JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. XXXX, XXX, 000–000 A
DOI:10.1021/jf9016338

Solubilization and Electrophoretic Characterization of Select Edible Nut Seed Proteins

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The solubility of almond, Brazil nut, cashew nut, hazelnut, macadamia, pecan, pine nut, pistachio, walnut, and peanut proteins in several aqueous solvents was qualitatively and quantitatively assessed. In addition, the effects of extraction time and ionic strength on protein solubility were also investigated. Electrophoresis and protein determination (Lowry, Bradford, and micro-Kjeldahl) methods were used for qualitative and quantitative assessment of proteins, respectively. Depending on the seed, buffer type and ionic strength significantly affected protein solubility. The results suggest that buffered sodium borate (BSB; 0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, 0.075 M NaCl, pH 8.45) optimally solubilizes nut seed proteins. Qualitative differences in seed protein electrophoretic profiles were revealed. For a specific seed type, these differences were dependent on the solvent(s) used to solubilize the seed proteins. SDS-PAGE results suggest the polypeptide molecular mass range for the tree nut seed proteins to be 3−100 kDa. The results of native IEF suggested that the proteins were mainly acidic, with a p*I* range from >4.5 to <7.0. Western immunoblotting experiments indicated that rabbit polyclonal antibodies recognized substantially the same polypeptides as those recognized by the corresponding pooled patient sera IgE.

KEYWORDS: Tree nut; protein; polypeptide; solubility; SDS-PAGE; immunoblot; IgE

INTRODUCTION

Diverse in their origins, cultivation practices, and end use (*1*), tree nuts are globally popular. Among edible nut seeds, peanuts or groundnuts (*Arachis hypogaea*), a legume, and nine tree nuts, almond (*Prunus dulcis*), Brazil nut (*Bertholletia excelsa*), cashew nut (*Anacardium occidentale*), hazelnut (*Corylus avellana*), macadamia (*Macadamia integrifolia*), pecan (*Carya illinoinensis*), pine nut (*Pinus pinea*), pistachio (*Pistachia vera*), and walnut (*Juglans regia*), are commercially important. The United States ranks first in global nut production and in 2005 was ranked first, second, second, third, and fourth in global almond, pistachio, walnut, hazelnut, and peanut (in shell) production (*2*), respectively. Edible nut seeds are typically high in lipids and proteins and are valued for their desirable sensory attributes—notably mild flavors and crunchy textures. The high lipid content, which is a contributor to satiety, coupled with desirable fatty acid profiles with respect to oleic, linoleic, and linolenic acid balance (*3*), has led to incorporation of edible nut seeds in weight management diets to suppress appetite and fat absorption, which have received increased attention in recent years (*4*). Nut seeds and nut seed derived ingredients, in various forms, are therefore being increasingly used to develop and manufacture value-added products to help improve economic returns.

Although safely enjoyed by most, peanuts and tree nuts have also received increased attention due to their inclusion in the "big 8" food groups responsible for the majority of food-induced allergies in humans (*5*, *6*). Several recent reviews have highlighted tree nut allergies (*7*−*11*). Type I IgE-mediated food allergies are caused by food proteins, with very rare exceptions. Investigating IgE-reactive nut seed proteins is therefore essential to improve our understanding of nut seed-induced allergies. Among edible nut seeds, peanuts have been the most well-characterized, including protein content and functional properties (*12*), protein type (*13*, *14*), and protein allergenic properties (*15*−*21*).

Many studies focus on defined single-protein molecular species for investigating food allergens, often using recombinant forms of the targeted protein. The study of recombinant proteins offers several advantages in characterizing targeted food allergens because recombinant proteins provide an unambiguous, consistently reproducible, and known amino acid sequence for the protein. When expressed in a suitable system, recombinant proteins can also provide an inexhaustible supply of defined protein species. However, when recombinant proteins do not

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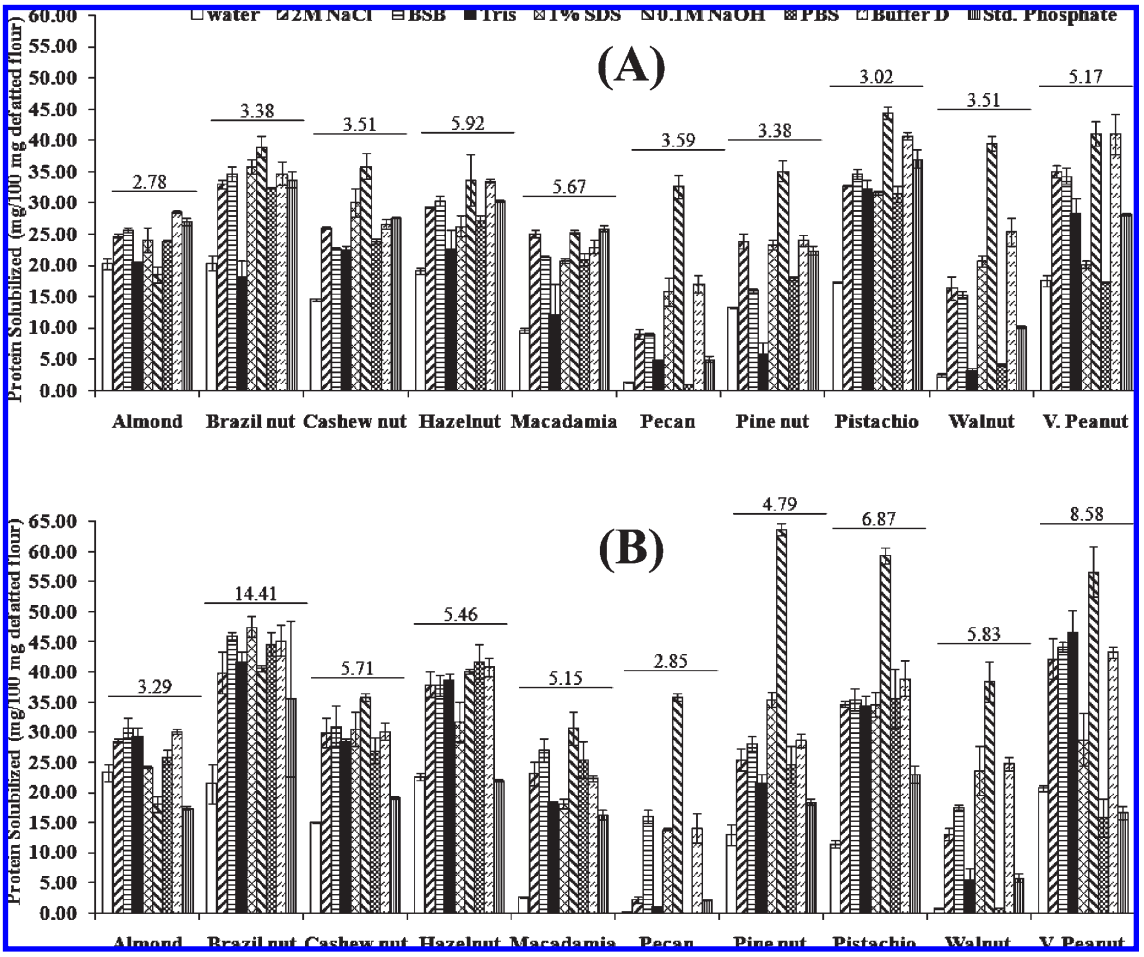

