JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antigenic Stability of Pecan [*Carya illinoinensis* (Wangenh.) K. Koch] Proteins: Effects of Thermal Treatments and in Vitro Digestion

MAHESH VENKATACHALAM,[†,‡] SUZANNE S. TEUBER,[§] W. RICH PETERSON,[§] KENNETH H. ROUX,[#] AND SHRIDHAR K. SATHE*[,†]

Department of Nutrition, Food and Exercise Sciences and Department of Biological Science, Florida State University, Tallahassee, Florida 32306, and Division of Rheumatology, Allergy and Clinical Immunology, University of California, Davis, California 95616

Rabbit polyclonal antibody-based inhibition ELISA as well as immunoblotting analyses of proteins extracted from variously processed pecans (cv. Desirable) indicate that pecan proteins are antigenically stable. Pecan antigens were more sensitive to moist heat than dry heat processing treatments. SDS-PAGE and immunoblotting analysis of the native and heat-denatured proteins that were previously subjected to in vitro simulated gastric fluid digestions indicate that stable antigenic peptides were produced. Both enzyme-to-substrate ratio and digestion time were influential in determining the stability of pecan polypeptides. The stable antigenic polypeptides may serve as useful markers in developing assays suitable for the detection of trace amounts of pecans in foods.

KEYWORDS: Pecans; proteins; inhibition ELISA; Western blotting; antigenic stability; digestibility

INTRODUCTION

Pecan is an economically important agricultural crop for the United States. The U.S. produces >80% of the world's pecans. In 2004, 186 million pounds of pecans valued at U.S. $327 million were produced in the United States (*1*). Pecans belong to the Juglandaceae family, which also includes other tree nuts such as walnuts, hickory nuts, heartnuts, and butternuts (*2*). Pecans enjoy ready consumer acceptance due their sweet flavor and crunchy texture. Pecans are popular as a snack (raw/roasted/salted) and are also commonly used in various food products including the widely enjoyed pecan pie, several baked goods, candy and confectioneries, toppings on desserts, in salads, and several main dishes.

Although enjoyed safely by millions of consumers, tree nuts represent a major class of food allergens. A random digit dial telephone survey indicated that ∼1.2% of Americans suffer from peanut or tree nut allergies (*3*). The survey also reported a disturbing 2-fold increase in peanut allergies in children when compared with the rate of peanut allergies reported in an earlier 1997 study (*4*). Although several studies have documented the antigenic nature of various food allergens, including tree nuts, there is a gap in research on pecan proteins in general and especially on those specific pecan proteins responsible for human allergies in particular (*5*).

For a food protein, native or denatured, to retain its allergenicity, the specific structures of the proteins to which the human IgE antibody is directed (i.e., the structural and linear epitopes) must survive food-processing treatments and in vivo digestion. A number of food allergens are known to be generally stable toward food-processing treatments (particularly heat processing) as well as the proteolytic enzymes encountered in the digestive tract in vivo (*6*, *7*). Ideally, double-blind placebo-controlled food challenges (DBPCFC) in humans should be conducted to assess the retention of allergenicity (whether toward food processing or enzymatic digestion). Because DBPCFC studies involve certain risks and are often time-consuming and expensive, alternative in vitro diagnostic methods are often substituted for such purposes.

A number of food allergens were shown to be stable to in vitro proteolysis when tested under simulated gastric fluid (SGF) conditions (*8*, *9*). For example, Astwood et al. (*8*) reported that soybean allergen β-conglycinin remained stable for at least 60 min under in vitro SGF conditions, whereas nonallergenic proteins, such as spinach ribulose bisphosphate carboxylase/oxygenase, were digested in SGF within 15 s under identical conditions. Astwood et al. (*8*) therefore suggested that "... the stability to digestion is a significant and valid parameter that distinguishes food allergens from nonallergens". Subsequent research (*10*−*13*) has yielded contradictory findings. The relevance of simulated digestion studies has therefore been questioned and discussed by Fu et al. (*11*) and Taylor (*13*). Some of the perceived limitations of in vitro digestion studies include (1) the use of extreme pepsin/protein ratios (such as 12.5:1 w/w), which may not accurately represent the in vivo ratio in the human gastrointestinal tract; (2) the use of an insufficient range

* Address correspondence to this author at 402 Sandels Bldg., College of Human Sciences, Florida State University, Tallahassee, FL 32306-1493 [telephone (850) 644-5837; fax (850) 645-5000; e-mail ssathe@mailer.fsu.edu].
[†] Department of Nutition, Food and Exercise Sciences, Florida State University.
[‡] Present Address: Whistler Center for Carbohydrate Research, Department of Food Science, Purdue University, West Lafayette, IN 47907.
[§] University of California.
[#] Department of Biological Science, Florida State University.

10.1021/jf0520802 CCC: $33.50
Published on Web 01/26/2006

of conditions to accurately represent the possible in vivo digestion conditions; (3) failure to monitor the loss of solubility of the allergenic protein(s) as a consequence of processing treatment; (4) failure to use the most sensitive and suitable diagnostic systems to follow and evaluate proteolysis; and (5) failure to probe the digested proteins to reveal the presence or absence of relevant IgE-binding epitopes in the protein digests. Despite such pitfalls, in vitro proteolysis of a targeted protein can furnish useful preliminary information in the assessment of stability potential of food allergens, particularly if the above issues are addressed.

Like several other tree nuts, pecans are often subjected to heat-processing treatments, either before or after they are added to food, and such processing may potentially modify pecan protein structure and solubility. These processing-induced structural changes in proteins may lead to modifications in their reactivity toward agents designed to detect these proteins. Consequently, sensitive methods relying on detecting targeted pecan proteins in foods may be compromised if the detecting agents fail to recognize these targeted but processing-altered proteins. It was therefore important to assess the stability of pecan antigens subjected to thermal processing treatments and to in vitro simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) digestion conditions.

MATERIALS AND METHODS

Thermal Processing of Pecans and Preparation of Nut Protein Extracts. In-shell pecans (cv. Desirable, 2002 crop, a gift from Dr. Tommy Thompson, USDA-ARS Pecan Breeding and Genetics, Somerville, TX) were used in the present study. Pecan halves/fragments (~20 g for each treatment) were subjected to different thermal processing methods, and defatted pecan flours were prepared as described by Venkatachalam et al. (*14*). Protein extracts from defatted pecan flour samples were prepared using buffered saline borate (BSB) (flour-to-solvent ratio of 1:10 w/v) as described by Venkatachalam et al. (*14*). Unless otherwise specified, soluble proteins were estimated as per the method of Lowry et al. (*15*). Standard curves were simultaneously prepared in appropriate buffers using bovine serum albumin (BSA) as the standard protein (0−200 μg range).

Rabbit and Human Antisera. Rabbit polyclonal antibody (pAb) production and characterization were carried out as described previously by Acosta et al. (*16*) with suitable modifications. In the present study, New Zealand female white rabbits were immunized with proteins (500 μg total proteins/dose) extracted from unprocessed defatted pecan flour in BSB using RIBI adjuvant (reconstituted in saline) as per the manufacturer's (Corixa Corp., Hamilton, MT) recommendation. Human antisera were from a pool of three pecan-allergic patients (with history of anaphylaxis to pecan) selected for the ability to recognize (together) the range of high, intermediate, and low molecular weight pecan polypeptides (unpublished data).

