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Biochemical Characterization of Soluble Proteins in Pecan [*Carya illinoinensis* (Wangenh.) K. Koch]

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Pecans (cv. Desirable) contained ~10% protein on a dry weight basis. The minimum nitrogen solubility (5.9−7.5%) at 0.25−0.75 M trichloroacetic acid represented the nonprotein nitrogen. Among the solvents assessed for protein solubilization, 0.1 M NaOH was the most effective, while borate saline buffer (pH 8.45) was judged to be optimal for protein solubilization. The protein solubility was minimal in the pH range of 3−7 and significantly increased on either side of this pH range. Increasing the NaCl concentration from 0 to 4 M significantly improved (~8-fold increase) protein solubilization. Following Osborne protein fractionation, the alkali-soluble glutelin fraction (60.1%) accounted for a major portion of pecan proteins followed by globulin (31.5%), prolamin (3.4%), and albumin (1.5%), respectively. The majority of pecan polypeptides were in the molecular mass range of 12−66 kDa and in the pI range of 4.0−8.3. The pecan globulin fraction was characterized by the presence of several glycoprotein polypeptides. Lysine was the first limiting essential amino acid in the defatted flour, globulin, prolamin, and alkaline glutelin fractions. Leucine and tryptophan were the first limiting essential amino acids in albumin and acid glutelin fractions, respectively. Rabbit polyclonal antibodies detected a range of pecan polypeptides in the 12−60 kDa range, of which the globulin fraction contained the most reactive polypeptides.

KEYWORDS: Pecan; protein; allergy; protein solubility; immunoblotting; rabbit polyclonal antibody; nonprotein nitrogen

INTRODUCTION

Pecan is an economically important tree nut crop in the United States. The nut crop typically has a biennial bearing pattern. The agronomic and economic importance of pecan trees is well-documented (*1*). Pecans, a member of the *Juglandaceae* family, in which walnuts and hickory nuts also belong, are used in a wide range of food products mainly due to their unique texture and flavor properties. While enjoyed safely by most consumers, tree nuts are responsible for several reported cases of food-induced allergies in humans (*2*−*8*). To protect sensitive individuals from unintended exposure and to further understand the stability of pecan proteins, we developed a rabbit anti-pecan protein polyclonal antibody (pAb)-based enzyme-linked immunosorbent assay (ELISA) with a detection sensitivity of 32−800 ng/mL. The assay permitted the assessment of the stability of proteins isolated from pecans subjected to various thermal and digestive treatments. These data were augmented by using pecan-sensitive patient sera IgE in Western blotting assays (*9*). The results indicated that regardless of the processing method to which pecans were subjected, the proteins/polypeptide retained their immunoreactivity.

A few studies have reported the effects of genetics, environment, and seed maturity on pecan kernel protein content (*10*−*13*). McWatters and Cherry (*14*) investigated protein emulsion, foaming, and solubility properties of defatted pecan flour. However, detailed biochemical studies on pecan protein fractions are lacking. The primary goal of the current study was to investigate pecan seed protein solubility and to determine biochemical properties—including polypeptide composition, amino acid composition, and immunoreactivity—of soluble proteins.

MATERIALS AND METHODS

In-shell pecans (cv. Desirable, 2002 crop, a gift from Dr. T. Thompson, U.S. Department of Agriculture—Agricultural Research Service Pecan Breeding and Genetics, Somerville, TX) were used in the present study. Pecan flour (full fat and defatted) preparation and storage were as described previously (*9*). Unless otherwise specified, all analyses were done at room temperature (RT, ~25 °C). Sources of chemicals were reported earlier (*9*).

Nonprotein Nitrogen (NPN). NPN was determined as described by Wolf (*15*) and Wolf et al. (*16*). Briefly, to 0.1 g of sample, 1.5 mL of aqueous trichloroacetic acid (TCA) solution of known strength (0, 0.25, 0.50, 0.75, 1.0, 2.0, 3.0, 4.0, and 5.0 M) was added, and the sample

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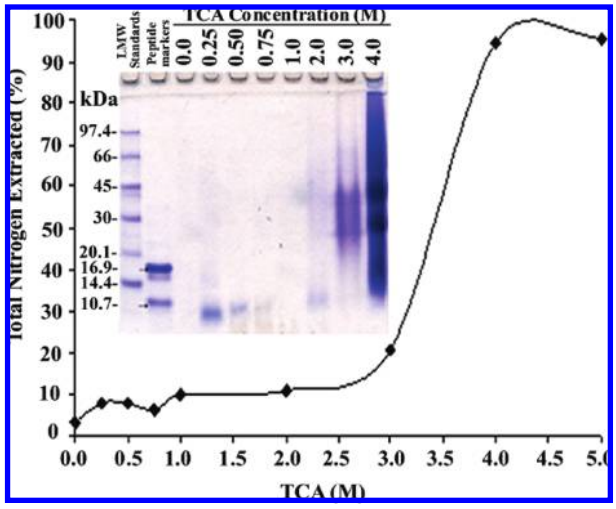

Figure 1. Effect of TCA (M) on defatted pecan meal nitrogen solubility. Inset: SDS-PAGE (with 2% v/v β-ME) for soluble nitrogen at indicated TCA concentrations.

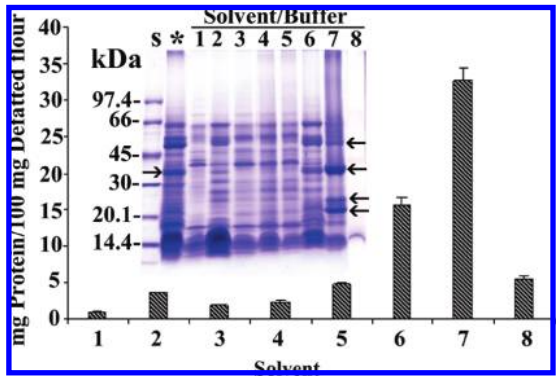

Figure 2. Effect of solvent on pecan protein solubility. The soluble protein was estimated by the procedure of Lowry et al. (*18*). Solvent (final pH of the dispersion): 1, DI H_2O (pH 6.65); 2, 1.0 M NaCl (pH 6.17); 3, 0.1 M sodium phosphate buffer (pH 7.5); 4, 0.1 M $NaHCO_3$ (pH 8.31); 5, 0.1 M Tris-HCl (pH 8.10); 6, 0.125 M BSB (pH 8.45); 7, 0.1 M NaOH (pH 12.90); and 8, 70% (v/v) ethanol (pH 6.12). Inset: SDS-PAGE (with 2% v/v β-ME) analysis of the solubilized proteins. The number on the top of the track indicates the solvent/buffer used. The protein load in each lane was 100 μg. S = LMW standards; * = proteins extracted in the SDS-PAGE sample buffer containing 2% (v/v) β-ME. After extraction, the sample was centrifuged, and 10 μL supernatant was loaded.

was extracted with continuous vortexing provided for 1 h at RT. Supernatants were collected after centrifugation (16100*g*, 15 min, RT) and analyzed for nitrogen according to AOAC method 950.48 (micro-Kjeldahl method) (*17*).

