Production and Characterization of Rabbit Polyclonal Antibodies to Almond (*Prunus dulcis* L.) Major Storage Protein

Martin R. Acosta,[†] Kenneth H. Roux,[‡] Suzanne S. Teuber,[§] and Shridhar K. Sathe*,[†]

Department of Nutrition, Food, and Exercise Sciences, College of Human Sciences, and Department of Biological Science, Florida State University, Tallahassee, Florida 32306-1493, and Division of Rheumatology, Allergy and Clinical Immunology, University of California, Davis, Davis, California 95616

Rabbits were immunized with purified almond major protein (AMP), the primary storage protein in almonds. Rabbit anti-AMP polyclonal antibodies (PA) could detect AMP when as little as 1–10 ng/mL were used to coat microtiter plates in a noncompetitive enzyme linked–immunosorbent assay (ELISA). Competitive inhibition ELISA assays detected the AMP down to 300 ng/mL. PA recognized the AMP in protein extracts from all U. S. major marketing cultivars of almonds (Mission, Neplus, Peerless, Carmel, and Nonpareil) with specific reactivity of 52.6–75% as compared to that of the AMP alone. Immunoreactivity of protein extracts prepared from commercial samples of blanched almonds, roasted almonds, and almond paste was respectively reduced by 50.0%, 56.6%, and 68.4% (noncompetitive ELISA) when compared to the immunoreactivity of the AMP. Moist heat (121 °C, 15 min) pretreatment of the AMP reduced the PA reactivity by 87% (noncompetitive ELISA). Exposing AMP to pH extremes (12.5 and 1.5–2.5) caused a 53% and 57% reduction in PA reactivity, respectively (noncompetitive ELISA). PA showed some cross-reactivity with the cashew major globulin, and to a lesser extent, the Tepary and Great Northern bean major storage protein (7S or phaseolin). The presence of almonds in a commercial food was detected using PA in a competitive ELISA.

INTRODUCTION

Almonds (*Prunus dulcis* L.) are one of the most popular tree nuts on a worldwide basis and rank number one in tree nut production. Almonds are typically used as snack foods and as ingredients in a variety of processed foods, notably, in bakery and confectionery products. The United States is the largest almond producer in the world and California typically generates about 70% of the world's almond supply in any given year (FAO, 1995). For example, in 1995/1996 the U.S. crop totaled 167,800 metric tons with a value of $1.048 $\times$ 10^9 and represented 64% of world production of 262 200 metric tons (USDA, 1997).

Almonds contain up to 25% protein and provide all of the essential amino acids, except methionine, in quantities equal to or greater than those recommended by the FAO guidelines (Sathe, 1993). Water-soluble proteins account for at least 95% of the total almond proteins. Regardless of the almond cultivar, a single storage protein appears to dominate the almond protein composition (Sathe, 1993). Earlier, Sathe (1992) had reported that this major protein in almonds (AMP) is composed of at least two types of polypeptides (38–41 and 20–22 kDa).

Tree nuts are one of the most important allergen sources associated with severe allergic reactions medi-

ated by IgE (Sicherer, 1999). With an increase in tree nut use in processed foods, unintentional dietary exposure to tree nut allergens is possible. The best defense against food allergies is to avoid the offending food. Such avoidance is not always possible because the offending substance may be present in a food in trace amounts and, for various reasons, may not be listed on the ingredient label. Errors in processing, including cross-contamination of equipment, and errors in preparation can also lead to contamination of one food with another to serve as a hidden allergen source. Therefore there is a need for developing sensitive, reproducible, and reliable methods that can detect the presence of important allergens, including almonds, in foods. Because AMP is by far the most abundant protein in almonds and we have shown the AMP to be a major allergen (unpublished observation), we investigated the possibility of using rabbit polyclonal antibodies raised against the AMP for this purpose. In addition, we were also interested in determining whether denaturation of AMP would eliminate or substantially decrease the binding between the AMP and the PA.

MATERIALS AND METHODS

Materials. Sources of electrophoresis chemicals have been reported earlier (Sathe, 1993). DEAE DE-53 was from Whatman, Hillsboro, OR. Molecular weight standards and Sephacryl S300 were from Pharamacia Inc., Piscataway, NJ. Whatman 3MM filter paper and Nitrocellulose paper (0.2 μm) were from Schleicher & Shuell Inc., Keene, NH. Acepromazine, bovine serum albumin (minimum purity 98% by electrophoresis, suitable for ELISA applications, Cat. No. A 7030), fentanyl, droperidol, Freund's complete adjuvant, incomplete Freund's adjuvant, goat anti-rabbit IgG, 5-bromo-4-chloro-3 indolyl