Electrophoresis and Immunoblotting. SDS-PAGE in the presence of β-mercaptoethanol (β-ME) was done according to the method of Fling and Gregerson (*17*) as described by Sathe (*18*). Protein transfer onto NC paper and immunoblotting were done as described by Su et al. (*19*) with suitable modifications when human sera were used for immunoblots. Rabbit antiserum dilution in Tris-buffered saline [10 mM Tris, 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20) (TBS-T)] was 1:50000 (v/v). Western blots using human sera IgE were done using 15% acrylamide gels. The blot was cut into strips, and the strips were blocked for 1 h at room temperature in PBS (pH 7.4)−3% nonfat dry milk−0.2% Triton X-100 (the blocking buffer). The strips were incubated overnight at 4 °C in sera diluted (1:10, v/v) in the blocking buffer, washed four times, 15 min each, in PBS−0.1% Triton X-100, blocked for 30 min in PBS (7.4)−3% nonfat dry milk−0.1% Triton X-100, and incubated for 1 h at room temperature in a 1:50000 (v/v) dilution of HRP−mouse anti-human IgE (clone B3102E8, SouthernBiotech, Birmingham, AL) in the blocking buffer. The strips were washed three times, 10 min each, in PBS 0.1% Triton X-100, two times, 10 min each, in PBS, incubated with SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL), and exposed to Fuji Super RX X-ray film (Fuji, Stamford, CT). The films were developed on a Konica SRX-101A processor (Konica Minolta Medical Imaging, Wayne, NJ).

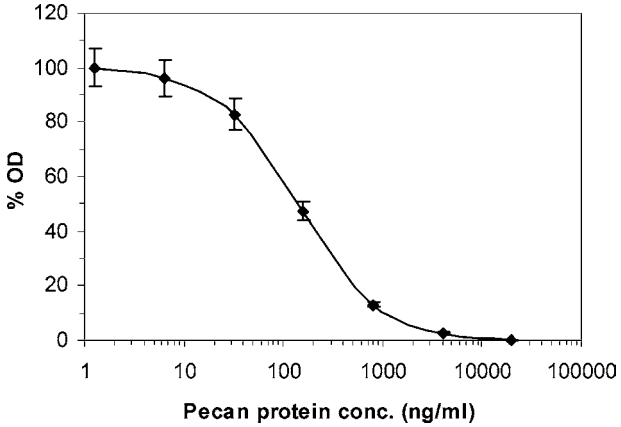

Figure 1. Inhibition ELISA standard curve for pecan protein detection using rabbit pAbs. Mean standard curve is based on 50 assays performed on different days. The IC_{50} (i.e., the amount of antigen needed to inhibit 50% of the OD signal) of the ELISAs is ~181 ng/mL. The detection range was 32–800 ng/mL. These values are based on estimation of total pecan proteins in BSB according to the method of Lowry et al. (*15*).

Competitive Inhibition ELISA. Competitive inhibition ELISA was developed and optimized as described by Acosta et al. (*16*). Microtiter plates were coated with 500 ng of BSB extracted pecan total proteins (in 50 μL)/well of microtiter plate for 1 h at 37 °C using BSB as the coating buffer. Coated plates were washed three times using BSB and then blocked by adding 100 μL of blocking buffer (0.5% BSA, 0.05% Tween-20, and 1 mM EDTA in PBS, pH 7.2). Pecan pAbs diluted 1:50000 v/v in 0.1% BSA−BSB were added to each well of a second uncoated plate. Standard pecan protein solution (unprocessed whole protein extract; 0.1 mg/mL) and processed sample inhibitory proteins (0.1 mg/mL) were serially diluted 5-fold into the successive wells of pecan pAbs containing plate and incubated for 1 h at 37 °C. Appropriate reagent blanks were included. Fifty microliters/well of the incubated solution was transferred to the coated plate and further incubated for 1 h at 37 °C. Plates were washed three times, color was developed using alkaline phosphatase labeled goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) and phosphatase substrate, and absorbance was read at 405 nm as described by Su et al. (*19*).

Protein in Vitro Digestibility and Determination of Antigen Stability. For simulated digestibility studies, total pecan proteins were extracted from defatted flour using BSB buffer. Stock solutions of 2 mg of pecan proteins/mL were prepared and used for all digestion experiments. To evaluate the influence of moist heat on in vitro digestibility of pecan proteins, protein samples were suitably diluted in distilled water and heated in microcentrifuge tubes in a boiling water bath (95 °C) for 30 min, cooled to room temperature, and then used for digestion. SGF and SIF protein digestion protocols were as described in U.S. Pharmacopoeia (*20*).

Simulated Gastric Fluid Digestion. SGF contained 0.32% w/v porcine pepsin (Sigma P6887, 3460 units/mg of solid) in 34 mM NaCl, 0.7% HCl, at pH 1.2. Final digestion conditions were as follows: 72 μg of pecan proteins per 225 μL of SGF; various enzyme concentrations to attain pepsin/protein ratios of 10:1, 1:1, 1:10, 1:25, 1:100, 1:500, and 1:1000, w/w; incubation temperature, 37 °C; and 0−4 h incubation times. Pepsin activity was stopped by adding 10 μL of 2 M NaOH followed by 125 μL of SDS-PAGE sample buffer (with 2% v/v β-ME) to the sample and heating the sample for 10 min in a boiling water bath.

Simulated Intestinal Fluid Digestion. SIF contained 10 mg/mL pancreatin (1 × USP, Sigma) in 50 mM potassium phosphate buffer, pH 7.5. Final digestion conditions were as follows: 100 μg of pecan

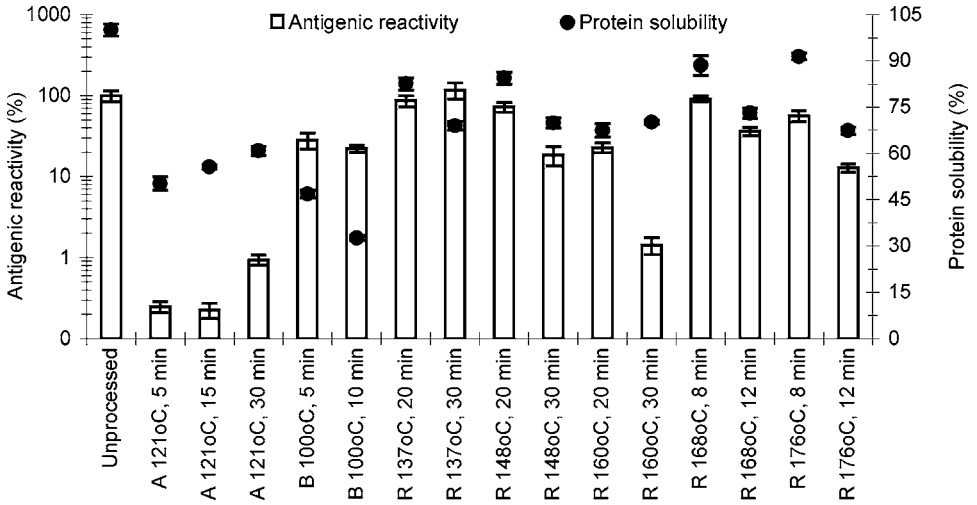

Figure 2. Effect of thermal processing on protein solubility and antigenic stability. Processing conditions: A, autoclaved; B, blanched; R, dry roasted, with temperature (°C) and time (min) of processing indicated. All data shown are mean ± SEM ($n = 8$). Protein solubility of processed samples is expressed as percent protein solubilized in BSB as compared to unprocessed control (100%). Antigenic reactivity (percent) of the processed samples is calculated as IC_{50} of unprocessed sample × 100/IC_{50} of processed sample and compared to unprocessed control (100% reactivity). Least significant difference (LSD) ($p = 0.05$, $n = 8$): differences between two means exceeding LSD value are significant. LSD values for antigenic reactivity = 37.61 and loss in protein solubility = 4.82.