Protein Estimation. Unless otherwise specified, soluble proteins were estimated by the method of Lowry et al. (*18*) using bovine serum albumin (BSA) as the standard protein (0−200 μg). When necessary, soluble proteins were also estimated by the Bradford assay (*19*), suitably modified to a microtiter plate assay as described by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) with BSA as a standard protein (0.05−0.5 mg/mL). Nut seed flour nitrogen as well as insoluble nitrogen were determined by micro-Kjeldahl method (N × 5.3) as per the AOAC Official method 950.48 (*17*).

Protein Solubility. *Influence of Solvent.* Protein solubility in different solvents was determined by extracting defatted pecan flour (100 mg) with 1.0 mL of each solvent for 1 h with continuous vortexing provided. The solvents used were as follows: 1, distilled deionized water DI H_2O (pH 6.65); 2, 1.0 M NaCl (pH 6.17); 3, 0.1 M sodium phosphate buffer (pH 7.5); 4, 0.1 M $NaHCO_3$ (pH 8.31); 5, 0.1 M Tris-HCl (pH 8.10); 6, 0.125 M borate saline buffer (BSB) (pH 8.45); 7, 0.1 M NaOH (pH 12.90); and 8, 70% ethanol (pH 6.12). Supernatants were collected after centrifugation (16100*g*, 10 min, RT) and analyzed for soluble proteins.

Effect of Extraction Time. Defatted pecan flour (100 mg) was extracted in 1.0 mL of BSB with continuous vortexing provided for the desired time intervals (15, 30, 45, 60, 75, 90, 105, and 120 min) and was centrifuged (16100*g*, 10 min, RT); the supernatants were collected and analyzed for soluble proteins.

Effect of Flour to Solvent Ratio. Defatted pecan flour (100 mg) was extracted in different volumes of BSB to obtain final flour-to-solvent ratios (w/v) of 1:5, 1:10, 1:20, 1:40, 1:60, 1:80, and 1:100. Extractions were done with continuous vortexing provided for 1 h at RT followed by centrifugation (16100*g*, 10 min, RT), and the supernatants were analyzed for soluble proteins.

Effect of pH. To 100 mg of defatted pecan flour was added 1.0 mL of DI H_2O or aqueous 2.0 M NaCl, and the final pH was adjusted to the desired value with 1.0 N NaOH or 1.0 N HCl. Extractions were done at RT with continuous vortexing provided for 1 h, and the supernatants were collected after centrifugation (16100*g*, 10 min, RT) and were analyzed for soluble proteins.

Effect of Ionic Strength. Defatted pecan flour (100 mg) was extracted with 1.0 mL each of 0.0, 0.2, 0.5, 1.0, 2.0, and 4.0 M NaCl, respectively (no pH adjustment), for 1 h with continuous vortexing provided at RT. The samples were centrifuged (16100*g*, 15 min, RT), and supernatants were analyzed for soluble protein content.

Protein Fractionation. Osborne protein fractions were prepared by sequentially extracting the defatted pecan flour with each (defatted flour-to-solvent ratio of 1:10 w/v) of 1.0 M NaCl (albumin + globulin), 70% v/v aqueous ethanol (prolamin), 0.1 M NaOH (glutelin), and 0.1 M HCl (acid glutelin) for 4 h at 4 °C with constant magnetic stirring provided. Following each extraction, the slurry was centrifuged (12600*g*, 20 min, 4 °C), and the supernatant was vacuum filtered using Whatman filter paper #4 to remove insoluble particles. Residues from centrifugation and filtration steps were pooled and used for the next extraction step. Filtrates containing desired protein fractions were dialyzed against DI H_2O for 36 h with six water (4 L each) changes. After dialysis, the albumin + globulin mixture was centrifuged (12600*g*, 20 min, 4 °C), and the precipitate (globulin) and supernatant (albumin) were separately lyophilized. Prolamin and glutelin fractions were lyophilized directly after dialysis. All lyophilized protein fractions were stored in airtight plastic bottles at −20 °C until further use.

Electrophoresis, Glycoprotein Staining, Isoelectric Focusing (IEF), and Immunoblotting. Nondenaturing nondissociating polyacrylamide gel electrophoresis (NDND-PAGE), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the absence or presence of a 2% (v/v) reducing agent β-mercaptoethanol (β-ME), and Western immunoblotting were done as described earlier (*9, 20, 21*). IEF in the presence of urea was done using 5% acrylamide gels as described earlier (*21*). Glycoproteins were visualized using a Gelcode Glycoprotein Staining (Pierce Chemical Co., Rockford, IL) procedure according to the manufacturer's instructions.

Size Exclusion Chromatography. A Sephacryl S 300 HR column (2.6 cm × 89.0 cm) was used to analyze pecan protein fractions and also to estimate the Stokes radii of the proteins. The equilibrium and elution buffer was 0.02 M Tris-HCl (pH 8.1) containing 0.1 M NaCl and 0 001 M NaN_3. Fractions were collected every 20 min. Protein elution was monitored by UV absorbance at 280 nm and electrophoresis. The column was calibrated using standard proteins (Pharmacia high and low molecular weight kits). The column flow rate was set at 23 mL/h. All column operations were carried out at 4 °C. When necessary, protein fractions were concentrated using YM-3 (3000 Da, MWCO) Microcon Centrifugal Filter Devices (Millipore Corp., Bedford, MA) prior to their use in gel electrophoresis. Protein samples were also analyzed by high-pressure liquid chromatography (HPLC) using a Beckman System Gold (Beckman Coulter, Fullerton, CA) equipped with a 126 programmable solvent delivery system, a 210A manual sample injection valve, and model 168 diode array detector. The column equilibrium and elution buffer was 0.1 M BSB buffer (pH 8.45). Protein samples were injected onto a Superdex 200 HR analytical column (10 mm × 30 cm, Amersham Pharmacia, Inc., Piscataway, NJ) maintained at a flow rate of 24 mL/h. The column effluent was monitored at 280 nm, and collected peak fractions were subjected to electrophoretic analyses.