Figure 1. Effect of solvent type on protein solubilization: soluble protein estimated by Lowry (**A**) and Bradford (**B**) methods. Data are represented as mean ± standard deviation (n = 2, each analyzed in triplicate). Numbers on the top of each data set for the seed type represent LSD for the set.

exhibit molecular behavior equivalent to their native counterparts, an investigation of native proteins becomes essential. Here the term "native" is meant to indicate the naturally occurring form of seed protein to distinguish it from its recombinant counterpart. The lack of concordance between the native and recombinant form(s) of the targeted protein(s) may arise from several factors including glycosylation, formation of multiple native isoforms due to either multiple genes coding for the same protein, post-translational protein modification, or a combination thereof (*22*−*25*). A recent report on Ara h 3 (*19*), a known peanut allergen, is instructive in this regard. The investigators identified a natural isoform of Ara h 3 exhibiting lower allergenicity than its original natural counterpart, demonstrating the importance of understanding native isoforms. Another reason why investigating native proteins is important is that protein extracts prepared from natural tissues are often of variable quality depending on the tissue maturity, tissue components, solvents used for protein extractions, and storage stability of the protein preparations (*26*−*31*), whether the protein preparations are used for diagnostic, clinical, or investigative purposes. Protein denaturation (including aggregation), protein degradation due to in situ enzymes, or introduction of active enzymes due to microbial contamination may also negatively influence the protein storage stability. Because allergic consumers may be exposed to nut allergens that are raw or variously processed, both undenatured and denatured forms of native proteins in the seed could be responsible for eliciting allergic reactions. For these reasons, it is important to identify and characterize allergenic proteins as they naturally occur in edible nut seeds.

Investigating native proteins requires knowledge of protein composition and protein solubility. To this end we recently published an account of the chemical composition of commercially important tree nut seeds (*3*). The current study reports findings on protein solubility and qualitative protein polypeptide composition. In addition, protein polypeptides recognized by corresponding rabbit polyclonal antibodies (pAbs) and pooled patient sera IgE were determined.

MATERIALS AND METHODS

Materials. Sources of seeds, chemicals, and reagents have been reported earlier (*3*). Brazil nuts, cashews, hazelnuts, macadamia nuts, pine nuts, Spanish peanuts, sesame seeds (polished white color), sunflower seeds, soybeans, and navy beans were purchased from local grocery stores. Almonds (Nonpareil marketing variety; Almond Board of California, Modesto, CA), pecans (cultivar Desirable, Dr. T. Thompson, USDA-ARS, Pecan Breeding and Genetics, Somerville, TX), pistachio (Paramount Farms, Inc., Los Angeles, CA), walnuts (Blue Diamond Growers, Sacramento, CA), and Virginia peanuts (VA 98R, Dr. Sean F. O'Keefe, VPI&SU, Blacksburg, VA) were gifts. The Inca peanut flour source has been described earlier (*32*).

Methods. *Preparation of Defatted Seed Flours.* Defatted seed flours were prepared and stored at −20 °C as described earlier (*3*). Briefly, a known weight of the sample (∼10 g/thimble) was defatted in a Soxhlet apparatus using petroleum ether (boiling point range of 38.2−54.3 °C) as the solvent (flour-to-solvent ratio of 1:10 w/v) for 8 h. Defatted samples were dried overnight (∼10−12 h) in a fume hood to remove residual traces of petroleum ether. Defatted samples were homogenized using a Sorvall blender (speed setting at 6−8) and stored in plastic screw-capped bottles at −20 °C until further use.

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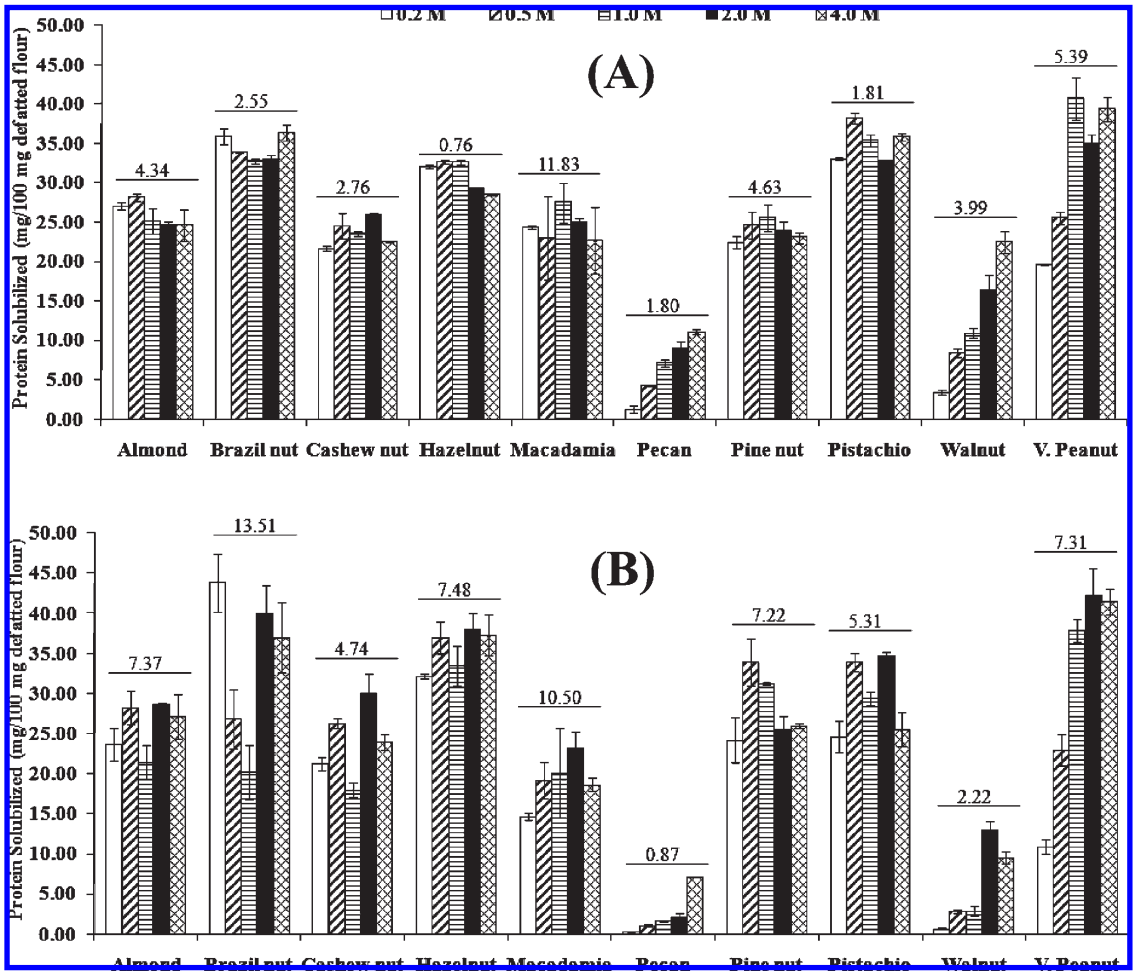

Figure 2. Effect of ionic strength on protein solubilization: soluble protein estimated by Lowry (**A**) and Bradford (**B**) methods. Data are represented as mean ± standard deviation (n = 2, each analyzed in triplicate). Numbers on the top of each data set for the seed type represent LSD for the set.