* Corresponding author. Tel: (850) 644-5837. Fax: (850) 645-5000. E-mail: ssathe@mailer.fsu.edu.
[†] Department of Nutrition, Food, and Exercise Sciences, Florida State University.
[‡] Department of Biological Science, Florida State University.
[§] University of California, Davis.

phosphate (BCIP), *N, N*-dimethylformamide (DMF), Ponceau S, and phosphatase substrate (*p*-nitrophenyl phosphate, diso-dium [PNPP]) were from Sigma Chemical Co., St. Louis, MO. Micro-ELISA plates (polyvinyl) were from Costar, Cambridge, MA. Nitroblue tetrazoleum (NBT), Tween 20, salts, and other chemical reagents were from Fisher Scientific, Pittsburgh, PA.

Methods. *Preparation of AMP and Whole Almond and Processed Food Protein Extracts.* The AMP was purified from defatted almond flour (Nonpareil cultivar) using anion ex-change (DEAE DE-53) and gel filtration (Sephacryl S300) column chromatographic procedures (Sathe, 1992). Briefly, defatted almond flour was extracted with 10 volumes of 0.02 M Tris-HCl (pH 8.1 buffer) with constant magnetic stirring, filtered through glass wool, and the filtrate was centrifuged (12000*g*, 20 min). The supernatant was loaded onto a DEAE DE-53 column (5.0 × 54.5 cm) equilibrated in 0.02 M Tris-HCl buffer (equilibrium buffer). The column was flushed with the equilibrium buffer until absorbance at 280 nm returned to baseline and then eluted with a 0–0.5 M NaCl gradient in the equilibrium buffer (3 L, each). Fractions were collected every 15 min, and column flow rate was 80 mL/h. Fractions containing the AMP were pooled and concentrated on an Amicon concentrator using a YM 10 membrane (MW cutoff, 10 000), and aliquots (<1% column volume) of the concentrated protein solution were loaded on a Sephacryl S300 column (1.6 × 93.5 cm) previously equilibrated with the equilibrium buffer containing 0.1 M NaCl. The column was eluted with the same buffer (column flow rate was ~20 mL/h). Protein elution from both columns was monitored by measuring absorbance at 280 nm and by SDS–PAGE. Fractions containing the AMP were pooled, dialyzed against distilled deionized water, and lyoph-ilized. All purification steps were done in a cold room (4 °C). The lyophilized protein preparations were stored at −20 °C in airtight plastic containers until further use.

For denaturation experiments, AMP was dissolved (1 μg/mL) in 0.1 M borate saline buffer, pH 8.45 (BSB), and subjected to heat denaturation by incubating the solution at 4, 25, 80, 90, 100, and 121 °C for 15 min prior to use in the ELISAs (described below).

To assess the influence of pH, BSB buffer pH was adjusted to the desired value (1.5–12.5) prior to dissolving the AMP. The AMP solution (of the desired pH) was then transferred to ELISA microtiter plates, and coating was allowed to continue for 20 h at 4 °C. ELISAs were then completed using 0.1 M BSB (pH 8.45) as described in the ELISA procedure.

Defatted flours of whole almonds from major U.S. marketing cultivars (Mission, Neplus, Peerless, Carmel, and Nonpareil), commercially processed blanched almonds, roasted almonds, almond paste, and food products [food 1, chocolate-containing sandwich cookies (cream filling); food 2, hamburger bun with sesame seeds on top; food 3, breakfast cereal containing almonds] were used. Proteins from the defatted samples were extracted with 0.1 M sodium phosphate buffer pH 7.0 (flour: buffer, 1:20 w/v) for 30 min at 25 °C with gentle shaking provided. Samples were centrifuged (15000*g*, 5 min), and the supernatants were used for further analysis.

Polyclonal Antibodies. Purified AMP (200 μg) in 1 mL of Freund's complete adjuvant was administered intradermally (id) to each of two New Zealand White rabbits (*Oryctulagus cuniculus*). Beginning 3 weeks after the initial immunization, six booster doses (200 μg of AMP in 1 mL of incomplete Freund's adjuvant per rabbit per booster dose, id) were administered at 3- or 6-week intervals (Harlow and Lane, 1988). Pre- and post-immunization blood was collected from the marginal ear vein. Blood was allowed to clot for 30 min and centrifuged at 5000*g* for 20 min, and the serum collected. Serum aliquots were kept at 4 °C for immediate use and at −20 °C for long-term storage.

