite of two 8−25% monomer acrylamide linear gradient gels.

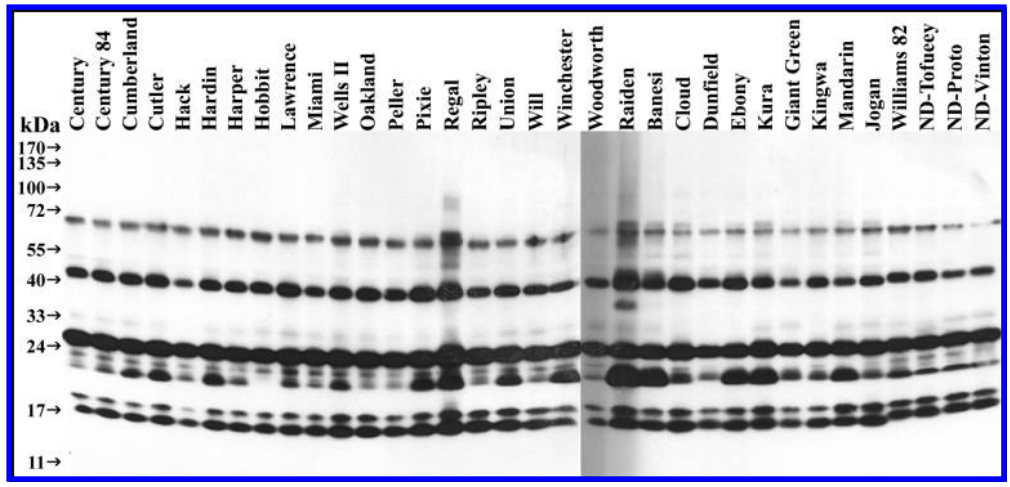

Figure 6. Western immunoblots for soybean varieties probed with rabbit anti-SRP pAbs. The protein load in each lane was 30 μg. This figure is a composite of two 8−25% monomer acrylamide linear gradient gels.