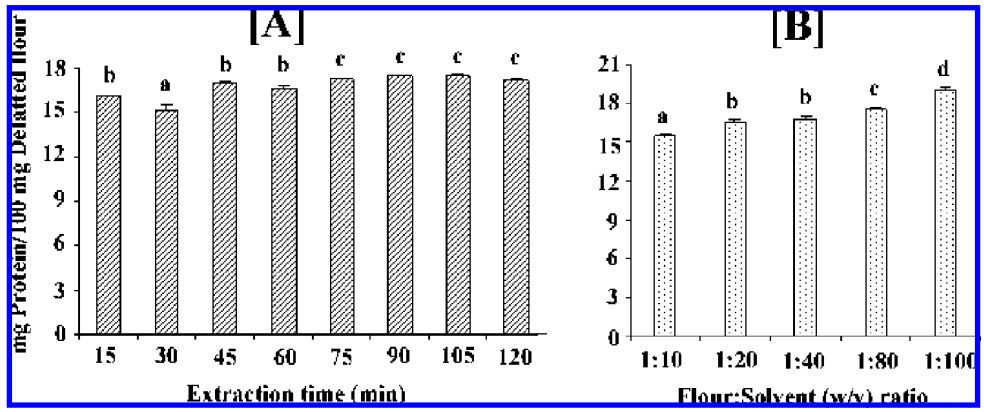

Figure 3. Effect of extraction time and flour-to-solvent ratio on pecan protein solubility. Data are expressed as mg of solubilized protein per 100 mg defatted flour (mean ± SEM). LSDs ($p = 0.05$, $n = 2$) were 0.59 and 0.68 for extraction time (**A**) and flour:solvent ratio (**B**), respectively. A different letter on the top of the bar indicates the significant difference.

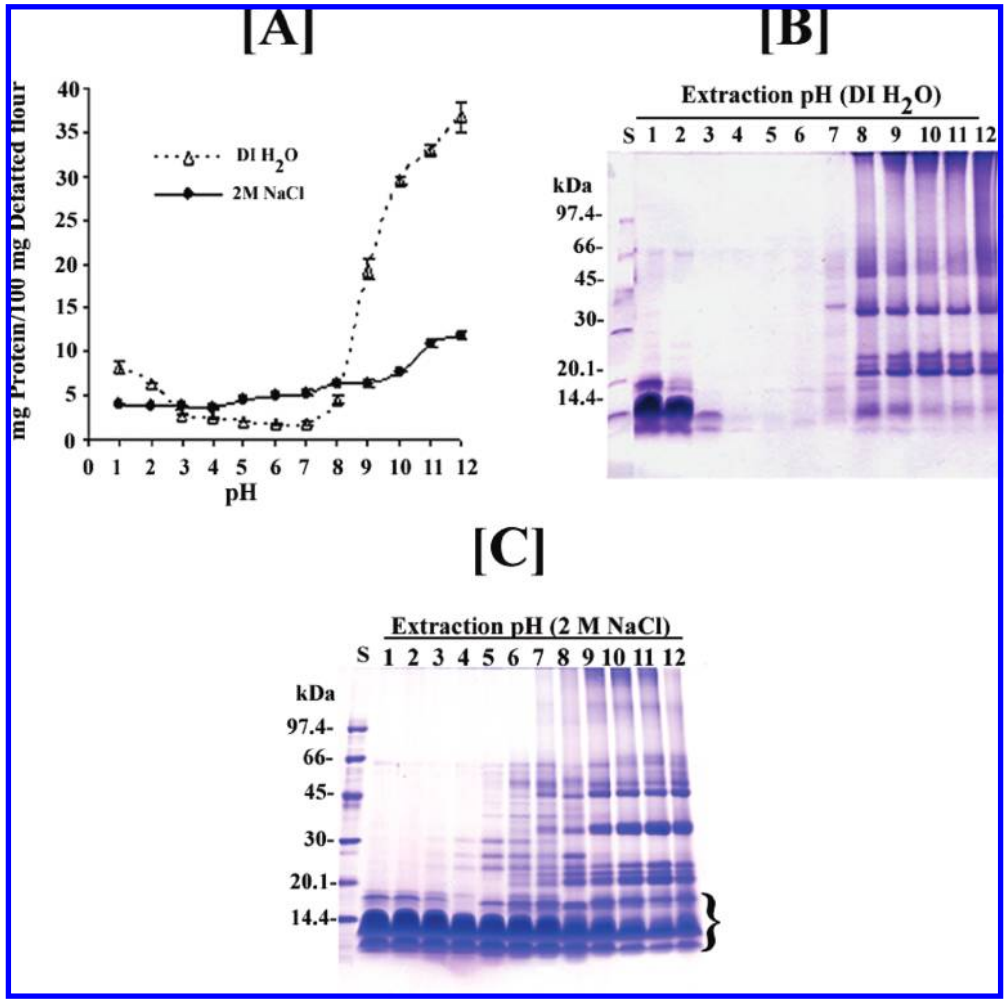

Figure 4. Effect of pH on pecan protein solubility (**A**) SDS-PAGE (in the presence of 2% v/v β-ME) analysis of proteins solubilized in distilled deionized water (**B**) and 2 M NaCl (**C**) at indicated pH. The protein load in each lane was 100 μg. S = LMW standards. Molecular masses of the standards indicated on the left side of the track containing the standards. Note the differences in polypeptide patterns of solubilized proteins in the alkali pH range (8−12).

Amino Acid Composition. The amino acid composition of the defatted pecan flour and protein fractions was determined using a Pico-Tag Column Amino Acid Analyzer (Waters Chromatograph Division, Milford, MA) as described previously (*9*). The tryptophan content was determined by the colorimetric method (no. 3) of Spies and Chambers (*22*). The amino acid composition was reported as g of amino acid per 100 g of protein.