Protein Solubilization. To assess protein solubilization and extraction efficiency of commonly used solvents, defatted flours were subjected to protein solubilization and extractions using the following solvents: (1) distilled deionized water (DI water); (2) aqueous NaCl at a range of M; (3) buffered saline borate (BSB; 0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, 0.075 M NaCl, pH 8.45); (4) 0.02 M Tris-HCl (pH 8.1); (5) 1.0% (w/v) aqueous sodium dodecyl sulfate (SDS); (6) 0.1 M aqueous NaOH; (7) PBS (0.1 M sodium phosphate buffer containing 0.85% NaCl, pH 7.2); (8) buffer D [Tris base 6.5 g + citric acid monohydrate 1.5 g + cysteine monohydrate 1.0 g + ascorbic acid 10.0 g + polyethylene glycol (PEG) + 0.5 M EDTA 4 mL + glycerol 220 mL + DI water 700 mL/liter, pH 8); (9) standard phosphate buffer [0.0325 M K_2HPO_4, 0.0026 M KH_2PO_4, 0.4 M NaCl, 0.01% (v/v) aqueous β-mercaptoethanol (β-ME)]; and (10) 70% aqueous ethanol (EtOH).

Protein Determination. Protein content was determined according to AOAC Official Method 950.48 as described earlier (*3*). Percent protein was calculated from protein nitrogen using appropriate conversion factors (*33*): 5.18 for almond, 5.46 for peanut, and 5.3 for the rest. Soluble protein content was determined using the methods of Lowry et al. (*34*) and Bradford (*35*).

Electrophoresis

(*a*) *Non-denaturing Non-dissociating Polyacrylamide Gel Electrophoresis* (*NDND-PAGE*) *(36,37)*. NDND-PAGE was used to separate proteins by their net negative electrical charge. Typically, NDND-PAGE gels were 1.5 mm thick, 3−30% linear acrylamide gradient gels [acrylamide/bis = 37:1 (w/w) with 90 mM Tris, 80 mM boric acid, 2.5 mM Na-EDTA, pH 8.5] with 3% monomer acrylamide stacking gels. Running buffer was 90 mM Tris, 80 mM boric acid, and 2.5 mM Na-EDTA, pH 8.4. Proteins were mixed with suitable volumes of NDND-PAGE sample buffer (2 volumes of 0.45 M Tris, 0.4 M boric acid, amd 12.5 mM Na-EDTA mixed with 1 volume of glycerol) containing 0.001% bromophenol blue as the tracking dye. Pharmacia (Pharmacia Co., Piscataway, NJ) high molecular weight (HMW) kit proteins were used as standards. For NDND-PAGE, seed proteins were extracted in 0.05 M Tris-HCl (pH 8.1).

(*b*) *Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis* (*SDS-PAGE*) *(37, 38)*. SDS-PAGE was used to characterize the polypeptide profile. Typically, samples were electrophoresed on an 8−25% linear monomer acrylamide gradient separating gel (14.5 cm × 16.5 cm × 1.5 mm) with a 4% monomer acrylamide stacking gel (1.0 cm × 16.5 cm × 1.5 mm). Proteins were mixed with suitable volumes of SDS-PAGE sample buffer (0.05 M Tris-HCl, pH 6.8; 1% SDS; 0.01% bromophenol blue as the tracking dye; and 30% glycerol) containing 2% (v/v) β-ME (for reducing gels), heated for 10 min in a boiling water bath, and cooled to room temperature, and aliquots were electrophoresed. Standard low molecular weight markers (Pharmacia) were used in each gel run. SDS-PAGE for subsequent immunoblotting of tree nut allergens by IgE in human sera differed in that 12% monomer acrylamide gels and broad range markers (Pharmacia) were used.

(*c*) *Isoelectric Focusing* (*IEF*). Native IEF was used to determine isoelectric points (p*I* values) of proteins soluble in 0.02 M Tris-HCl, pH 8.1. The gels, 5% linear monomer acrylamide containing broad p*I* range (pH 3.5−10) ampholines (Pharmalyte), were run using the Multiphor II flat-bed electrophoresis system according to the manufacturer's (Amersham Biosciences, Piscataway, NJ) instructions. Protein standards with known p*I* values were used in each run. Cooling (10 °C) was provided during the gel runs. Urea IEF with 5.4% monomer acrylamide gels containing 8 M urea was used for determining isoelectric points of polypeptides. Proteins were solubilized in 0.02 M Tris-HCl (pH 8.1) containing 8 M urea [with and without 10 mM dithiothreitol (DTT)] for urea IEF.

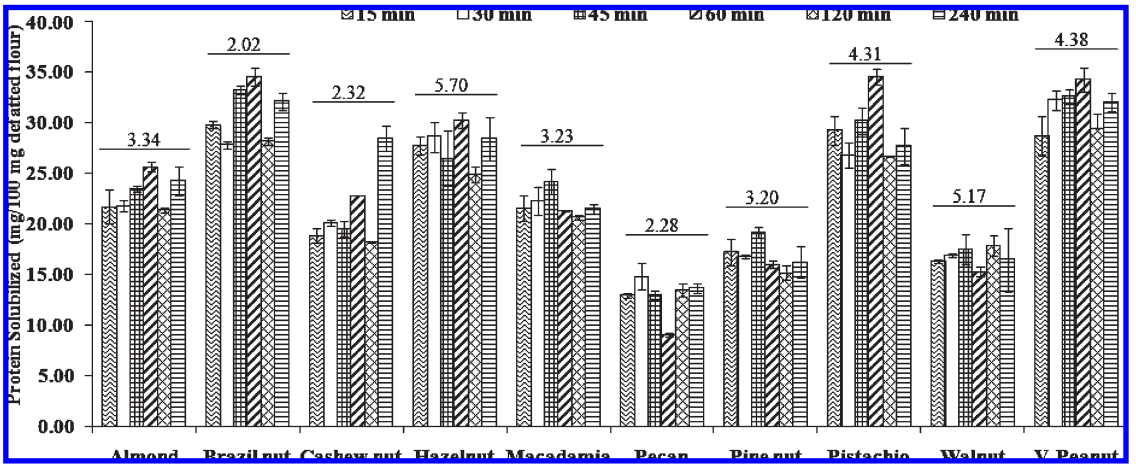

Figure 3. Effect of extraction time on protein solubility: soluble protein estimated by Lowry method. Data are represented as mean ± standard deviation ($n = 2$, each analyzed in triplicate). Numbers on the top of each data set for the seed type represent LSD for the set.