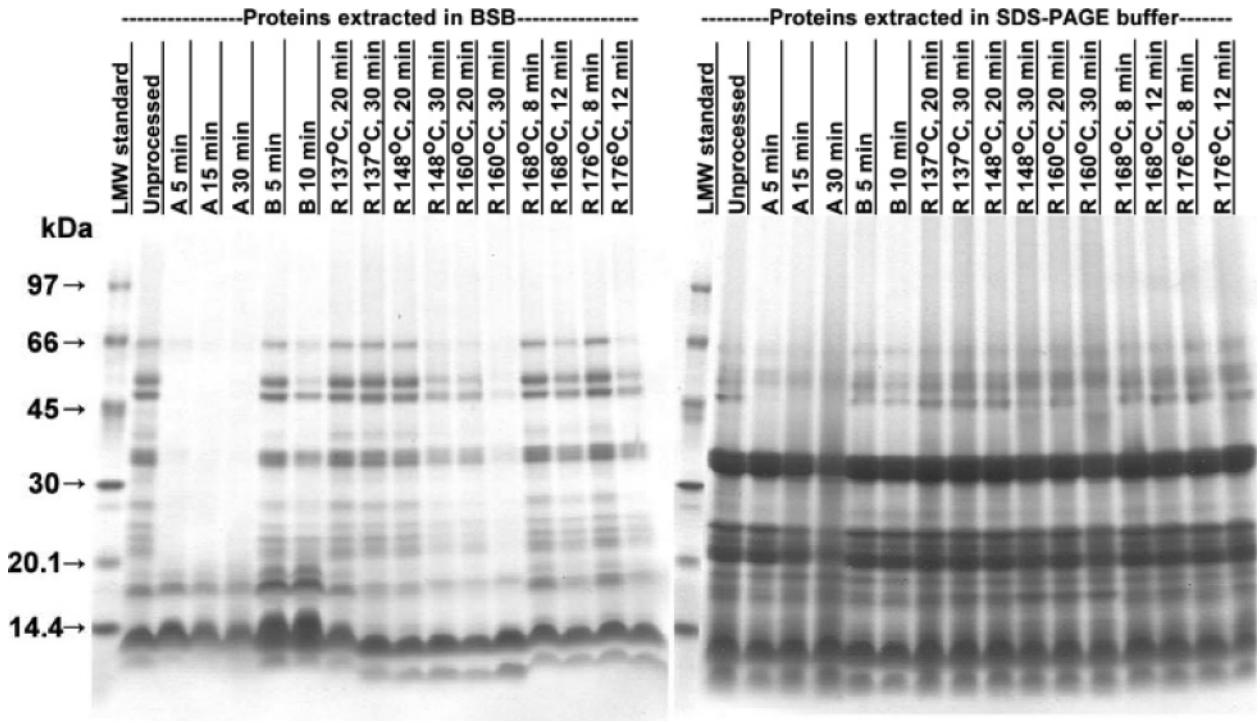

Figure 3. SDS-PAGE (with 2% β-ME) profiles for unprocessed and processed pecan proteins. Protein load for samples extracted in BSB in each lane was 30 μg. For proteins extracted in SDS-PAGE sample buffer, 4 μL of supernatant was loaded per lane. A, autoclaved; B, blanched; R, dry roasted.

proteins per 200 μL of intestinal solution; various enzyme concentrations to get pancreatin/protein ratios of 10:1, 1:1, 1:10, and 1:100, w/w; incubation temperature of 37 °C; and 0−4 h incubation times. Enzyme activity was terminated by the addition of 100 μL of SDS-PAGE sample buffer containing (2% v/v β-ME) and heating in a boiling water bath for 10 min. Appropriate substrate and enzyme blanks were included in all experiments.

Data Analysis and Statistical Procedures. All statistical analyses were performed using SPSS statistical software (version 10; Chicago, IL). All experiments were carried out at least in duplicate, and data are expressed as the mean ± SEM. One-way ANOVA and Fisher's least significant difference (LSD) test as described by Ott (*21*) were used to determine statistical significance ($p = 0.05$).

RESULTS AND DISCUSSION

Protein Detection Using ELISA and Immunoblotting. The Desirable pecan cultivar used in this study is a crop mainly from the southeastern United States and is gaining a premium market mainly due to the large size of the kernel, the ease of shelling, and its pest-resistant properties (*22*; personal communication with Dr. T. Thompson). The competitive inhibition ELISA could detect pecan proteins in the range of 32−800 ng/mL with an average IC_{50} value (inhibitor concentration required to inhibit 50% signal in ELISA) of 181 ng/mL (**Figure 1**). Immunoblotting of native extracted proteins using the anti-pecan

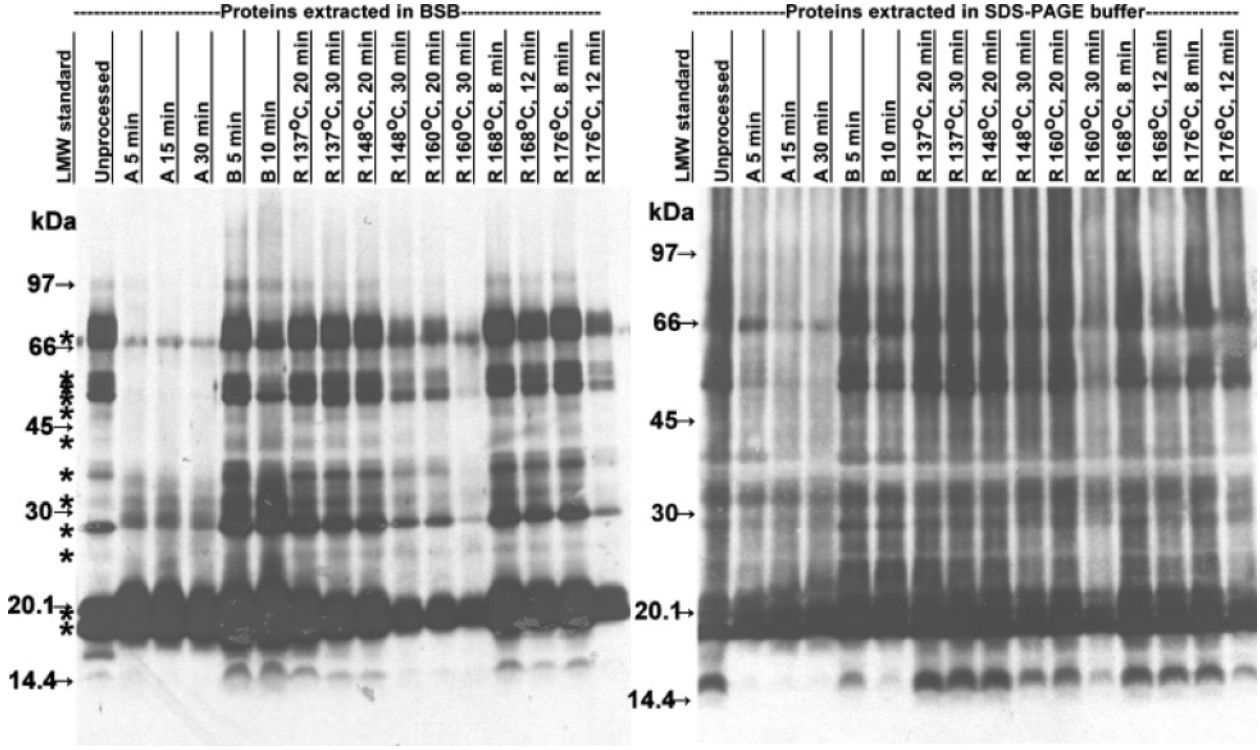

Figure 4. Immunoblots for unprocessed and processed pecan proteins probed with anti-pecan rabbit pAb. Samples were electrophoresed on SDS-PAGE (with 2% β-ME) gels. A, autoclaved; B, blanched; R, dry roasted. Asterisks (*) indicate pecan polypeptide(s) recognized by sera IgE from patients allergic to pecans. Protein load in each lane was 15 μg.