Rabbit Antisera. Rabbit pAb production and characterization were as described previously (*9*).

Statistics. All analyses were done at least in duplicate, and data are reported as means ± standard errors of the mean (SEM). Where appropriate, data were analyzed for significance using analysis of variance and Fisher's least significant difference (LSD at $p = 0.05$).

RESULTS AND DISCUSSION

Protein and NPN. Pecan seeds contained 10.0 mg protein/100 mg full-fat flour, and the defatted flour contained 34.8 mg

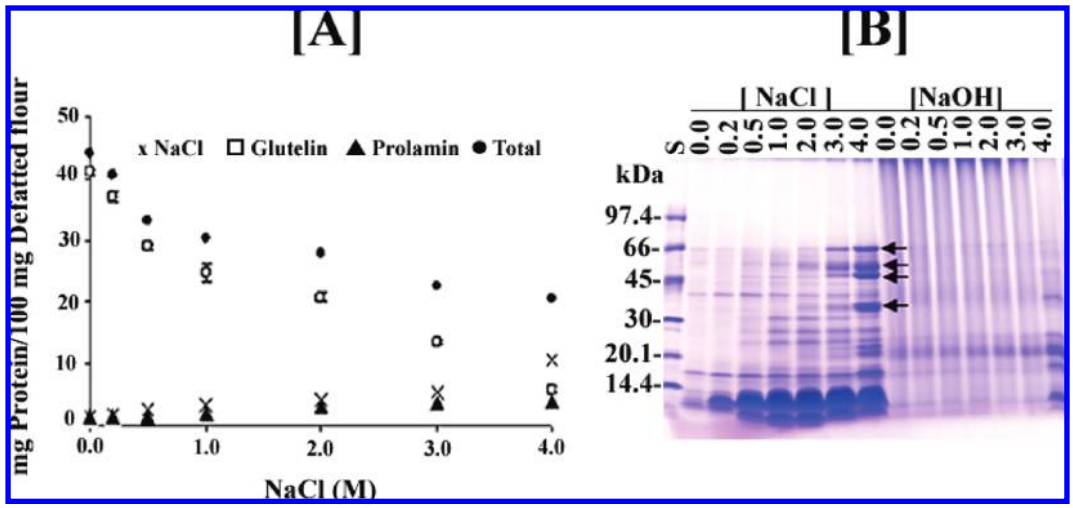

Figure 5. (**A**) Effect of NaCl (M) on pecan protein solubility NaCl = albumin + globulin. (**B**) SDS-PAGE (with 2% v/v β-ME) analysis of NaCl and NaOH solubilized proteins. The protein load in each lane was 60 μg. S = LMW standards (MWs indicated in the left margin).

Table 1. Osborne Protein Fractions[a]

solubility fraction	Lowry	Bradford	micro-Kjeldahl	LSD[b]
DI H_2O (albumins)	2.04 ± 0.11	1.82 ± 0.26	1.46 ± 0.01	0.52
2.0 M NaCl (globulins)	22.13 ± 1.52	26.24 ± 0.70	31.46 ± 0.83	3.44
70% (v/v) ethanol (prolamins)	9.00 ± 0.04	1.57 ± 0.14	3.43 ± 0.26	0.55
0.1 M NaOH (alkali glutelins)	64.94 ± 1.59	68.95 ± 1.06	60.14 ± 0.75	3.78
0.1 M HCl (acid glutelins)	1.89 ± 0.08	1.42 ± 0.04	3.51 ± 1.30	2.39
BSB solubilized proteins[c]	157.28 ± 7.08	143.89 ± 9.47	74.20 ± 0.81	21.88

[a] Osborne fraction data are expressed as a percent of total solubilized proteins (mean ± SEM) ($n = 2$). [b] Differences between means in the same row exceeding the corresponding LSD value are significant. [c] Comparison of the BSB solubilized pecan protein content estimated by the Lowry, Bradford, and micro-Kjeldahl protein estimation methods are expressed as mg of solubilized protein per 1.0 g defatted flour (mean ± SEM, $n = 4$).

protein/100 mg of flour [micro-Kjeldahl (N × 5.3)] expressed on a dry weight basis. The seed meal nitrogen solubility (**Figure 1**) was 5.94−7.54% of total nitrogen in the TCA concentration range 0.25−0.75 M. The nitrogen solubility gradually increased from 9.8 to 10.9% when the TCA concentration was increased from 1 to 2 M and sharply increased when TCA was >3 M. The results suggest that the NPN in pecans is small (<10% total N) and may introduce an error of ~11.1% if the seed protein content was calculated by micro-Kjeldahl method using the equation protein = total N × 5.3. At low molarity (0.25 and 0.5 M), TCA mainly extracted small (<10 kDa) molecular mass polypeptide(s) (**Figure 1**, inset).

Protein Solubility. *Effect of Solvent Type.* The protein solubility was quantitatively dependent on the solvent used (**Figure 2**). Among the solvents tested, 0.1 M NaOH was the most effective protein solubilizer as indicated by its ability to solubilize ~100% seed proteins (seed protein = micro-Kjeldahl N × 5.3). BSB buffer (pH 8.45) was the second with a much lower efficiency of protein solubilization (~50%). The remaining tested solvents typically solublized <15% defatted seed flour proteins.

SDS-PAGE (**Figure 2**, inset) analysis of the solubilized proteins indicated that the polypeptide patterns were qualitatively dependent on the solvent as well. A sample of total proteins extracted from pecan flour directly in SDS-PAGE sample buffer (a reducing buffer that affords nearly complete protein solubilization) was also included in the SDS-PAGE analysis to compare with the polypeptides profiles of the solvent tested. Among the solvents tested, 0.1 M NaOH yielded a distinctly different polypeptide profile. For example, 0.1 N NaOH solubilized proteins contained polypeptides with marked higher intensity (indicated by the arrows) as compared to the other polypeptides. Qualitatively, these polypeptides appeared to be better solubilized by 0.1 M NaOH than by any other buffer, perhaps with the exception of the SDS-PAGE sample buffer. Sodium chloride (1.0 M) solubilized several polypeptides in the low molecular mass range (estimated molecular masses <20 kDa) more efficiently than the other solvents tested (subjectively judged by the bandwidth and Coomassie blue staining intensity). Sodium phosphate, $NaHCO_3$, and Tris-HCl buffers yielded closely matched polypeptide profiles. These three profiles were distinctly different from the profile of the total proteins extracted with the SDS-PAGE sample buffer (lane indicated by an *), not only with respect to the staining intensity of the polypeptides extracted but also with respect to the type of polypeptides solubilized. For example, the doublet polypeptide between markers 30 and 45 kDa that stained intensely in the SDS-PAGE sample buffer-extracted protein sample was not well-stained in protein extracts using these three buffers (compare lanes 3, 4, and 5 with the one marked with an * for the doublet marked with an arrow on the left side of the track marked with an *). Among the solvents tested, BSB-extracted proteins most closely resembled the polypeptide profile of the SDS-PAGE sample buffer-extracted proteins (compare the lanes 6 and *), the latter ostensibly representing the total soluble proteins in the nut seeds.