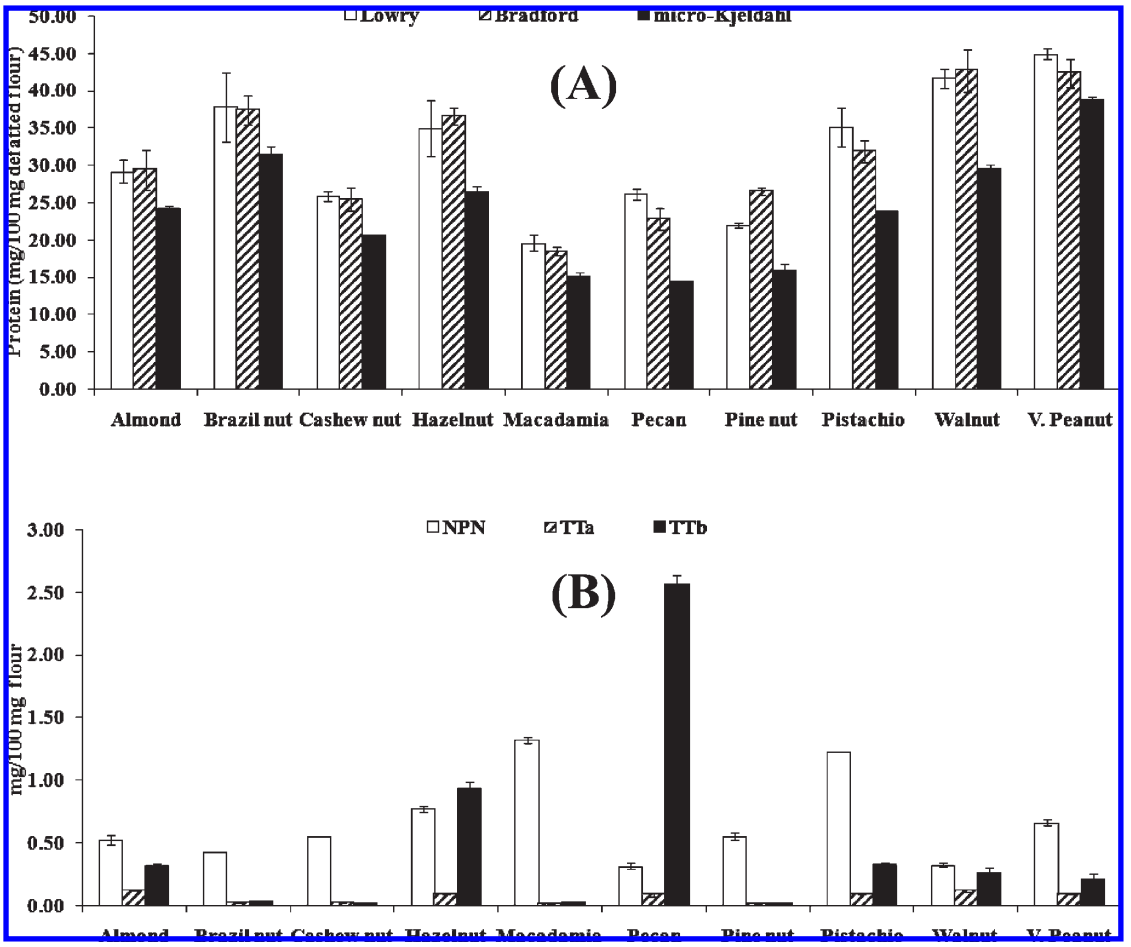

Figure 4. BSB-soluble proteins estimated by Lowry, Bradford, and micro-Kjeldahl protein assays (**A**). NPN, TT[a], and TT[b] (**B**), respectively, represent nonprotein nitrogen, total tannins present in BSB solubilized proteins, and total tannins (in full-fat flour) solubilized by acidified methanol [1% (v/v) HCl]. Data are expressed as mean ± standard deviation ($n = 2$, each analyzed in duplicate except Bradford—in triplicate).

All gels, except IEF gels, were run at a constant current (8−12 mA/gel) until the tracking dye reached the gel edge. Running tap water cooling (∼15 °C) was provided during the gel run. Gels were stained overnight with 0.25% (w/v) Coomassie Brilliant Blue R (CBBR) in 50% (v/v) aqueous methanol containing 10% (v/v) glacial acetic acid (*37*) and destained for 4 h in 50% (v/v) aqueous methanol containing 10% (v/v) glacial acetic acid, followed by destaining in 25% (v/v) aqueous methanol containing 5% (v/v) glacial acetic acid until the blue background was clear. Silver staining of the gels was done as described (*39*). Briefly, the gels were immersed in aqueous fixing solution containing 11.4% w/v trichloroacetic acid/3.4% w/v sulfosalicylic acid, and 30% (v/v) MeOH for 2 h followed by five washes (5 min each) with DI water. The gels were then incubated in an aqueous solution consisting of 25% v/v MeOH and 8% v/v acetic acid for 1 h. The gels were subsequently treated with 10% v/v aqueous glutaraldehyde for 2 h followed by overnight washing with DI water. The gels were then treated with a 200 mL of aqueous solution containing 1 mL of 15% w/v freshly prepared NaOH, 8 mL of 20% w/v silver nitrate, and ∼6 mL of concentrated ammonium hydroxide solution for 15−20 min,

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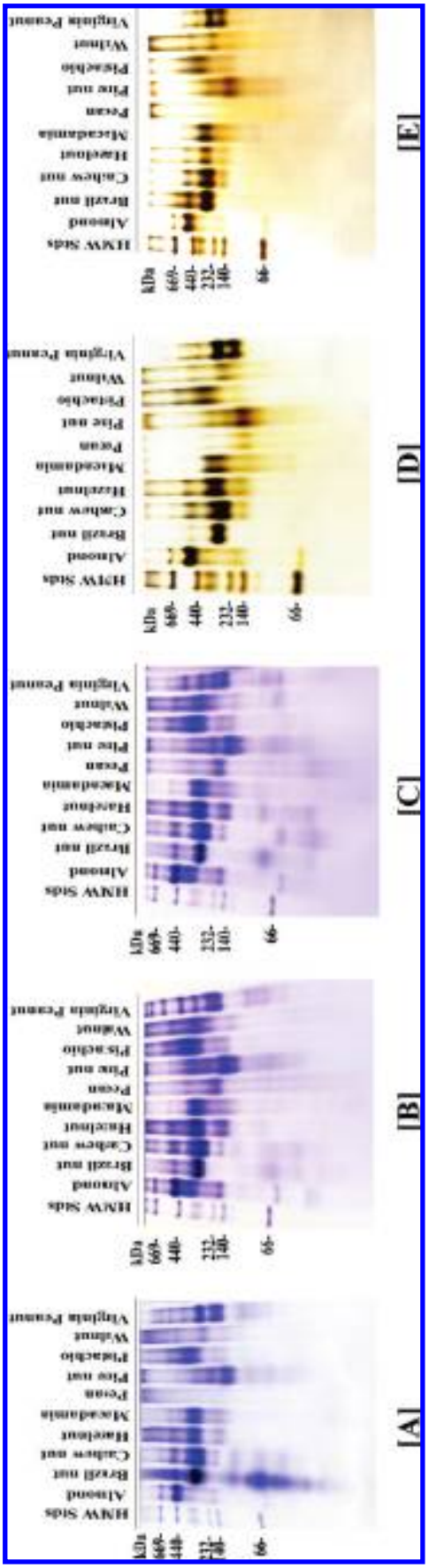

Figure 5. NDND-PAGE, 3−30% linear monomer acrylamide gradient, for soluble tree nut proteins in the absence (**A**, **C**−**E**) and presence (**B**) of 2% (v/v) β-mercaptoethanol. Gels were stained with CBBR (**A**−**C**) or silver stain (**D**, **E**). Solvents used for protein solubilization were 0.05 M Tris-HCl, pH 8.1 (**A**, **E**), 0.1 M BSB, pH 8.45 (**B**, **C**), and DI water, dispersion adjusted to pH 9.0 (**D**). Protein loads, except for the marker proteins, were 60 μg/lane (**A**−**C**) and 10 μg/lane (**D**, **E**).

and the gels were washed three times with DI water. Protein bands were visualized by immersing the gels in 200 mL of aqueous developing solution containing 20 mL of 0.05% w/v citric acid and 100 μL of formaldehyde. The development was stopped with 200 mL of 0.05% w/v aqueous citric acid solution containing 100 μL of 35% w/v aqueous methylamine. The gel background was cleared with Kodak fixer solution (Kodak, Rochester, NY). Proteins transferred onto the membranes were visualized by brief ($\leq$5 min) staining with 0.1% (w/v) Ponceau S in 5% (v/v) aqueous acetic acid, as required.