rabbit pAbs revealed several polypeptides (molecular masses ranging from 10 to 120 kDa) including some that were also detected by sera IgE (indicated by asterisks in **Figure 4**) from patients known to be allergic to pecans (**Figure 5**). Furthermore, similar immunoblotting profiles of several commercially important pecan varieties screened using the anti-pecan rabbit pAbs ensured that the pecan pAbs used in the present study were suitable for the detection of pecan polypeptides in most or all commercial pecan cultivars (data not shown).

Protein Solubility. The effect of heat processing on pecan protein solubility (in BSB buffer) is shown in **Figure 2**. Heat-processing treatments often cause protein denaturation that can lead to loss in protein solubility. As expected, the loss in protein solubility (0−67%) after processing was dependent on the severity and duration of processing. A decrease in pecan protein solubility was consistent with the results of several recent studies on tree nut and peanut subjected to various thermal processing treatments (*14*, *19*, *23*−*25*). Pecan proteins seemed to be particularly sensitive to moist heat as compared to dry heat processing. Among the moist heat treatments, blanching treatment (5 and 10 min) showed higher loss in protein solubility (53−67%) as compared to that for the autoclave treatment (39−50%). This result was unexpected as blanching is generally considered to be a milder form of heat processing than autoclaving and roasting.

Assessment of Antigenic Stability of Pecan Proteins by Competitive Inhibition ELISA. To compensate for the decrease in protein solubility, all protein extracts were normalized to 1 mg/mL in BSB prior to use in ELISA assays. The loss in antigenic reactivity of the processed samples in ELISA assays typically was not significantly different when compared with the unprocessed control with the exception of all autoclaved samples and one roasted (160 °C, 30 min) sample (**Figure 2**). It is important to note that the pecans subjected to extreme processing conditions such as autoclaving (121 °C for 5, 15, and 30 min) and roasting (160 °C for 20 and 30 min) resulted in a dark unappealing external appearance, and these extreme conditions are unlikely to be used in commercial pecan processing. Pecan protein reactivity, just as protein solubility, was more sensitive to moist heat than dry heat processing (**Figure 2**). Losses in protein solubility were 50, 44, and 39%, respectively, for pecans subjected to autoclaving for 5, 15, and 30 min, with the corresponding loss in antigenic reactivity being ∼99% for all three processed samples. Blanching for 5 and 10 min, on the other hand, resulted in 53 and 67% losses in protein solubility with the corresponding losses in antigenic reactivity of 72 and 78%. Unlike autoclaving, when proteins are likely to remain inside the seed during the treatment, proteins may leach

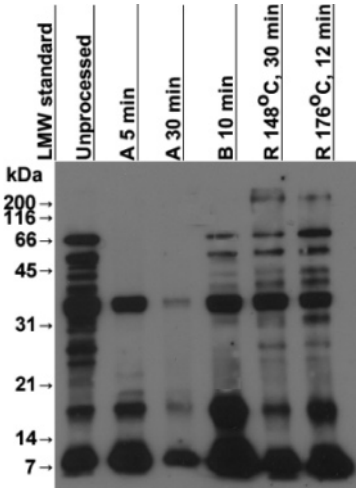

Figure 5. Immunoblots for unprocessed and processed pecan proteins probed with pooled sera IgE from patients allergic to pecans. Samples were electrophoresed on SDS-PAGE (with 2% β-ME) gels. A, autoclaved; B, blanched; R, dry roasted. Protein load in each lane was 10 μg.

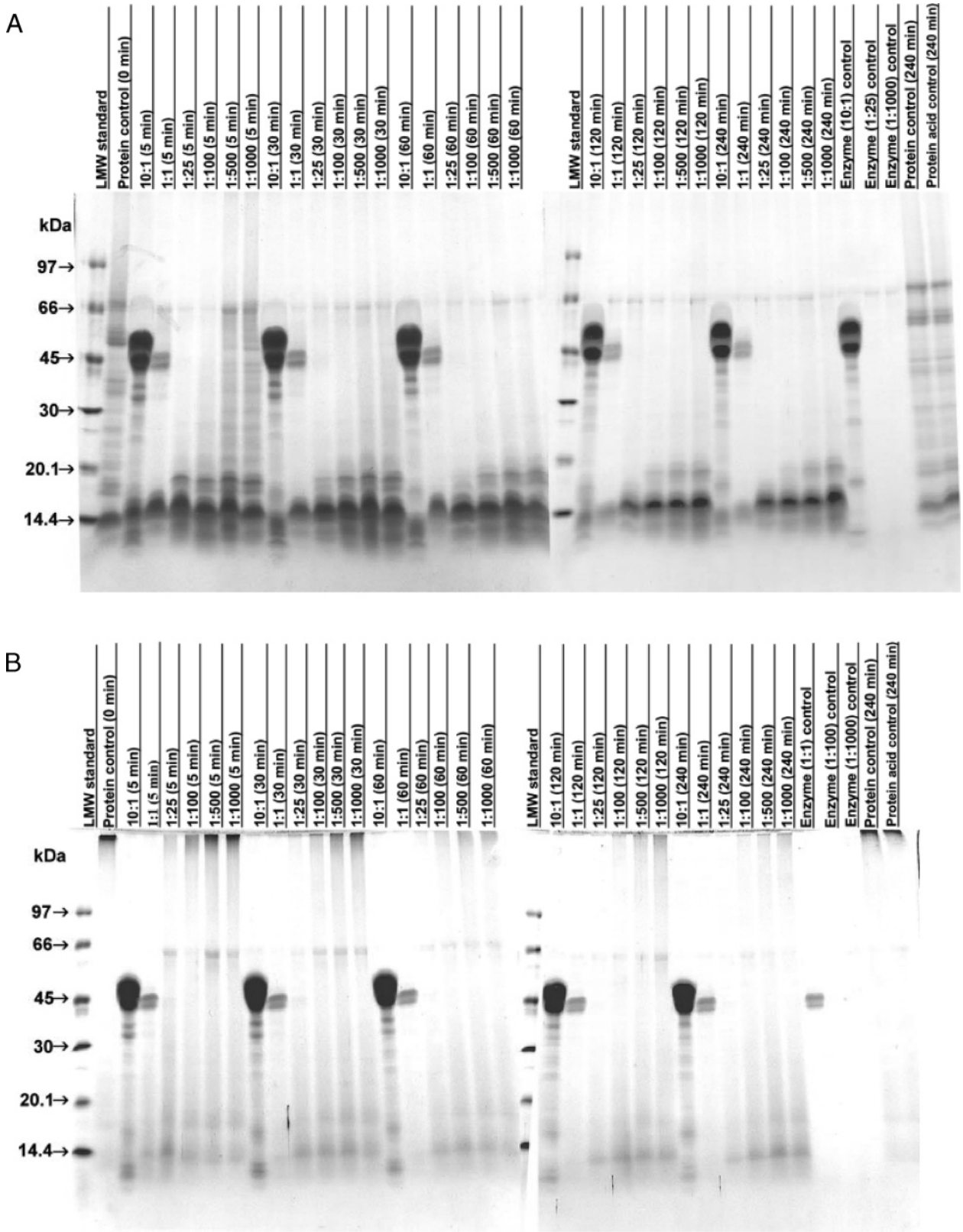

Figure 6. SDS-PAGE (with 2% β-ME) analysis of (**A**) native and (**B**) heat-denatured pecan proteins digested in SGF (pepsin). Pecan protein load in each lane was 15 μg.