The difference in polypeptide pattern as a function of the solvent used for protein extraction (from the same seed type) is important when such protein extracts are used for a variety of experiments and may partly explain variations in bioactivities assessed by different laboratories. For example, protein extracts prepared using aqueous buffers are often used in immunoassays (employing various formats) to assess immunoreactivity of such extracts. Depending on the solvent used for preparing protein extracts destined for use in such bioassays, the presence of clinically relevant allergens may or may not be detected. In a recent study, Wallowitz et al. (*8*) have similarly addressed the importance of using appropriate solvent in the preparation of walnut protein extracts intended for use in clinically relevant diagnostic testing for walnut allergies.

Effect of Extraction Time, Solvent Ratio, pH, and Ionic Strength. Using BSB as the optimum solvent, a defatted flour-to-solvent ratio of 1:10 (w/v) and an extraction time of 1 h were judged to be sufficient for efficient protein solubilization (**Figure 3**). Pecan

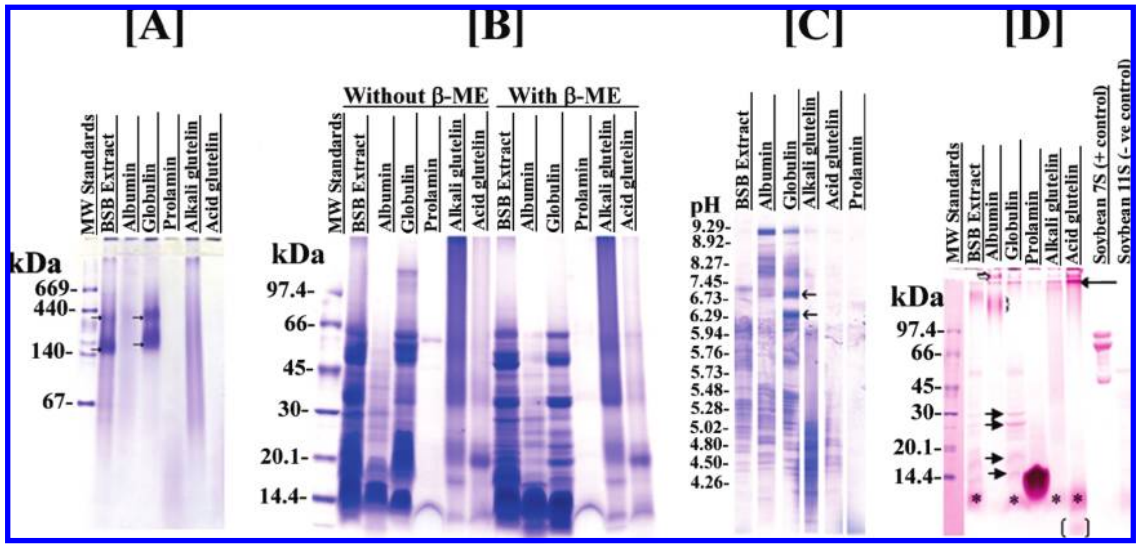

Figure 6. Electrophoretic analyses of pecan protein fractions. (**A**) NDND-PAGE, (**B**) SDS-PAGE, (**C**) urea IEF, and (**D**) glycoprotein staining of SDS-PAGE with 2% v/v β-ME. The protein load for each fraction was 60 (**A**), 100 (**B**), 20 (**C**), and 100 (**D**) μg.

Table 2. Pecan Protein Amino Acid Composition[a]

amino acid	defatted flour	albumin	globulin	prolamin	alkaline glutelin	acid glutelin	FAO/WHO[b]
aspartic acid	8.93	7.09	7.40	9.49	6.69	8.45	
glutamic acid	26.30	28.94	33.28	25.78	23.59	25.79	
serine	4.59	4.20	5.10	5.55	5.02	5.10	
glycine	4.18	4.54	4.02	5.57	5.05	6.09	
histidine[b]	2.71	2.77	2.58	2.64	3.07	2.65	1.9
arginine	12.30	9.67	13.01	9.74	11.89	9.95	
threonine[b]	2.37	3.49	2.59	3.36	3.46	3.16	3.4
alanine	4.73	4.61	3.55	4.72	5.03	5.04	
proline	5.12	5.82	5.25	5.65	6.22	5.46	
valine[b]	4.44	4.29	3.38	4.25	4.93	2.38	3.5
methionine[b]	2.00	1.74	2.05	1.49	2.41	4.83	2.5
cysteine	0.21	0.57	0.31	0.22	0.21	1.77	
isoleucine[b]	3.70	3.19	2.93	3.48	3.88	0.20	2.8
leucineb	6.91	5.62	4.99	7.01	7.27	3.69	6.6
phenylalanine[b]	4.75	3.23	3.81	4.15	5.61	7.13	6.3
tyrosine	2.91	3.48	3.36	2.77	3.75	4.78	
lysine[b]	3.08	5.50	1.91	2.02	1.21	3.22	5.8
tryptophan[b]	0.82	1.31	0.51	2.14	0.72	0.37	1.1

LEAA[c]	defatted flour	albumin	globulin	prolamin	alkaline glutelin	acid glutelin
first	Lys	Leu	Lys	Lys	Lys	Trp
second	Thr	Met/Cys	Trp	Met/Cys	Trp	Lys
third	Trp	Lys	Leu	Thr		Met/Cys
E/T (%)[d]	30.97	31.69	25.04	30.75	32.76	31.79