Enzyme-Linked Immunosorbent Assay (ELISA). Noncom-petitive (direct binding) ELISAs were done using the protocol described by Watterson et al. (1993). A 96-well polyvinyl microtiter ELISA plate was coated with 50 μL/well of the indicated AMP solution in BSB, pH 8.45, and incubated overnight (20 h) at 4 °C. After the incubation, wells were washed three times with BSB and blocked with 50 μL of 1%

bovine serum albumin in BSB (1% BSA–BSB) for 1 h at 25 °C, followed by three washings with BSB. The PA, suitably diluted in 0.9% NaCl, was then incubated for 2 h at 25 °C (50 μL/well) followed by four washings with BSB. Alkaline phos-phatase-labeled goat anti-rabbit IgG (secondary antibody) was diluted 1:32 000 v/v in 0.1% (w/v) BSA–BSB and added to each well (50 μL/well). The plates were incubated for 1 h at 25 °C and then washed four times with BSB. Phosphatase substrate (50 μL/well, 1 mg/mL) [500 mg of *p*-nitrophenyl phosphate in a final volume of 500 mL containing 48 mL of diethanolamine and 24.5 mg of $MgCl_2$, pH adjusted to 9.8 with 5 M HCl] was added to each well, and the color development was allowed to proceed for 12 min at 25 °C. Color development was stopped by adding 50 μL/well of 3.0 M NaOH. The optical density (OD) of each well was read at 405 nm using an ELISA reader (Model EL 307, Bio-Tek Instruments Inc., Riverton, NJ). When appropriate, ELISA data were calculated to express the relative amount of PA binding as a function of the protein concentration used to coat the ELISA wells:

$$\text{specific activity} = \frac{\left[\dfrac{\text{net OD}_{405\,\text{nm}}\text{ of AMP}}{(0.65 \times \mu\text{g of protein in sample/50 }\mu\text{L})}\right]}{\left[\dfrac{\text{net OD}_{405\,\text{nm}}\text{ of AMP}}{(0.05\ \mu\text{g of AMP/50 }\mu\text{L})}\right]}$$

This formula is based on the assumption that the AMP comprises ~65% of the total proteins in almonds (Wolf and Sathe, 1998).

Competitive inhibition ELISAs were similarly performed with the exception that serial dilutions of known concentra-tions of inhibitor were preincubated at 37 °C for 1 h in the presence of PA (at 10^{-4} dilution) prior to transfer to AMP-coated plates. Once transferred, the PA, in the presence of inhibitor, was incubated and washed, the second antibody was added, and the color development was completed as described above.

All OD readings for all ELISAs are the net OD values (sample OD − appropriate blank OD). Percent inhibitions (cross-reactivity) were calculated as follows:

$$\text{IC}_{50}\text{ for AMP/IC}_{50}\text{ for the inhibitor}$$

Electrophoresis and Electroblotting. Electrophoresis using nondenaturing nondissociating polyacrylamide gel electro-phoresis (NDND–PAGE) was done according to Andrews (1986), and sodium dodecyl sulfate polyacrylamide gel elec-trophoresis (SDS–PAGE) was done according to the method of Fling and Gregerson (1986). Details of gel electrophoreses were the same as described earlier (Sathe, 1993).

Proteins from electrophoresis gels were transferred on to 0.2 μm nitrocellulose (NC) paper according to the method described by Towbin et al. (1979), and the NC paper was cut into appropriate strips. Proteins were visualized by soaking appropriate NC strips in 0.1% (w/v) Ponceau S in distilled water. Unbound sites on the NC paper strips were blocked using Tris-buffered saline [10 mM Tris, 0.9% (w/v) NaCl, 0.05% (v/v) Tween-20] (TBS) containing 5% (w/v) nonfat dry milk powder (DMBS) for 45 min at 25 °C with gentle shaking. The NC strips were then incubated with the appropriate dilution of the rabbit antiserum in TBS–DMBS solution at 25 °C for 1 or 4 h. An orbital shaker was used to provide gentle shaking. At the end of the incubation period, the NC strips were washed three times with TBS (5 min per washing) and then incubated at 25 °C for 1 h with alkaline phosphatase-labeled goat anti-rabbit IgG (secondary antibody) diluted in DMBS (1:10 000 v/v). The NC strips were washed three times with TBS (5 min per washing) and then reacted with alkaline phosphatase substrate solution [41 μL of BCIP (1 mg of BCIP/mL of dimethylformamide) + 82 μL of NBT (50 mg of NBT/mL of 70% aqueous dimethylformamide) + 0.1 M Tris containing 0.1 M NaCl and 0.5 mM $MgCl_2$ to a final volume of 25 mL] for 10 min at 25 °C. Color development was stopped with phosphate-buffered saline (10 mM sodium phosphate, 0.9% NaCl) solution (PBS) containing 20 mM ethylenediaminetetraacetic acid (EDTA).
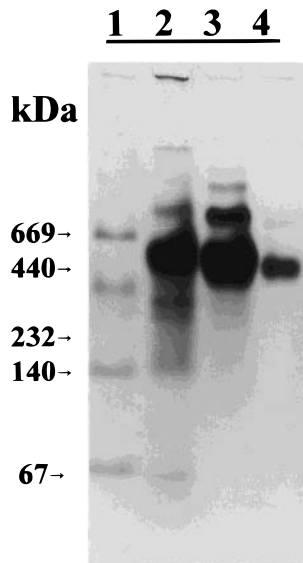
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Figure 1. NDND−PAGE (3−30% linear gradient acrylamide gel, acrylamide:bis = 37:1 w/w) for the AMP purification. (Lane 1) Protein standards (Pharmacia HMW Kit) were as follows: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and albumin (67 kDa). (Lane 2) Defatted almond (Nonpareil) flour proteins extracted with 20 mM Tris-HCl, pH 8.1, buffer (flour:buffer, 1:10 w/v). (Lane 3) Protein fractions containing AMP pooled from DEAE DE-53 column. (Lane 4) Protein fractions containing pure AMP pooled from Sephacryl S300 HR column. Protein load in lanes 2−4 was, respectively, 100, 100, and 30 μg.