out in the processing water during blanching. If water-soluble proteins were to account for the bulk of pecan protein antigenicity, the protein loss in water (used for blanching) due to leaching can be expected to result in a significant loss in the seed protein antigenic reactivity. However, on the basis of the antigenic reactivity values, the loss in reactivity upon autoclaving was much higher than the blanching process, thereby suggesting that the proteins lost during blanching did not account for a major portion of total seed protein antigenic reactivity. Antigen recognition by pAbs used in the ELISA may be affected not only by the loss of proteins in blanch water but also by the stability of relevant structural and linear epitopes in the proteins

that were retained within the seeds subjected to the stresses of processing conditions employed. For these reasons, although ELISA is useful in assessing the overall antigenicity of the total proteins in the seed, it does not allow assessment of stability of specific proteins (or polypeptides) because they may have been removed during processing.

Assessment of Antigenic Stability of Pecan Proteins by SDS-PAGE and Immunoblotting. Because normalizing samples (to be used in ELISA) for protein concentration does not ensure that variously processed pecan samples will have the same extracted polypeptide profiles, both SDS-PAGE and immunoblotting were used to qualitatively visualize and compare the processed samples with the unprocessed control. With few exceptions, the SDS-PAGE polypeptide profile of BSB extracted soluble proteins from processed samples was similar to that for the unprocessed control (**Figure 3**). The exceptions were the polypeptide profiles for samples subjected to autoclave (5, 15, and 30 min) and roasting (160 °C for 30 min), for which a significant difference in the extractable polypeptides, especially in the region of ~66−20 kDa, was seen. These results would suggest that excessive heat processing caused an irreversible protein denaturation or degradation resulting in significant loss of protein solubility. Interestingly, blanching did not cause a significant qualitative change in either SDS-PAGE (**Figure 3**) or immunoblotting (**Figure 4**) profiles compared with that of the unprocessed control, regardless of buffer used for protein extraction. These results are consistent with the ELISA results noted earlier and confirm that proteins lost during blanching treatment are not major contributors (at least qualitatively) to the total antigenicity of pecan proteins.

When proteins were extracted in denaturing/reducing buffer (SDS-PAGE sample buffer with 2% v/v β-ME), polypeptide profiles of all samples qualitatively matched closely (with minor differences). Immunoblotting analysis of these samples using rabbit and human antisera was performed to qualitatively assess the antigen stability of processed samples with the unprocessed control (**Figures 4** and **5**). Overall, processed samples probed with rabbit pAbs registered polypeptide profiles similar to the one for the control, albeit with decreased intensity (but not a complete loss of recognition) in the banding pattern (66−25 and 14.4 kDa) for the autoclaved samples and one roasted (160 °C, 30 min) sample. The decrease observed in these bands in immunoblotting is likely due to irreversible loss of protein solubility rather than protein epitope (especially the linear ones) destruction. Immunoblotting analysis of select processed samples probed using sera IgE compared similarly to that of samples probed by rabbit pAbs, thereby confirming that pecan antigens are thermally stable (**Figure 5**). These results are consistent with the antigenic stability of almond (*14*, *23*), cashew nut (*24*), and walnut (*19*) proteins in processed samples. The results also suggest that polypeptide(s) in the molecular mass range of 20−66 kDa appear to be sensitive to thermal processing, depending on the severity of the treatment. The variable effect of thermal processing on the same set of proteins has been noted by other researchers as well. For example, using human serum IgE binding inhibition assay, Monduelet et al. (*26*) have recently shown that the IgE binding capacity of protein extracts from boiled peanuts was half that of the extracts prepared from raw or roasted peanuts. No significant difference was noted between the protein extracts prepared from raw and roasted peanuts. The two major allergenic peanut proteins, Ara h 1 and Ara h 2, when purified from roasted peanuts, showed higher IgE reactivity than the corresponding counterparts purified from either raw or boiled peanuts. The authors concluded that raw and roasted peanuts did not differ in their allergenicity, whereas the allergenicity reduction in boiled peanuts was attributable to the loss of low molecular weight soluble allergens in the cooking water. Impaired protein extraction efficiency due to either the loss in protein solubility or the use of suboptimal extraction buffer may significantly hamper efforts to monitor trace quantities of offending agents in foods. A recent study (*25*) on peanut protein solubility loss of 50−80% upon roasting is illustrative in this regard. These investigators compared 17 different in-house and commercial buffers for protein solubilization efficiency in the pH range of 3−11. These investigators reported that not only the type of buffer but the buffer pH was also an important factor in determining protein solubilization efficiency as demonstrated by the fact that TBS buffer (20 mM Tris and 150 mM NaCl) at pH 8.2 extracted ~35% more peanut proteins than the same buffer at pH 7.4.

Unlike the loss of low molecular mass allergenic proteins in peanuts (*26*), low molecular mass proteins (16−20 kDa range) in pecans were antigenically stable toward all processing treatments. These results suggest that the 16−20 kDa polypeptides recognized by pecan pAbs may serve as excellent marker polypeptides for the development of immunoassays designed to detect trace quantities of pecans in foods.

Pecan Protein in Vitro Digestibility and Determination of Antigen Stability. Native and heat-denatured pecan proteins were readily (within 5 min.) digested by pepsin at pH 1.2 and 37 °C when pepsin/protein ratios were between 10:1 and 1:100, w/w, and incubation times were up to 4 h (**Figure 6**). However, at lower pepsin concentrations (pepsin/protein ratios of 1:500 and 1:1000, w/w) under the same incubation conditions, significant proteolysis occurred only after 30 min of incubation, especially for the native proteins. A similar trend was observed for heat-denatured proteins with one major difference. Heat-denatured proteins were hydrolyzed at a faster rate, judged qualitatively on the basis of band intensity and width, as indicated by the disappearance of the low molecular mass (10−20 kDa) polypeptides range (compare corresponding lanes in **Figure 6B** with those in **Figure 6A**), a result that was expected because heat denaturation generally improves protein digestibility. The ease of pecan protein digestion observed in the current investigation is consistent with the high in vitro pepsin digestibility of almond (*18*), cashew nut (*27*), and walnut (*28*) proteins. Compared to pepsin, pancreatin enzymes were less effective in hydrolyzing pecan proteins (**Figure 8**), an expected result because pepsin has a much broader specificity when compared with the specificity of pancreatic proteolytic enzymes.