AAD[e] (%)	defatted flour	albumin	globulin	prolamin	alkaline glutelin	acid glutelin
hydrophobic	36.83	34.89	30.77	38.67	41.32	39.34
hydrophilic	9.87	11.17	11.05	11.68	12.23	10.63
acidic	35.23	36.02	40.68	35.27	30.28	34.24
basic	18.09	17.93	17.50	14.40	16.17	15.82

[a] All data are expressed as an average of two determinations. Compositions are expressed as g amino acid/100 g protein. [b] Essential amino acid [recommendation by FAO/WHO for weaned (2−5 years) child]. [c] LEAA, limiting essential amino acid. [d] E/T (%), ratio of total essential amino acids to total amino acids. [e] AAD (%), amino acid residue distribution.

proteins were not easily solubilized in DI H_2O at neutral and acidic pH (**Figure 4A**) and were minimally soluble in the pH range 3−7. Just as in case of walnut proteins (*23*), pecan proteins were more soluble at pH >8 than at acid or neutral pH. However, as compared to walnut proteins (*23*), pecan proteins appeared to be less soluble in the acid pH range 1−4. In the pH range 1−4, solubilized polypeptides were mainly <20 kDa (**Figure 4B**). The polypeptide profile of proteins solubilized in the pH range 1−4 was distinctly different than those solubilized in the pH range 9−12. The main difference is the relative staining intensity of the polypeptides >20 kDa in the pH range 8−12 as compared to those in the acid pH range. One possible reason for such differences in solubility may be the presence of nonprotein components (notably phenolics) that may interact with the proteins and thereby alter protein solubility. The phenolic content of pecan flour is almost twice that of English walnuts (*24*). A close examination of protein solubility in the absence and presence of salt at various pH values (**Figure 4A**) indicates that in the absence of salt, low molecular mass polypeptides (**Figure 4**B), mainly representing albumin and globulin fractions, were quite soluble at pH 1 and 2. The addition of NaCl improved the solubility of these polypeptides (**Figure 4C**). At higher pH, higher molecular mass polypeptides (**Figure 4C**) were more readily solubilized. The protein solubility at pH >9 (**Figure 4A**) in the presence of 2 M NaCl was lower than in DI H_2O at the same pH. The observed decrease in protein solubility may result from salt promoting hydrophobic interactions within and between proteins leading to protein aggregation and/or the promotion of hydrophobic interaction between proteins and phenolics. To learn the role of ionic strength (NaCl) in protein solubilization, proteins were first extracted at different salt concentrations. The residue was successively extracted using 70% (v/v) aqueous ethanol and alkali (**Figure 5A**). Results suggested that protein solubility significantly (LSD = 0.53, $n = 2$, $p = 0.05$) increased from 1.39 to 10.61 mg/100 mg defatted flour as the NaCl concentration increased from 0 to 4.0 M. Of interest was the simultaneous and statistically significant decrease in alkali-soluble proteins. If a lack of sufficient ionic strength (NaCl) resulted in incomplete globulin solubilization, protein yield from extraction of the residue using alkali (alkali glutelin fraction) solvent should increase. As can be seen from **Figure 5A**, results suggested the opposite (yield of alkali glutelins decreased). SDS-PAGE analyses (**Figure 5B**) of the corresponding 0−4 M NaCl indicated that salt helped increase protein solubility. Qualitatively, the banding intensity of NaCl-soluble polypeptides increased. The polypeptide profile of NaOH solubilized proteins using flour residue from 4 M NaCl extraction did not suggest alkali glutelin to be equivalent to insoluble globulin fraction [e.g., compare the polypeptide profiles of proteins solubilized by 4 M NaCl and 4 M NaOH (post 4 M NaCl extraction)].