Preparation of Nonalmond Proteins. The following seed and nut proteins were prepared as described in the indicated reference and were used as inhibitors to test for cross-reactivity in competitive ELISA: Great Northern bean (*Phaseolus vulgaris* L.) phaseolin and tepary bean (*Phaseolus acutifolius* L.) phaseolin (Sathe and Sze-Tao, 1997), Basmati rice albumin and globulin fractions (Steenson and Sathe, 1995), Inca peanut albumin (Hamaker and Sathe, 1996), cashew globulin (Sathe et al., 1997), walnut albumin and globulin fractions (Sze and Sathe, 1995), and soybean glycinin (11S) and β-conglycinin (7S) (Nagano et al., 1992).

Protein Determination. Soluble protein was determined by the method of Lowry et al. (1951). Appropriate blanks were used in all assays. Bovine serum albumin was used as the standard protein. Standard curves for BSA were prepared in appropriate buffer and were done for every assay.

Statistics. All experiments were done in duplicate, and mean ± standard deviations were reported. Data were analyzed for linear regression when appropriate. For statistical significance ($p = 0.05$), one-way analysis of variance and Fisher's least significant difference test were used.

RESULTS AND DISCUSSION

AMP Purification. The AMP used in these experiments was biochemically pure (Figure 1). Ultracentrifugal analysis of the AMP indicated that the AMP was at least 99.8% homogeneous (Sathe et al., unpublished results).

AMP Detection and Quantitation. Western blotting experiments (Figure 2) indicated that the native AMP was recognized by the PA in the dilution range of $10^{-4}-10^{-7}$. The middle point (4.0×10^{-4} dilution) between the strongest (10^{-4} antibody dilution) and the weakest (10^{-7} antibody dilution) reactivity between the PA and the AMP was considered as the optimum dilution. For routine ELISAs and Western blotting experiments, we typically used the 10^{-4} dilution of the antibodies. Western blotting experiments (Figure 3)
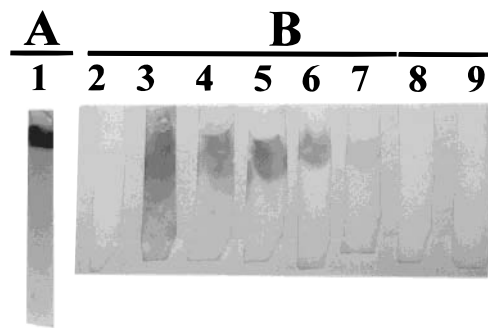

Figure 2. Western blotting for the AMP titer determination. NDND−PAGE (6% acrylamide gel, acrylamide:bis = 37:1 w/w) (A) AMP. (B) Western blotting. (Lane 2) Preimmune serum; (lanes 3−9) immune serum serial dilutions 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10}, respectively. AMP load in each lane was 5 μg.
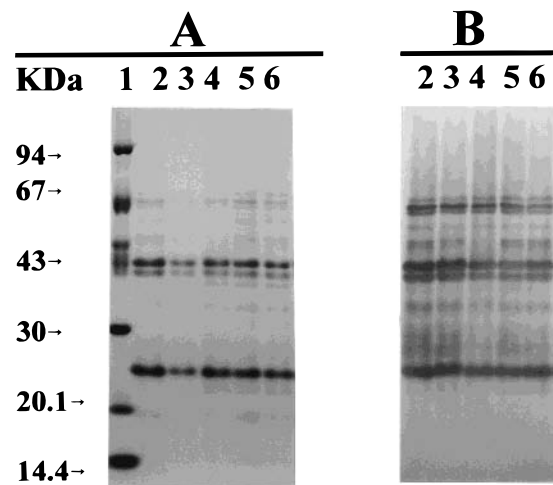