Immunoblotting profiles of SGF-digested pecan proteins, both native and heat-denatured, displayed several low molecular mass (~16−20 and 28 kDa) antigenic peptides that persisted throughout the 4 h of digestion used in the present study (**Figure 7**). At a pepsin-to-protein ratio of 10:1, w/w, almost complete loss of antigenic peptides was observed within a short time (5 min). However, such loss was not evident at other pepsin/protein ratios investigated, suggesting that unless there is an overwhelming amount of pepsin produced in response to incoming substrate protein, some of the antigens may escape pepsin action. To ascertain whether the stable antigenic peptides were relevant to pecan allergies, select protein digests were also probed with patient sera IgE (**Figure 10**). Of particular note was the observation that at a pepsin/protein ratio of 1:1, w/w, residual antigenic peptides could be detected by patient sera IgE after 60 min of digestion and by pecan pAbs even after 4 h of digestion (**Figures 7** and **10A**). If gastric emptying takes place in ≤4 h, such stable peptides may escape gastric digestion and

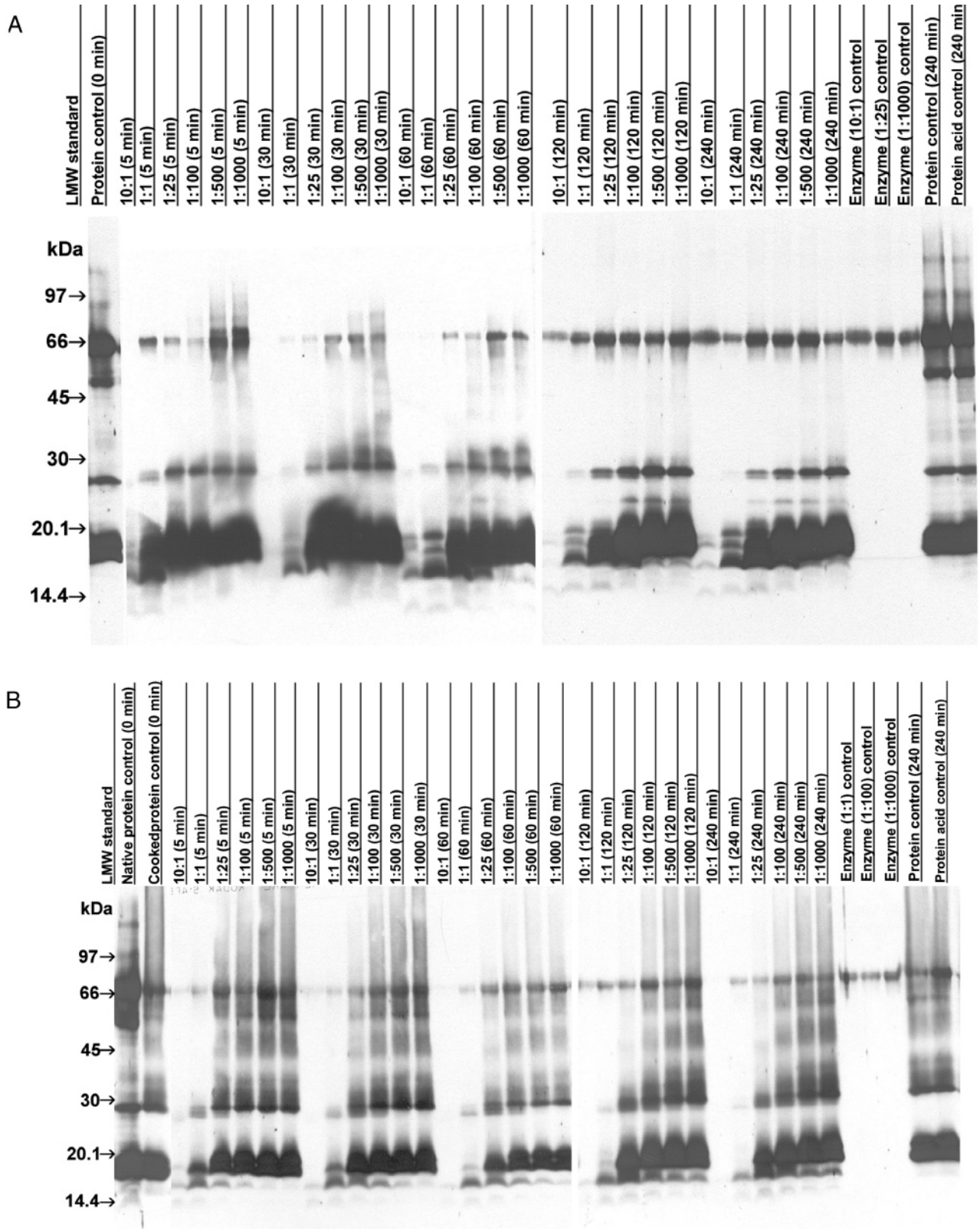

Figure 7. Immunoblots for (**A**) native and (**B**) heat-denatured pecan proteins digested in SGF (pepsin) probed with anti-pecan rabbit pAb. Samples were electrophoresed on SDS-PAGE (with 2% β-ME) gels. Pecan protein load in each lane was 10 μg.

may therefore be able to trigger allergenic response. Although these results do not reveal whether the remaining antigenic polypeptides were a result of incomplete proteolysis of the original substrate proteins or were newly formed antigenic polypeptides that were not present at the beginning of the substrate digestion, they do indicate that unless a substantial quantity of pepsin (in relation to substrate proteins) is present, complete digestion of antigenic polypeptides may not be feasible by pepsin alone. Results of separate SIF digestions and subsequent immunoblots of the digests (**Figures 9** and **10B**) further demonstrated that pancreatin enzymes were less effective than pepsin in digesting the antigenic peptides. Tagaki et al. (*29*) similarly reported that the rate of digestion of chicken egg white ovomucoid (OVM), an allergenic protein, depended on