Glycoprotein Staining. Glycoprotein staining was done on SDS-PAGE gels using the Gelcode Glycoprotein staining (Pierce Chemical Co., Rockford, IL) procedure per the manufacturer's instructions.

Tannins. The BSB-solubilized proteins, described under Protein Solubilization, were analyzed for soluble tannins. In a separate set of experiments, whole nut full-fat seed flours were extracted with 10 volumes of acidified methanol (MeOH) at room temperature with constant magnetic stirring, samples were centrifuged, and aliquots of the supernatants were used for total tannin determination according to a vanillin method described earlier (*40*).

Immunoblotting. Protein extracts prepared from defatted seed flours were probed with rabbit pAbs or pooled allergic patient sera IgE (two or three patient sera per nut) using the protocols described earlier (*23*,*41*). All participants gave informed consent. The study was approved by the Institutional Review Board of the University of California, Davis. Serum samples were obtained from patients with convincing repeated histories of systemic allergic reactions to peanuts or the specific tree nuts and positive ImmunoCAP assays (>0.35 kU/L) (Pharmacia, Uppsala, Sweden) and IgE immunoblotting. ImmunoCAP assays were performed in a hospital commercial laboratory (University of California, Davis, Medical Center, Sacramento, CA).

Data Analysis and Statistical Procedures. All statistical analyses were performed using SPSS statistical software (version 15; Chicago, IL). All experiments were carried out at least in duplicate, and data are expressed as the mean ± standard deviation. One-way ANOVA and Fisher's least significant difference test as described by Ott (*42*) were used to determine statistical significance, and results were considered to be significant if $p \leq 0.05$.

RESULTS AND DISCUSSION

Protein Solubility. Depending on the tree nut type, solvent (**Figure 1**), ionic strength (**Figure 2**), and extraction time (**Figure 3**), soluble protein contents varied over a wide range. Protein quantification was dependent on the method used for protein determination (**Figures 1**, **2**, and **4**). For a specified solvent and nut seed type, the Bradford assay typically resulted in higher protein estimation when compared to that obtained by the method of Lowry et al. As both of these protein estimation methods are widely used, it was of interest to determine if the true protein content of the sample was accurately reflected by either of these methods. For this reason, defatted nut seed flours were first extracted at pH 8.5 in DI water and centrifuged as described under Protein Solubilization, and the supernatants were subjected to protein determination by micro-Kjeldahl (total nitrogen and non- protein nitrogen), Lowry, and Bradford assays (**Figure 4**). The Lowry method typically overestimated protein by a factor of 1.16 (Virginia peanut) to 1.81 (pecan). Soluble protein content of the samples was also overestimated by the Bradford method by a factor of 1.26 (Virginia peanut) to 1.92 (pine nut). Phenolic compounds are often suggested to be one of the major causes for protein overestimation by Lowry and Bradford assays (*43*). Total tannin analyses of protein extracts and tannin extracts prepared from equivalent amounts of seed flours using acidified (1% v/v, HCl) MeOH generally did not support this suggestion. For example, pine nut seeds did not exhibit high amount of tannins, and yet the overestimation factor for both Bradford (1.92) and Lowry (1.38) methods was significant. Additionally, although almonds had higher tannins compared to pine nuts, protein

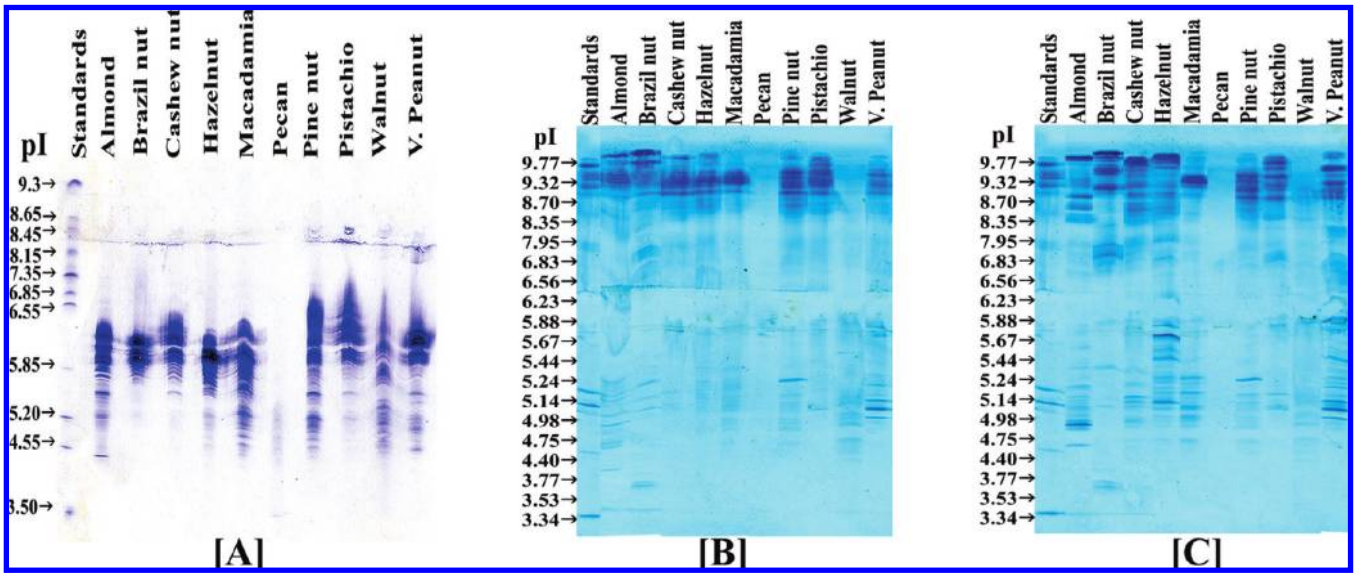

Figure 6. IEF for 0.02 M Tris-HCl buffer (pH 8.1) soluble nut seed proteins in the absence of denaturants 8 M urea and 10 mM DTT (**A**), in the presence of 8 M urea (**B**), and in the presence of 8 M urea and 10 mM DTT (**C**). Sample protein loads in each lane were 100 (**A**), 80 (**B**), and 80 (**C**) μg, respectively.

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overestimation factors for Lowry (1.20) and Bradford (1.40) methods were lower for almond than for the pine nut.