Figure 3. Reactivity of PA against the major marketing varieties of almonds. (A) SDS−PAGE (8−25% linear acrylamide gradient, acrylamide:bis = 37:1 w/w) in the presence of 2% β-mercaptoethanol (β-ME). (Lane 1) Protein standards phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). (Lanes 2−6) Mission, Neplus, Peerless, Carmel, and Nonpareil protein extracts, respectively. (B) Western blotting. The protein load in lanes 2−6 was 15 μg each.

using SDS−PAGE in the presence of 2% β-ME indicated that PA recognized several polypeptides present in the AMP. Recognition of multiple bands by PA was expected since AMP is a multimeric protein that, upon SDS−PAGE electrophoresis in the presence of 2% β-ME, is resolved in as many as 25 polypeptides (unpublished data). The PA could detect the AMP from all the major U.S. marketing cultivars of almonds by using Western blotting analysis (Figure 3). Earlier studies by Bargman et al. (1992) had suggested that IgE in sera from sensitive individuals with known almond allergies react with almond polypeptides (30−70 kDa) and that two polypeptides (45−50 and ∼70 kDa) were the major allergenic polypeptides. These investigators used whole protein extracts for their Western blotting experiments. The reactivity of polypeptides 38−42 and 20−22 kDa with the PA in our investigations is consistent with the results of Bargman et al. (1992).

Noncompetitive ELISA assays using native AMP (Figure 4) indicated that the PA anti-AMP reactivity remained linear when ELISA plates were coated with AMP in the concentration range of 1−100 ng/mL (n =
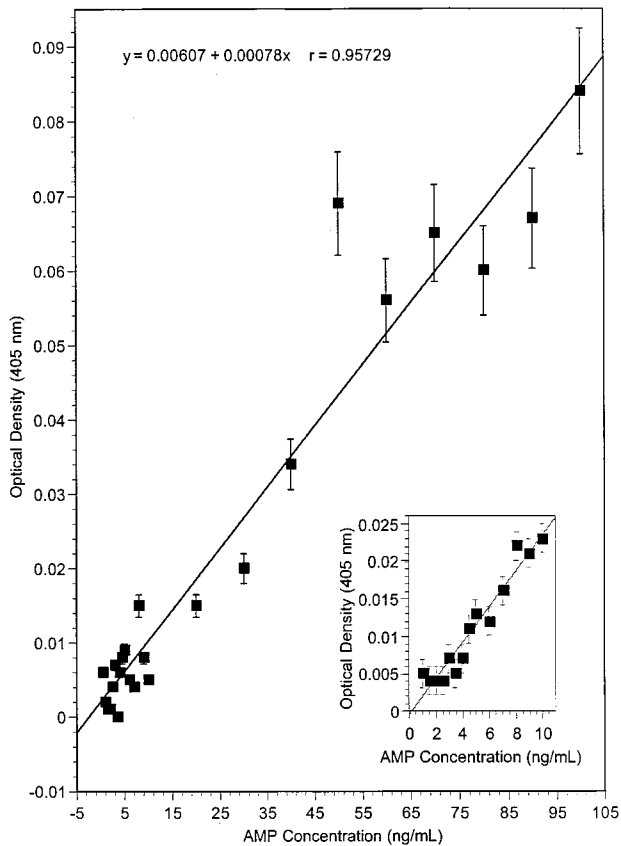
Figure 4. Effect of AMP concentration on noncompetitive ELISA assay linearity.

Figure 5. Competitive inhibition ELISA assay for the detection of AMP in foods.

Table 1. Cross-Reactivity of PA with Non-AMP Proteins

inhibitor	$IC_{50}AMP/IC_{50}$ inhibitor
cashew globulin (13S)	2.7×10^{-3}
tepary bean phaseolin (7S)	1.8×10^{-3}
great Northern bean phaseolin (7S)	1.0×10^{-3}
walnut albumin	2.1×10^{-4}
Basmati rice globulin	1.7×10^{-4}
walnut globulin	1.0×10^{-4}
Basmati rice albumin	$<5.6 \times 10^{-5}$
soybean β-conglycinin (7s)	$<5.6 \times 10^{-5}$
soybean glycinin (11s)	$<5.6 \times 10^{-5}$
Inca peanut albumin (2S)	$<5.6 \times 10^{-5}$

3, $r = 0.96$; for inset, $n = 9$, $r = 0.97$). A positive reaction with wells coated with as little as 1–10 ng of AMP/mL is comparable to the detection levels of 1 ng of protein/mL for α-, β-, and γ-kafirins (Watterson et al., 1993); less sensitive than the 1 ng of meal/mL for α-, β-, and γ-zeins (Wallace et al., 1989); and more sensitive than the 5 μg/mL level reported for crude peanut extract (Hefle et al., 1994). The differences in the detection limits may partly arise from the differences in the protein molecular sizes [sorghum and corn protein polypeptides, 10–40 kDa, are much smaller than the native AMP (460 kDa) and the native peanut proteins (150–200 kDa)], the inherent molecular structural differences in proteins, and the species used for antibody production.