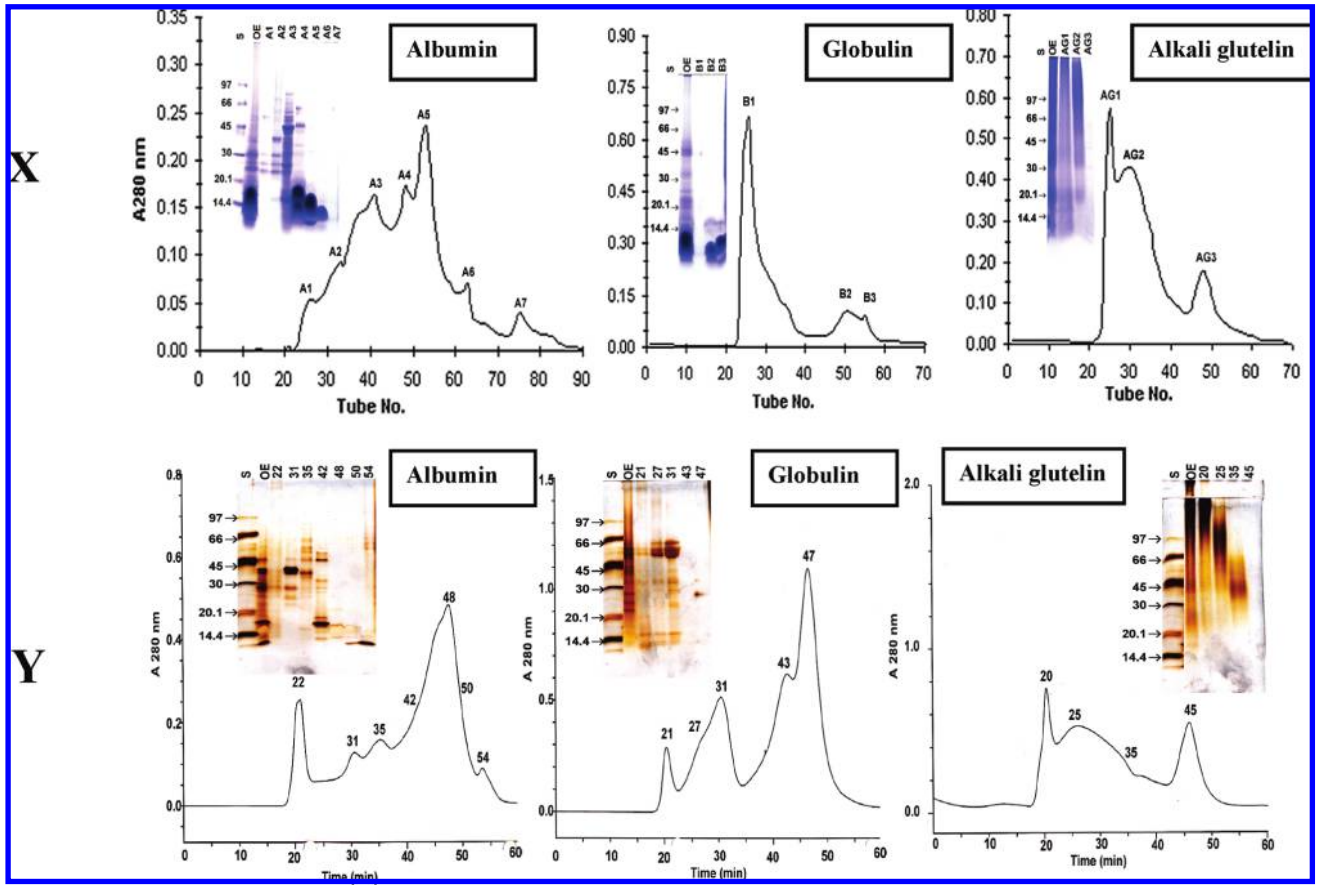

Figure 7. Size-exclusion chromatographic separation and corresponding SDS-PAGE (with 2% v/v β-ME) for pecan proteins using low-pressure Sephacryl S 300 HR gel filtration (**X**) and high-pressure liquid chromatography (HPLC) on Superdex S 200 HR (**Y**), respectively; OE = original protein fraction loaded on column. Peak fractions were collected, and aliquots were mixed with an equal volume of SDS-PAGE sample dye and used for SDS-PAGE. Gels in parts **X** and **Y** were stained with Coomassie Blue and silver stain, respectively.

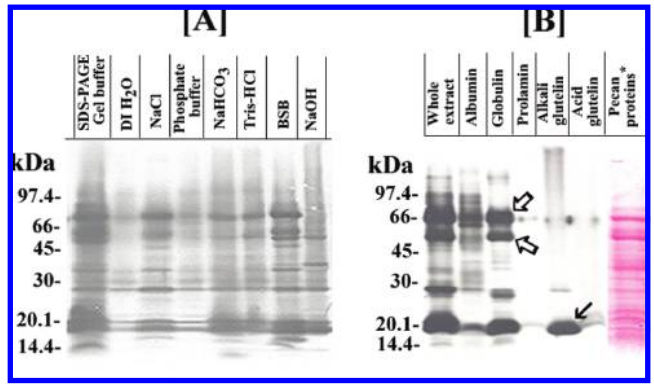

Figure 8. Immunoblots of pecan proteins (30 μg per lane) separated on SDS-PAGE (in the presence of 2% v/v β-ME). (**A**) Proteins solubilized in different solvents were probed using rabbit anti pecan pAbs. (**B**) Osborne protein fractions were probed using rabbit anti-pecan pAbs. Whole extract = BSB solubilized proteins. Pecan proteins* (the total pecan seed proteins stained with Ponceau S stain after the proteins were transferred on to the nitrocellulose paper) are included for comparison purposes.

Thus, ionic strength, pH, and phenolic compound interactions with proteins may all be important in determining the pecan protein solubility in aqueous media.

Protein Fractionation and Gel Electrophoretic Analyses. From the results of Osborne protein fractionation (**Table 1**), it was apparent that alkali glutelins are the predominant seed protein (64.94%) followed by globulins (22.13%), prolamins (9%), albumins (2.04%), and acid-soluble glutelins (1.89%). Glutelins are also the major protein fraction in walnuts (*23*), a finding not surprising as both pecans and walnuts belong to the *Juglandaceae* family.

The staining intensity and band thickness observed (judged subjectively) in NDND-PAGE (**Figure 6A**) analyses of the Osborne protein fractions suggest that the globulin fraction is the predominant pecan protein, a result that would be inconsistent with the quantitative data on protein fractionation as summarized in **Table 1**. NDND-PAGE separates proteins based on their negative electrical charge. Failure of prolamin and glutelin fractions to enter the separating gels suggested a lack of negative charge on these proteins under the experimental conditions and was unexpected as, in the absence of salt, the seed proteins were quantitatively solubilized at pH >8 (**Figure 4A**). SDS-PAGE analyses under

denaturing as well as denaturing plus reducing conditions (**Figure 6B**) facilitated the polypeptide migration into the gel system. Reduction of disulfide bonds resulted in particularly significantly altering the globulin polypeptide profile. Neither denaturation nor disulfide reduction was of much help in improving the sharpness of glutelin polypeptides. We have previously observed similar behavior for walnut glutelins (*23*). SDS-PAGE polypeptide analyses of alkali- and acid-soluble glutelins exhibited mobilities of constituent polypeptides in these two fractions and were similar, but not identical, to those in the globulin fraction.

Osborne protein fractions were analyzed for protein content determination by Lowry, Bradford, and micro-Kjeldahl methods (**Table 1**). We (*24*) have earlier reported that acidified methanol extraction did not significantly increase phenolic extraction from pecan flour, indicating the pecan phenolics to be predominantly nonpolar. If the phenolics bound to the globulins were to interfere in Lowry protein determination, one would expect a higher protein estimation by Lowry method as compared to the micro-Kjeldahl method, which is not the case in the current investigation. As compared to the micro-Kjeldahl method, both Lowry and Bradford methods overestimated the protein content of the alkali glutelin fraction. The alkali glutelin fraction had a dark amber color and contained ~4.6 mg phenolics/100 g when extracted with absolute methanol. The Lowry method is based on Folin−Ciocalteu reagent reacting with amino acids tyrosine and tryptophan to produce the blue color (*18*); however, the Folin−Ciocalteu reagent also reacts with many phenolic compounds and may have reactivity toward other nonprotein and nonphenolic compounds (*25, 26*). In addition, as a recent report documents, the reactivity of the Folin−Ciocalteu reagent toward primary, secondary, and aromatic amines and aromatic amine pyrrole and indole but not imidazole and benzimidazole derivatives has been noted. The study reported that tertiary aliphatic amines, but not primary, secondary, and quaternary aliphatic amines, were also strongly reactive toward Folin−Ciocalteu reagent (*27*). The results from the current investigation thus do not unequivocally support the possible loss of globulin solubility as a result of globulin−phenolic interactions, leading to high alkali-soluble glutelin fractions.