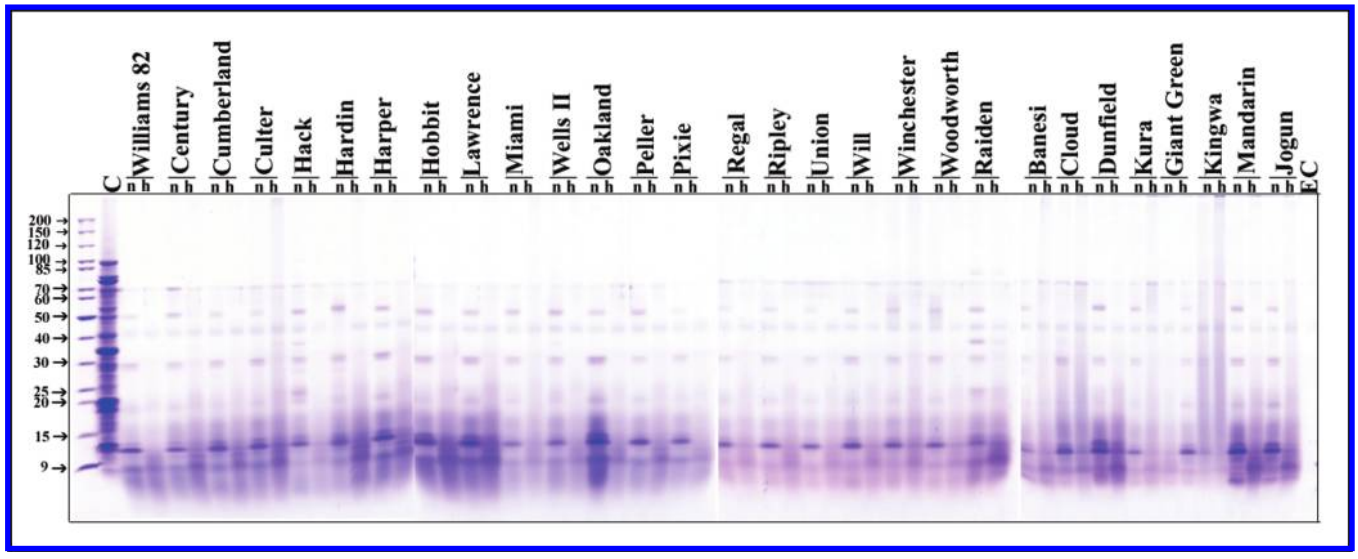

Figure 7. SDS−PAGE profiles for pepsin-digested soybean proteins. This figure is a composite of four separate 8−25% monomer acrylamide linear gradient gels. *In vitro* digestion was for 30 min at 37 °C using a substrate protein/pepsin ratio of 100:1 (w/w). C, Williams 82 soybean protein extract (unheated protein and no pepsin) used as a control; n, unheated protein extract digested with pepsin; h, heat-denatured protein extract digested with pepsin; EC, pepsin control. The soybean protein load in each lane was 30 μg, and the soybean protein load for pepsin control (EC) was 0.3 μg.

protein in soybeans is nutritionally important because SRP contains a higher amount of methionine than 11S and 7S (*42, 45*). Recently, we have reported the presence of SRP-like proteins in several dry bean seeds (*48*). Increasing the

synthesis of SRP and SRP-like proteins in dry legume seeds may potentially improve sulfur amino acid content of legume seeds that are known to be deficient in sulfur amino acids, thereby helping to improve the essential amino acid balance. For these reasons, BSB buffer soluble proteins were reduced, denatured, and subjected to SDS−PAGE and the corresponding Western blots were probed with anti-whole soybean + SRP rabbit pAbs (**Figure 5**). As indicated by Ponceau S stain (**Figure 4**), CBBR staining (**Figures 1** and **2**), and immunoblotting (**Figures 5** and **6**), the qualitative profile for the tested samples remained stable whether or not the sample was stored (e.g., ND soybeans). Similarly, Western blots probed with anti-SRP rabbit pAbs indicated that the SRP polypeptides are also stable in all of the tested samples (**Figure 6**). Together, these results demonstrate the stability of seed protein immunoreactivity. The stability of seed proteins during storage is important for several reasons, including the development of detection methods based on the ability to detect targeted seed proteins or polypeptides and the maintenance of seed protein nutritional quality.

***In Vitro* Protein Digestibility.** Soybeans are valued for their high protein and oil content (*5*, *50*), and by some estimates as much as 65% of the value of soybean seeds is attributed to their protein content (*51*). Soybean proteins are considered to be easily digestible and of high nutritional value (*52*). *In vitro* pepsin digestion experiments (**Figure 7**) indicated the seed proteins to be highly digestible, regardless of the cultivar. Native proteins were easily hydrolyzed to several small-molecular-weight (<20 kDa) polypeptides. All subunits of both 7S and 11S appeared to be degraded. Interestingly, peptide(s) with electrophoretic mobility approximately halfway between the 15 and 9 kDa standard protein markers appeared to be resistant to pepsin. Moist heat denaturation helped improve *in vitro* digestibility, as indicated by hydrolysis of much of the protein to small-molecular-weight (≤15 kDa) polypeptides. Thus, apparent *in vitro* digestibility of soybean seed proteins was not negatively affected by the long-term storage. Pinto et al. (*53*) reported that, during 1 year of storage from −18 to 42 °C, soybean protein *in vitro* digestibility remained unaffected.

The results of the current investigation suggest that, when soybean flours were stored for 20 years at −20 °C, (a) the major storage protein polypeptides remained stable, (b) total seed proteins retained their immunoreactivity, (c) SRP was stable as indicated by the retention of its immunoreactivity (dot and Western blot assay results), and (d) *in vitro* pepsin digestibility of seed proteins was not adversely affected.

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