Competitive inhibition ELISA assays (Figure 5, $n = 3$) indicated that the AMP could be detected down to 300 ng/mL when assayed in pure form. These assays were also able to detect the presence of almonds in a commercially processed food (food 3, a breakfast cereal known to contain almonds but not cashew nuts) and were negative for foods 1 and 2 that did not contain almonds (food 1, chocolate-containing sandwich cookies with cream filling; food 2, hamburger bun with sesame seeds on top). These data indicate the potential for immunobased assays in detecting almonds in processed foods.

Specificity of PA. Competitive ELISA assay results (Table 1) in which various purified seed and nut proteins were used as soluble inhibitors in the PA anti-AMP/AMP solid phase assays show that some proteins were slightly cross-reactive with AMP, the greatest activity being shown by cashew globulin and the tepary and Great Northern bean phaseolins. Western blotting
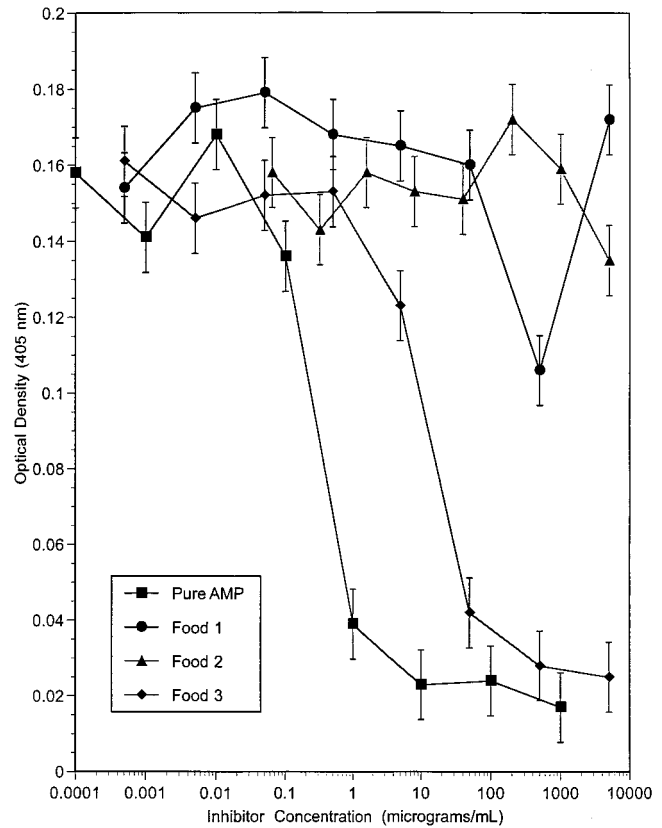
experiments (data not shown) confirmed this cross-reactivity profile. These results suggest that almond major proteins may share common antigenic features with some other seed storage proteins. Such cross-reactivity is not uncommon. Simultaneous allergies to more than one type of nut in human population are not uncommon (Ewan, 1996). A recent study using human subjects (Fernandez et al., 1995) has reported human IgE-mediated cross-reactivity between pistachio, cashew, and mango seed proteins (but not mango pulp proteins) by using Western blotting, RAST, and skin tests. In view of the cross-reactivity of the PA with non-AMP proteins, further work will be needed in the development and refinement of an immunobased assay to specifically detect the presence of AMP in food products.