IEF (**Figure 6C**) revealed the pecan proteins to be mainly acidic in nature. Alkali exposure may cause a shift toward the acidic range for alkali-soluble glutelins. The amino acid composition of the protein solubility fractions (**Table 2**) revealed that the alkali glutelin fraction contained 10% more hydrophobic amino acids as compared to the globulin fraction. However, alkali glutelin had 10% less acidic amino acids and approximately the same amount of total basic amino acids when compared with the corresponding values for the globulin fraction. Therefore the glutelin and globulin solubility behavior cannot be explained solely on amino acid composition either. The mechanism by which increased ionic strength improved globulin solubility with a concurrent drop in alkali-soluble glutelins therefore needs further investigation.

With the exception of the albumin fraction, all other solubility fractions had one band staining positive for glycoproteins marked with an * in **Figure 6D**. The albumin fraction had two major glycoprotein staining polypeptides of molecular mass >100 kDa (indicated by "}" and an open arrow). The globulin fraction had at least four polypeptides in the 20−50 kDa range (indicated by the solid arrows on the left of the globulin track). Of particular note was the difference between the globulin and the glutelin staining patterns. While globulin and glutelin (both alkali- and acid-soluble) fractions contained high molecular mass glycoproteins (>200 kDa) (i.e., the bands that barely entered the stacking and the separating gels), there was a distinctly different profile for globulin, especially with respect to the glycoprotein polypeptides with molecular mass >14.4 kDa but <66 kDa. These polypeptides appeared to be present only in globulin fraction and, under the experimental conditions, were undetectable in the glutelin fraction. The acid glutelin fraction also had a small molecular mass (<14.4 kDa) peptide (indicated by []) that could not be detected in globulin and alkali glutelin fractions. The acid glutelin fraction was characterized by protein that barely entered the separating gel, indicated by the solid arrow on the right side of the acid glutelin track and staining much more intensely (judged subjectively) than in either the globulin or the alkali glutelin fractions.

Chromatography. Separation on high-resolution gel filtration matrix coupled with SDS-PAGE (**Figure 7X**) further illustrated the complexity of protein fractions. The regression equation for Stokes radius was $Y = 0.0087X + 0.3145$ ($r = 0.987$, $n = 2$) and that for estimating molecular mass was ($Y = -0.3219X + 1.972$ ($r = 0.981$, $n = 2$). The gel filtration column chromatography separated albumin, globulin, and alkali glutelin into 7, 3, and 3 fractions, respectively. The first peak in the albumin fraction (A1) eluted near the column void volume, suggesting the presence of a very large protein or an aggregate made up of smaller polypeptides (the gel inset suggests the latter). The estimated molecular mass and the Stokes radii of the next three fractions were A2 (362950 Da; 62.68 Å), A3 (73,870 Da; 35.88 Å), and A4 (18,250 Da; 17.96 Å). The corresponding values for A5−7 could not be determined as their elution volumes were outside the range of standards used for column calibration. Peak A5 contained polypeptides 10−18 kDa, while A6 and A7 polypeptides were <10 kDa. Globulin resolved in three peaks with the first fraction (B1) eluting close to column void volume. This fraction was mainly composed of a faintly stained 55 kDa polypeptide with minor amounts of smaller molecular mass polypeptides. Globulin fractions B2 and B3 were mostly composed of polypeptides ≤15 kDa. Sephacryl S 300 HR resolved the alkali glutelin into three major fractions (AG1, AG2, and AG3), none of which exhibited sharp polypeptide banding pattern in SDS-PAGE. Peak AG1 eluted close to the column void volume, while peak AG2 had an estimated Stokes radius of 67.5 Å and molecular mass 445190 Da. These values were very close to the corresponding values of 61 Å and 486800 Da earlier reported values for walnut glutelin by Sze-Tao and Sathe (*23*).

HPLC analysis of albumin, globulin, and glutelin fractions also confirmed the polypeptide diversity within the protein fractions (**Figure 7Y**). The albumin fraction was resolved into seven peaks, each with a distinct polypeptide profile, an observation consistent to that seen using low-pressure gel filtration (**Figure 7X**). HPLC separated the globulin fraction into five peaks, unlike low-pressure gel filtration where the majority of proteins eluted in the void volume (peak B1 in **Figure 7X**). The alkaline glutelin fraction was resolved into four fractions by HPLC, each of which showed a diffused SDS-PAGE polypeptide banding pattern. Generally, SDS-PAGE profiles of peak fractions separated by low- and high-pressure chromatography failed to register concordance.

Amino Acid Composition. As compared to the FAO/WHO pattern, pecan proteins are deficient in lysine (**Table 2**). Although phylogenetically quite distant from many cereals (*Gramineae*), it is interesting to note that pecans are deficient in lysine, the first limiting amino acid in many edible cereals such as rice, corn, and wheat. The pecan amino acid composition is consistent with the reported literature values (*24*). Among the protein fractions, albumins and acid glutelins were limiting

in leucine and tryptophan, respectively, while all of the other fractions were deficient in lysine. However, as compared to the needs of an adult, pecans contain all of the essential amino acids in adequate quantities. Ahrens et al. (*28*) have similarly noted that almond proteins are also adequate with respect to adult human amino acid requirements.

Immunoblotting. Because BSB was found to be an optimal solvent for pecan protein extraction, BSB-soluble proteins were used to raise polyclonal antibodies (pAbs) in rabbits. Western blots probed with rabbit pAbs (**Figure 8A**) exhibited polypeptide profile qualitatively representative of most proteins soluble in the tested solvents. Western blots of Osborne solubility fractions (**Figure 8B**) indicate globulin polypeptides to be highly immunoreactive with prolamin polypeptides being the least. The alkali glutelin fraction registered polypeptide immunoreactivity profile distinctly different than the one for the globulin fraction. On the basis of electrophoretic mobility, the low molecular mass (~20 kDa range indicated by a solid arrow in alkali glutelin lane) polypeptide appeared to be common in both globulin and alkali glutelin fractions. However, two strongly polypeptides (open arrows in the globulin lane) appeared to be absent in the alkali glutelin fraction. These differences in the immunogenic profiles of the two fractions warrant further investigations to clarify the relationship (or the lack of it) between these two major protein fractions in pecans.

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