Depending on the seed type, NaCl significantly influenced protein solubility as seen in **Figure 2**. For example, increasing NaCl from 0.2 to 4 M increased pecan protein solubility by 9.24-fold and that of walnut proteins by 6.65-fold. Improved protein solubility as a consequence of increased salt in the protein extraction buffer has been reported for Brazil nut (*44*), pecan (*45*), and walnut (*46*). Increased protein solubility with increased NaCl suggests a higher proportion of globulins (compared to albumins) in the seeds, improved solubility of albumins as a result of disruption of ionic interactions between albumins and phenolics, or a combination of both. On the other hand, increasing NaCl to 4 M decreased hazelnut protein solubility significantly (from 32.02 g/100 g of defatted flour at 0.2 M to 28.48 g/100 g of defatted flour at 4 M NaCl), indicating a higher proportion of water-soluble albumins compared to salt-soluble globulins in hazelnut seeds. Almonds registered a slightly decreased protein solubility at 4 M NaCl compared to solubility in the absence of NaCl, although the decrease was not statistically significant. This observation is consistent with the earlier reported solubility of almond proteins in DI water containing 1 mM NaN_3 (31.26 ± 1.05 mg of protein/100 mg of defatted flour) to be not significantly different from solubility in 1 M NaCl (30.08 ± 2.89 mg of protein/100 mg of defatted flour) (*37*), and the ionic strength did not significantly influence almond protein solubility or composition as revealed by ultracentrifugal analysis (*47*).

Electrophoresis. *NDND-PAGE.* NDND-PAGE separates proteins on the basis of their negative electrical charge. As can be seen from **Figure 5**, regardless of the solvent used for protein extraction, the staining method used, or the presence or absence of a reducing agent, only a limited number of protein species were evident in each of the tested seed samples. Improved solubility of pecan and walnut proteins in BSB, compared to Tris-HCl, pH 8.1, buffer or DI water, was evident in NDND-PAGE gels (compare the pecan or walnut tracks in panels **A**, **D**, and **E** with the corresponding tracks in panels **B** and **C**). Pecan (*45*) and walnut (*46*) proteins are characterized by dominance of globulins and glutelins and therefore often require salt and alkali pH for efficient protein solubilization. Note from **Figure 1** that 0.1 M aqueous NaOH was actually the best solvent for walnut and pecan. Pecan and walnut proteins did not stain well in NDND-PAGE whether CBBR or silver stain was used for protein visualization. Adding a reducing agent did not seem to significantly alter the electrophoretic mobility of proteins (compare corresponding profiles in **Figure 5B**,**C**), suggesting disulfide bonds may not be easily accessible to the reducing agent in non-denatured BSB soluble proteins. Difficulty in staining pecan and walnut proteins in NDND-PAGE gels may primarily be due to their low solubility in aqueous buffers, lacking the required salt concentration for effective solubilization of these proteins.

IEF. The results of IEF in the absence of a denaturant (**Figure 6A**) suggested that the proteins solubilized by 20 mM Tris-HCl, pH 8.1, buffer were mainly acidic, p*I* range from >4.5 to <7.0. As can be seen from this figure, pecan proteins were not efficiently solubilized. IEF in the presence of 8 M urea (**Figure 6B**) registered distinctly different patterns compared to the corresponding profiles in its absence. The main difference was the appearance of protein/polypeptide bands in both its acidic and alkali ranges with a major shift toward alkali p*I*. Qualitatively, 10 mM DTT addition did not significantly alter the IEF profile (**Figure 6C**) compared to the corresponding pattern observed in the presence of 8 M urea, suggesting that disulfide linkages, although important, were not as influential as the ionic interactions which are normally disrupted by urea.

SDS-PAGE. The need for preparation of standardized allergen extracts for a variety of purposes is well recognized (*48*−*50*). Comparative polypeptide profiles for proteins solubilized from the specific seed flour using different solvents are summarized in **Figure 7**. Depending on the seed type and the solvent used for protein extraction, polypeptide profiles for the same seed flour exhibited significant differences. For example, aqueous EtOH (70% v/v), typically used for solubilization of prolamins, extracted low molecular mass (<30 kDa) polypeptides with some exceptions (e.g., almond, Brazil nut, cashew nut, hazelnut, and pistachio) for which additional polypeptides of >30 kDa were extracted. Aqueous EtOH was the least effective protein solubilizer, suggesting that prolamins are a minor component of the total proteins in tested nut seeds. With the exception of cashew nut and pistachio, SDS-solubilized polypeptides exhibited a

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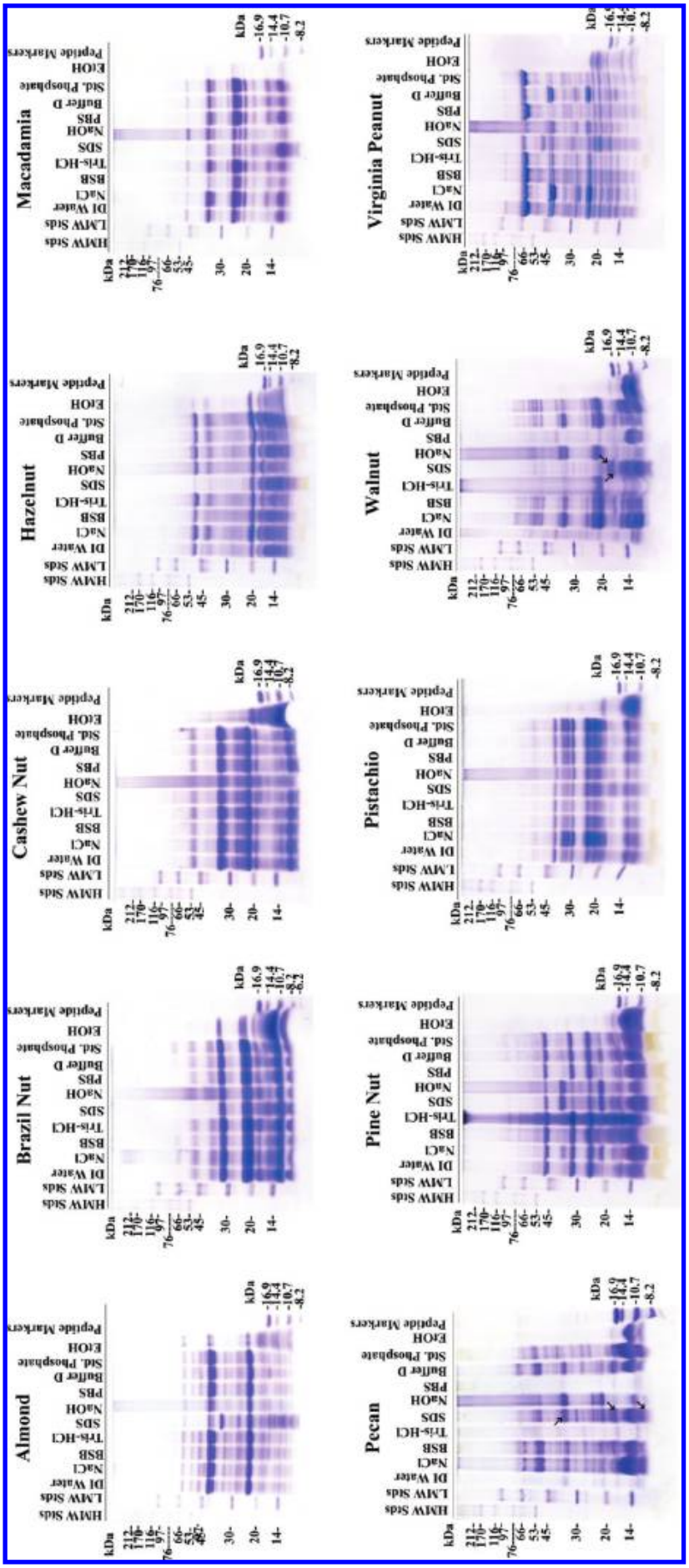

Figure 7. SDS-PAGE in the presence of 2% (v/v) β-ME for tree nut proteins solubilized in different solvents. Pecan and walnut proteins lacked solubility in PBS. Note the distinct polypeptide profile for 70% (v/v) EtOH extracted proteins.