Effects of Processing Treatments on the Reactivity with PA. Earlier studies by Bargman et al. (1992) using commercially processed almond protein extracts in Western blotting assays showed that thermal processing of almonds (blanching and roasting) abolished the immunoreactivity of the 15 and 70 kDa polypeptides with the IgE in the sera from almond-allergic human subjects. In our studies, thermal treat-
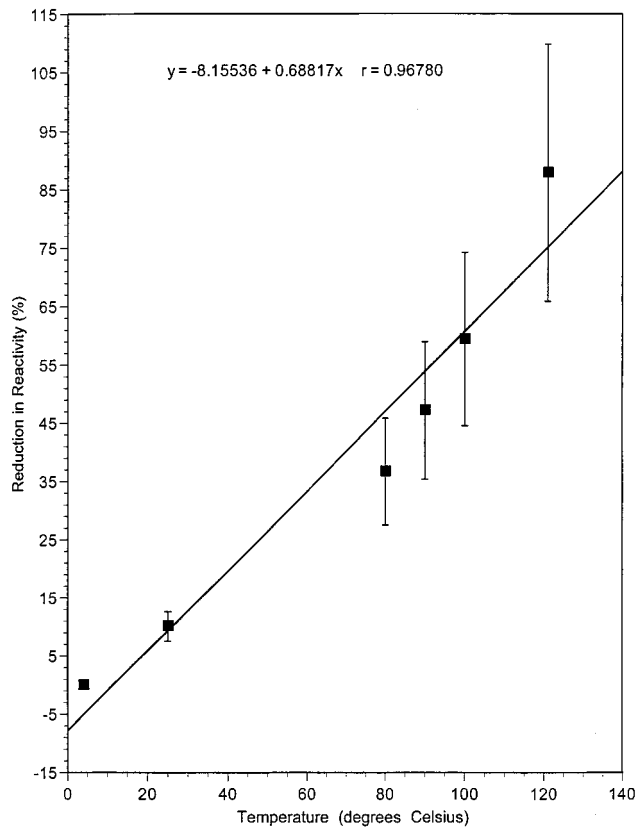
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Figure 6. Effects of heat pretreatment on the ability of AMP to react with PA (AMP at 0.05 μg/50 μL in noncompetitive ELISA).

Figure 7. PA reactivity (noncompetitive ELISA) against protein extracts of various almond almond cultivars and processed almonds. Activity is expressed on a per microgram of AMP basis with the assumption that AMP comprises 65% of the total proteins in almonds. (Lane 1) AMP (0.5 μg/50 μL). (Lanes 2–6) Protein extracts prepared from defatted almond flours using Mission, Neplus, Peerless, Carmel, and Nonpareil varieties, respectively. (Lanes 7–9) Protein extracts prepared from defatted flours of commercially prepared blanched almonds, almond paste, and roasted almonds, respectively. Protein load for these assays was 0.05 μg/50 μL, each.

ment of the AMP prior to coating ELISA wells (Figure 6, n =13) caused a significant reduction (up to 87%) in the PA reactivity, and as would be expected, the reduction in PA reactivity increased with an increase in treatment temperature. Thermal treatment of proteins promotes protein unfolding, denaturation, and/or aggregation, which disrupts the protein structure. Sathe and Sze-Tao (1997) have shown that, when almond protein isolate was subjected to heat treatment at 100 °C for less than 2 min in an aqueous solution, 80–85% of almond proteins were quickly denatured to the point of precipitation. This loss in protein structural integrity often leads to reduction or loss in epitope recognition (Tukur et al., 1996). The fact that a dilute AMP solution subjected to autoclaving (121 °C for 15 min) did not completely lose its PA reactivity suggests that at least some of the AMP epitopes are based on primary amino acid sequence. We cannot rule out the possibility that some structural features of the AMP may require more severe heat treatment (especially in dilute solutions) to completely abolish their reactivity with the PA. Reduction in the immunoreactivity due to thermal processing of almonds is encouraging and suggests that further reduction may be accomplished with additional treatment to enable the production of hypoallergenic almonds/ almond products if binding to allergic patients' sera IgE is similarly abrogated.

Protein extracts of defatted flours prepared from major marketing varieties of almonds (Figure 7, $n = 6$) reacted similarly with PA. The specific reactivity (noncompetitive, direct binding assay) of the protein extracts from Mission, Neplus, Peerless, Carmel, and Nonpareil was in the range 52.6–75% when compared with the reactivity of the AMP alone (Figure 7). This range of
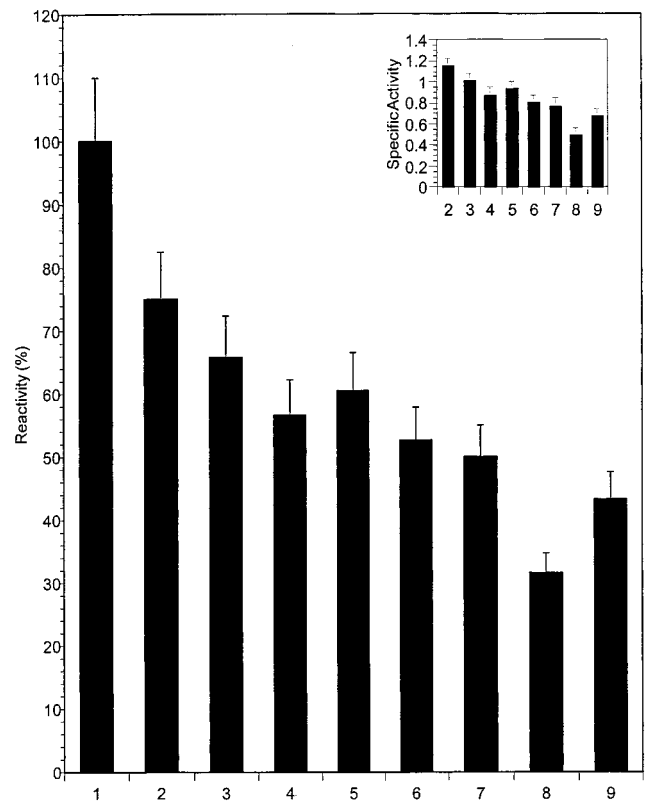
decreased reactivity of PA for AMP in the protein extracts was expected since AMP accounts for ~65% of the total almond proteins and non-AMP proteins present in the almond protein extracts may also nonspecifically bind with PA thereby reducing available PA for binding with the AMP or may compete with PA for binding to the surface of the ELISA plates.