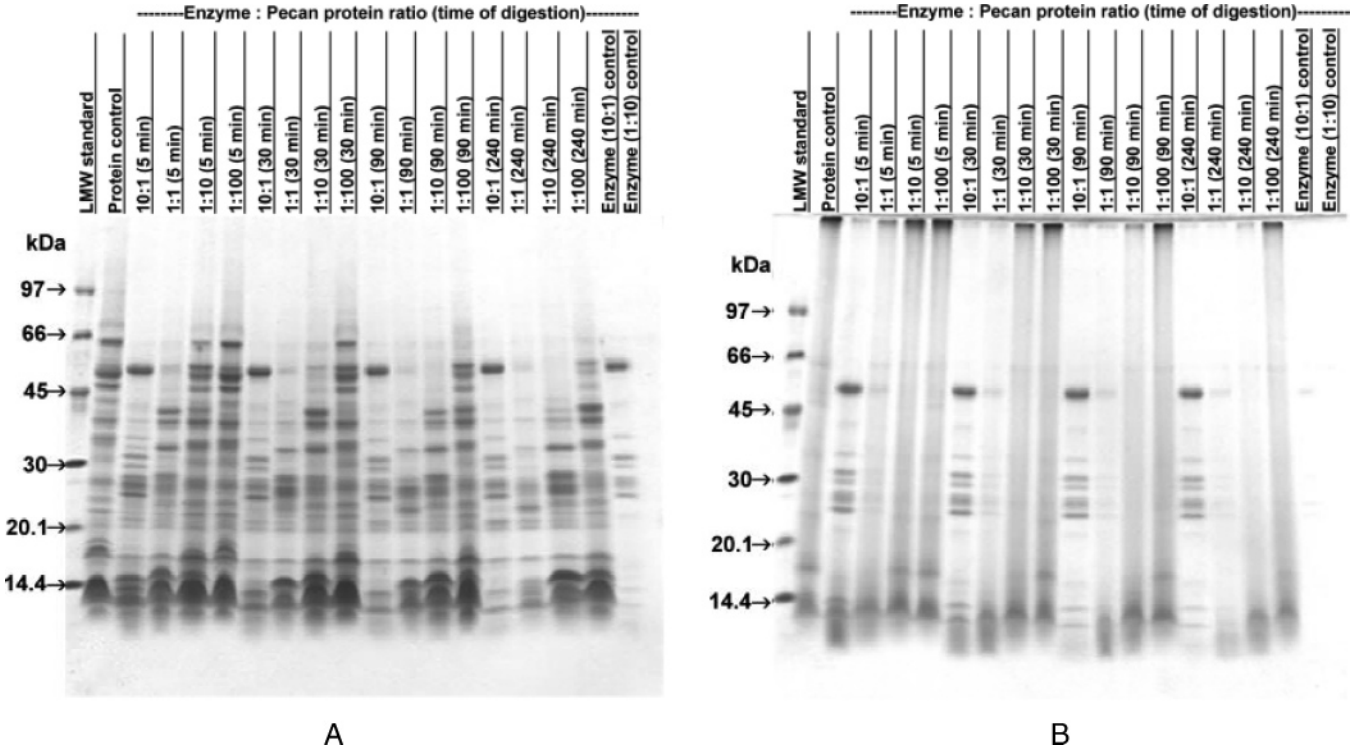

Figure 8. SDS-PAGE (with 2% β-ME) analysis of (**A**) native and (**B**) heat-denatured pecan proteins digested in SIF (pancreatin). Pecan protein load in each lane was 25 μg.

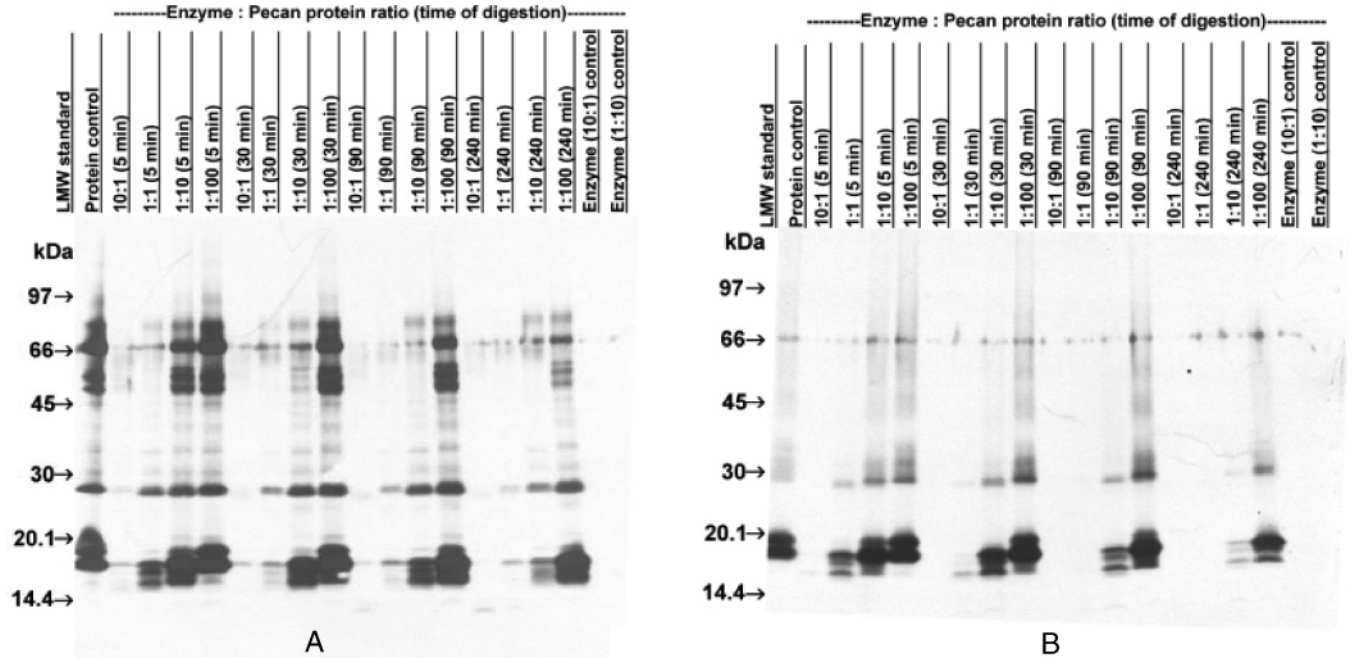

Figure 9. Immunoblots for (**A**) native and (**B**) heat-denatured pecan proteins digested in SIF (pancreatin) probed with anti-pecan rabbit pAb. Samples were electrophoresed on SDS-PAGE (with 2% β-ME) gels. Pecan protein load in each lane was 10 μg.

the pepsin-to-OVM ratio and that several allergenic peptides [93% of the OVM-reactive sera ($n = 24$) bind 23.5−28.5-kDa fragments, 21% react with the smaller 7- and 4.5-kDa fragments] persisted after SGF digestion.

Allergens belonging to the 2S albumin proteins class are believed to be resistant to proteolysis (*30*, *31*). Whether the stable low molecular mass antigenic peptides (16−20 kDa) observed in the immunoblots in the present investigation are 2S albumins in pecans or simply polypeptides generated during proteolysis with molecular masses similar to those of the 2S albumins remains to be determined. Nonetheless, these polypeptides may be useful stable markers in the assessment of the pecan protein stability. It is important to note that the pH of the gastric fluid can change after consumption of foods and thereby affect pepsin activity. We have observed that the change in pH of SGF strongly influences in vitro assessment of the antigenic stability of several tree nut allergens (unpublished data). It is therefore desirable that caution be exercised when the utility of in vitro models employing simulated gastrointestinal systems is investigated for the purpose of predicting protein allergenicity.

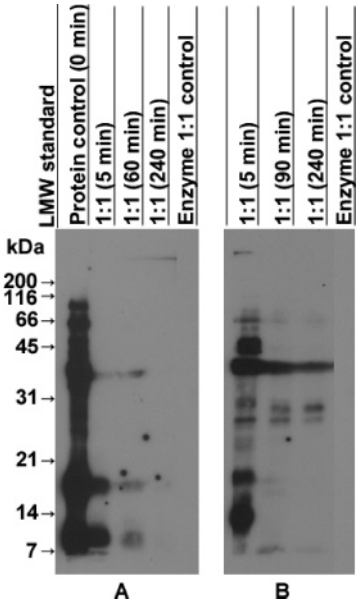

Figure 10. Immunoblots for native pecan proteins digested in (**A**) SGF and (**B**) SIF probed with pooled sera IgE from patients allergic to pecans. Samples were electrophoresed on SDS-PAGE (with 2% β-ME) gels. Pecan protein load in each lane was 10 μg.

Conclusions. Pecan proteins were thermally stable. Both protein solubility and reactivity in inhibition ELISA revealed that pecan proteins were more sensitive to moist heat than dry heat treatments. However, protein solubility and antigenic reactivity were not directly correlated, suggesting that loss in protein solubility during the extraction and testing phase alone may not be always reliably and predictably related to the loss in antigenicity. Also, caution has to be exercised when the results of loss in protein solubility and/or antigenic reactivity of some extreme processing conditions are interpreted with respect to their antigenic potential.