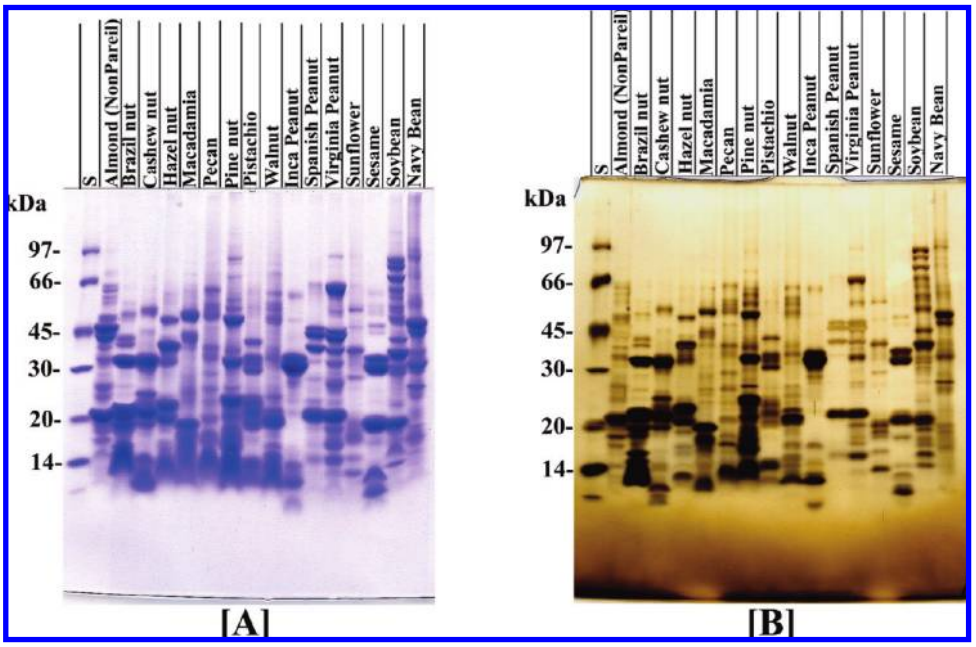

Figure 8. SDS-PAGE for tree nut proteins in the presence of 2% (v/v) β-ME: (**A**) CBBR staining; (**B**) silver staining. S, standard protein markers (molecular mass of each standard indicated in the left margin). Inca peanut, Spanish peanut, Virginia peanut, sunflower, sesame, soybean, and navy bean proteins were included for comparative purposes. Except for the standard proteins, protein load in each lane was 40 μg. The same gel was subjected to consecutive silver and CBBR staining procedures, in that order.

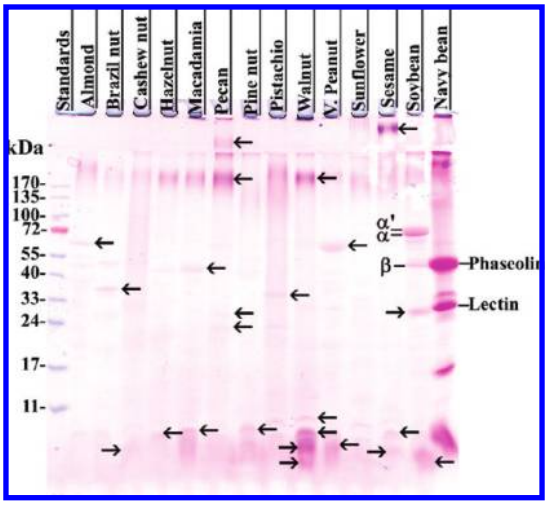

Figure 9. Glycoprotein staining for the seed protein extracts. Pharmacia LMW standards were used as marker proteins (molecular mass indicated in the left margin). Sunflower, sesame, soybean, and navy bean proteins were included for comparative purposes. Soybean β-conglycinin (7S) protein subunits α'-, α-, and β-, soybean lectin, navy bean major globulin (phaseolin), and lectin are known glycoproteins and were used as positive controls. Note the presence of several low molecular mass (<14 kDa) polypeptides as well as some high molecular mass polypeptides staining positive (arrows), indicative of glycopeptides. Pecan proteins contained two additional polypeptides in the range 24−33 kDa that stained positive for glycoprotein stain.

distinctly different profile compared to the polypeptide extracted using non-SDS solvents. Polypeptide profiles for PBS-solubilized pecan and walnut proteins also deserve mentioning, as in both cases the profiles were qualitatively distinctly different when compared with those for other solvents (except aqueous EtOH) used. In the case of pecan, the relative band intensity of certain polypeptides (e.g., polypeptide ~17 and ~19 kDa, indicated by arrows in the SDS track) was significantly higher (compare tracks for BSB vs SDS, for example). Similarly, note the significant intensity difference between SDS- and BSB-extracted polypeptides (note the polypeptides indicated by arrows in the SDS track). The low solubility of pecan and walnut proteins in PBS, both qualitative and quantitative, is of concern as PBS is a widely used buffer in protein extraction and characterization. A lack of protein solubility in PBS may lead to failure to detect trace amounts of pecans and walnuts in foods when this buffer is used. Of potential clinical importance, a lack of solubility in PBS or DI water may be an important factor in the performance of commercial in vitro specific IgE assays and skin test reagents. Qualitatively, BSB and NaOH were judged to provide representative polypeptide profiles for a targeted seed. Although NaOH is an efficient protein solubilizer, the accompanying high pH (usually >10) and NaOH molarity used may irreversibly denature certain proteins. In addition, alkali pH may also cause deamidation of glutamine and asparagine side chains and breakage of scissile bonds (e.g., ionic interactions), thereby causing undesirable/unacceptable alterations in the native protein properties. Exposure to extreme pH conditions has been demonstrated to result in substantial loss of almond major storage protein antigenicity (*51*). Potential loss of clinically important IgE-reactive epitopes as a consequence of protein insolubility, protein modification, or both may limit the utility of such protein extracts. As BSB-extracted proteins seemed to be representative of nut seed protein profiles, BSB was judged as the optimum solvent for routine protein extractions.

When SDS-PAGE gels were first silver stained followed by restaining of the same gels with CBBR staining (after clearing the silver stain), no qualitative difference in polypeptide profile was observed (**Figure 8**). These results suggest that either staining procedure may be effectively used to investigate protein polypeptide composition.

Glycoprotein Analysis. Glycoprotein staining of SDS-PAGE-separated polypeptides (**Figure 9**) indicated the presence of glycopeptides in certain seed proteins. With the exception of pecan and walnut, the majority of glycoprotein positive polypep-

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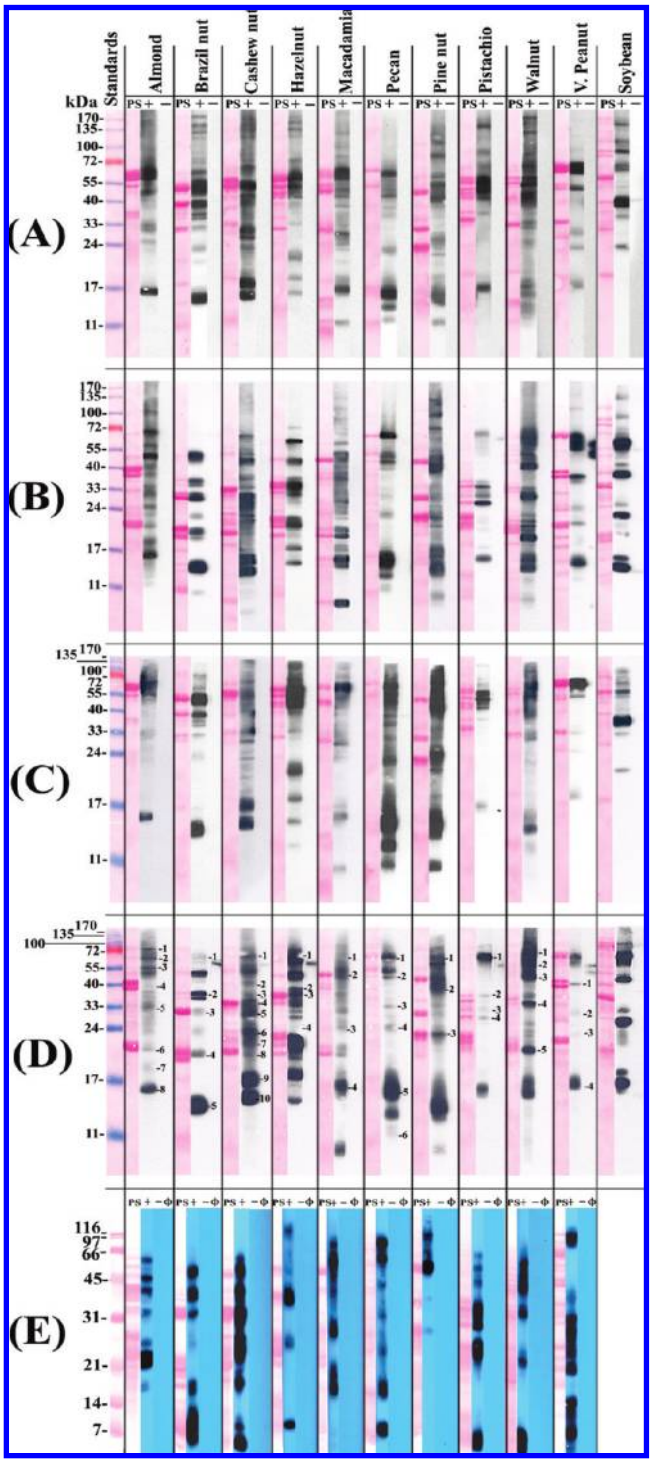

Figure 10. Western blotting for tree nut extracts probed with corresponding rabbit antiserum (**A**−**D**) and human sera (**E**) in the absence (**A**, **C**) and presence (**B**, **D**, **E**) of 2% (v/v) β-ME using 8−25% linear gradient (**A**, **B**), 15% (**C**, **D**), and 12% (**E**) monomer acrylamide gels. Protein load, except for marker proteins, in each lane was 30 μg. PS, Ponceau S stained; +, probed with corresponding rabbit pAb (**A**−**D**) or serum IgE (**E**); −, negative control; Φ, probed with secondary antibody only. The numbers on the right of the tracks in **D** are the polypeptides recognized by corresponding rabbit pAb and IgE. Molecular mass of marker proteins is indicated in the left margin (kDa). Estimated molecular mass of polypeptides identified by rabbit pAb and IgE in the targeted nut seed protein extracts are summarized in **Table 1**.

Table 1. Estimated Molecular Mass of Tree Nut Protein Polypeptides Recognized by Pooled Human Sera IgE and Rabbit Anti-Nut Seed Protein pAbs

	polypeptides (kDa) corresponding to **Figure 10D**									
seed	1	2	3	4	5	6	7	8	9	10
almond	68.5	62.4	56.8	46.1	37.8	23.9	19.2	15.4		
Brazil nut	63.7	42.8	35.5	22.4	13.0					
cashew nut	63.7	48.1	42.8	39.4	34.8	28.8	25.7	22.4	17.1	14.0
hazelnut	63.7	44.7	42.0	24.9						
macadamia	63.7	53.3	29.4	15.7						
pecan	63.7	50.6	37.4	30.0	15.1	9.7				
pine nut	65.0	45.6	27.6							
pistachio	63.7	42.0	35.9	33.4						
walnut	65.7	58.6	50.6	38.2	23.4					
Virginia peanut	47.1	34.8	27.6	16.4						

tides stained weakly. Further studies are warranted to determine the identity, the sugar type(s), and the number of sugar residues involved in protein glycosylation.

Western Blotting. Western blotting experiments (**Figure 10**) indicated that there was a strong correspondence between the nut seed protein polypeptides recognized by rabbit IgG pAbs and those recognized by patient sera IgE. The patient IgE immunoblots probably do not represent the full spectrum of IgE reactivity that can occur, as pools of sera from two to three patients reporting allergy to each nut seed were used. Such correspondence between rabbit pAb and patient sera IgE reactivity for almond (*52*) protein polypeptides has been reported earlier. The polypeptides recognized by both rabbit pAb and allergic patient human sera are identified in **Table 1**. The molecular mass of these polypeptides was measured by plotting standard protein relative mobility (R_f) versus $\log_{10}$ molecular mass ($r = 0.973$ and 0.986 for pAb and human IgE blot, respectively). Note the molecular mass shown in **Table 1** may not match exactly with the corresponding standard protein in **Figure 10D** due to the differences in relative mobility of the polypeptide depending on the acrylamide concentration in the SDS-PAGE. However, the majority of polypeptides recognizing human sera IgE were also recognized by rabbit pAb raised against respective nut seed proteins. The results suggest that rabbit pAbs may be used to study tree nut proteins described in the current investigation.

Conclusions. In summary, pecan proteins were the least soluble seed proteins in the tested aqueous buffers. Among the solvents tested, BSB efficiently solubilized nut seed proteins. However, 0.1 M aqueous NaOH was the best solvent for pecan and walnut, but due to concerns over protein stability in this highly basic solution, BSB is also the recommended solvent of choice for these two tree nuts. Aqueous EtOH was the least efficient in solubilizing nut seed proteins. Total soluble proteins were typically overestimated by the Lowry et al. and Bradford procedures as compared to conventional micro-Kjeldahl nitrogen determination method. PBS, a commonly used mild buffer for protein solubilization, may not be an adequate solvent for effective solubilization of pecan and walnut seed proteins. High salt concentration (4 M) significantly increased pecan and walnut protein solubility.

Rabbit IgG pAbs recognized many of the same polypeptides in a tree nut protein extract that were recognized by pooled patient sera IgE.

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Received May 15, 2009. Revised manuscript received July 10, 2009. Accepted July 19, 2009. Financial support from the Department of Nutrition, Food and Exercise Sciences, Florida State University; Almond Board of California, Modesto, CA; and the USDA NRICGP (9901530, 2003-01212, 2004-35503-14117, and 135000 520 019281) is gratefully acknowledged.

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