Native and heat-denatured pecan proteins were readily hydrolyzed by pepsin and pancreatin enzymes. However, complete proteolysis and loss of antigenicity were not observed under all digestion conditions. Factors including enzyme-to-protein ratio and digestion times were important in the determination of the antigenic stability of pecan polypeptides.

LITERATURE CITED

(1) USDA. *Fruit and Tree Nuts Situation and Outlook Yearbook*; Market and Trade Economics Division, Economic Research Service, U.S. Department of Agriculture: Washington, DC, Oct 2005.

(2) Rosengarten, F. R. *The Book of Edible Nuts*; Walker: New York, 1984.

(3) Sicherer, S. H.; Munoz-Furlong, A.; Sampson, H. A. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J. Allergy Clin. Immunol.* **2003**, *112*, 1203−1207.

(4) Sicherer, S. H.; Munoz-Furlong, A.; Burks, A. W.; Sampson, H. A. Prevalence of peanut and tree nut allergy in the United States of America determined by a random digit dial telephone survey. *J. Allergy Clin. Immunol.* **1999**, *103*, 559−562.

(5) Roux, K. H.; Teuber, S. S.; Sathe, S. K. Tree nut allergens. *Int. Arch. Allergy Immunol*. **2003**, *131*, 234−244.

(6) Sathe, S. K.; Teuber, S. S.; Roux, K. H. Effects of food processing on the stability of food allergens. *Biotechnol. Adv.* **2005**, *23*, 423−429.

(7) Taylor, S. L.; Lehrer, S. B. Principles and characteristics of food allergens. *Crit. Rev. Food Sci. Nutr*. **1996**, *36S*, S91−S118.

(8) Astwood, J. D.; Leach, J. N.; Fuchs, R. L. Stability of food allergens to digestion *in vitro*. *Nat. Biotechnol*. **1996**, *14*, 1269−1273.

(9) Besler, M.; Steinhut, H.; Paschke, A. Stability of food allergens and allergenicity of processed foods. *J. Chromatogr. B: Biomed. Sci. Appl*. **2001**, *756*, 207−228.

(10) Yagami, T.; Haishima, Y.; Nakamura, A.; Osuna, H.; Ikezawa, Z. Digestibility of allergens extracted from natural rubber latex and vegetable foods. *J. Allergy Clin. Immunol*. **2000**, *106*, 752−62.

(11) Fu, T. J.; Abbott, U. R.; Hatzos, C. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluids—a comparative study. *J. Agric. Food Chem.* **2002**, *50*, 7154−7160.

(12) Bannon, G.; Fu, T. J.; Kimber, I.; Hinton, D. M. Protein digestibility and relevance to allergenicity. *Environ. Health Perspect*. **2003**, *111*, 1122−1124.

(13) Taylor, S. L. Comment on digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid—a comparative study. *J. Agric. Food Chem.* **2003**, *13*, 51, 5183−5184; author reply, 5185−5187.

(14) Venkatachalam, M.; Teuber, S. S.; Roux, K. H.; Sathe, S. K. Effects of roasting, blanching, autoclaving, and microwave heating on antigenicity of almond (*Prunus dulcis* L.) proteins. *J. Agric. Food Chem.* **2002**, *50*, 3544−3548.

(15) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(16) Acosta, M. R.; Roux, K. H.; Teuber, S. S.; Sathe, S. K. Production and characterization of rabbit polyclonal antibodies to almond (*Prunus dulcis* L.) major storage protein. *J. Agric. Food Chem*. **1999**, *47*, 4053−4059.

(17) Fling, S. P.; Gregerson, D. S. Peptide and protein molecular weight determination by electrophoresis using a high-molarity tris buffer system without urea. *Anal. Biochem*. **1986**, *155*, 83−88.

(18) Sathe, S. K. Solubilization, electrophoretic characterization and *in vitro* digestibility of almond (*Prunus amygdalus*) proteins. *J. Food Biochem*. **1993**, *16*, 249−264.

(19) Su, M.; Venkatachalam, M.; Teuber, S. S.; Roux, K. H.; Sathe, S. K. Impact of γ-irradiation and thermal processing on the antigenicity of almond, cashew nut and walnut proteins. *J. Sci. Food Agric*. **2004**, *84*, 1119−1125.

(20) Simulated Gastric Fluid, TS. In *The United States Pharmacopoeia 23, The National Formulary 18*; United States Pharmacopoeial Convention: Rockville, MD, 1995; p 2053.

(21) Ott, L. *An Introduction to Statistical Methods and Data Analysis*; Duxbury Press, Wadsworth Publishing: Belmont, CA, 1977; pp 1−208.

(22) Worley, R. E. Pecan production. In *Pecan Technology*; Santerre, C. R., Ed.; Chapman and Hall: New York, 1994; pp 13−38.

(23) Roux, K. H.; Teuber, S. S.; Robotham, J. M.; Sathe, S. K. Detection and stability of the major almond allergen in foods. *J. Agric. Food Chem*. **2001**, *49*, 2131−2136.

(24) Wei, Y.; Sathe, S. K.; Teuber, S. S.; Roux, K. H. A sensitive sandwich ELISA for the detection of trace amounts of cashew (*Anacardium occidentale*) nut in foods. *J. Allergy Clin. Immunol.* **2002**, *109*, 928S.

(25) Poms, R. E.; Capelletti, C.; Anklam, E. Effect of roasting history and buffer composition on peanut protein extraction efficiency. *Mol. Nutr. Food Res*. **2004**, *48*, 459−464.

(26) Monduelet L.; Paty, E.; Drumare, M. F.; Ah-Leung, S.; Scheinmann, P.; Willemot, R. M.; Wal, J. M.; Bernard, H. Influence of thermal processing on the allergenicity of peanut proteins. *J. Agric. Food Chem.* **2005**, *53*, 4547−4553.

(27) Sathe, S. K.; Sze-Tao, K. W. C.; Wolf, W. J.; Hamaker, B. R. Biochemical characterization and in vitro digestibility of the major globulin in cashew nut (*Anacardium occidentale*). *J. Agric. Food Chem*. **1997**, *45*, 2854−2860.

(28) Sze-Tao, K. W. C.; Sathe, S. K. Walnuts (*Juglans regia* L): proximate composition, protein solubility, protein amino acid composition and protein *in vitro* digestibility. *J. Sci. Food Agric*. **2000**, *80*, 1393−1401.

(29) Takagi, K.; Teshima, R.; Okunuki, H.; Itoh, S.; Kawasaki, N.; Kawanishi, T.; Hayakawa, T.; Kohno, Y. Kinetic analysis of pepsin digestion of chicken egg white ovomucoid and allergenic potential of pepsin fragments. *Int. Arch. Allergy Immunol*. **2005**, *136*, 23−32.

(30) Moreno, F. J.; Mellon, F. A.; Wickham, M. S.; Bottrill, A. R.; Mills, E. N. Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant in vitro gastrointestinal digestion. *FEBS J*. **2005**, *272*, 341−52.

(31) Murtagh, G. J.; Archer, D. B.; Dumoulin, M.; Ridout, S.; Matthews, S.; Arshad, S. H.; Alcocer, M. J. C. In vitro stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8. *Clin. Exp. Allergy*. **2003**, *33*, 1147−1152.

Received for review August 23, 2005. Revised manuscript received December 2, 2005. Accepted December 16, 2005. This work was partly supported by the College of Human Sciences (CHS) Research Initiative Program; scholarships (M.V.), and the Council on Faculty Research (COFRs) at the Florida State University, Tallahassee, FL.

JF0520802