ELISA (Figure 7) and Western blotting data (Figure 8) indicated that the reactivity of PA with the protein extracts prepared from processed almonds decreased but was not eliminated. The decrease in reactivity, as expected, was dependent on the type of processing treatment almonds had received. Bargman et al. (1992), using radioallergosorbent tests (RASTs) and Western blotting, also found that immunoreactivity of protein extracts from almond butter, blanched almond, and roasted almond proteins decreased but was not eliminated. Because of the proprietary nature, exact commercial processing conditions are often difficult to know. Generally, almond thermal processing involves minimal (e.g., paste), moderate (e.g., blanching), or high (e.g., roasting) temperatures (Bargman et al., 1992; Schmidt, 1984; Woodroof, 1967). Almond paste manufacture typically involves adding sugar (10–40% w/w in the final product) to almonds and grinding the mixture at high shear rates at 250 °F for 1–3 min (Faid et al., 1995; Cunningham, 1997). Almonds are typically blanched for 3–5 min in a boiling water bath where the internal
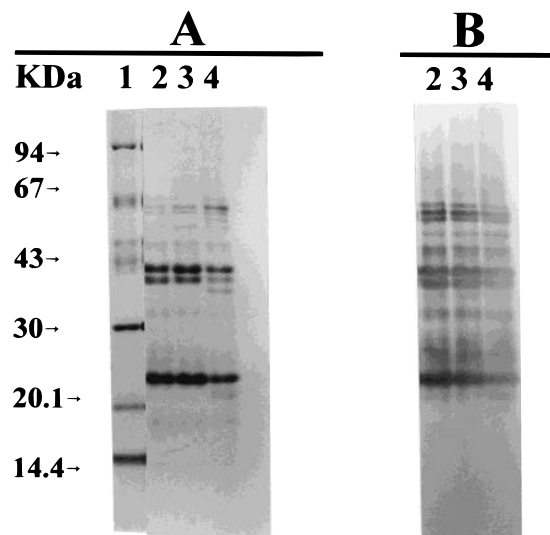
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Figure 8. Reactivity of PA against protein extracts prepared from processed almonds. (A) SDS−PAGE (8−25% linear acrylamide gradient, acrylamide:bis = 37:1 w/w). (Lane 1) Protein standards (94−14.4 kDa, as in Figure 3). Protein extracts from defatted processed almond samples. (Lane 2) Blanched almonds. (Lane 3) Almond paste. (Lane 4) Roasted almonds. (B) Western blotting, lanes 2−4, respectively, protein extracts from defatted processed almonds, blanched, paste, and roasted. Protein load in lanes 2−4 was 20 μg each.

Figure 9. Effects of pre-exposure of AMP to different pH values on PA-AMP reactivity (noncompetitive ELISA in BSB, pH 8.45).

temperature of the seed reaches to 190 °F (Schmidt, 1984; Cunningham, 1997). Almond roasting temperature is variable (range 280−350 °F) depending on the manufacturer; typically as the roasting temperature increases, the roasting time decreases. For example, roasting may be done at 280−300 °F for 20−30 min or at 350 °F for 8−12 min (Woodroof, 1967; Cunningham, 1997). Almonds are often coated with butter, margarine, or vegetable oil prior to roasting (Woodroof, 1967). Since blanching as well as paste preparation involves moist heat, blanched almonds and almond paste protein extracts were expected to lose immunoreactivity more than the roasted almonds. Almond paste was expected to lose immunoreactivity less than blanched and roasted almonds because sugars have a protective effect on protein denaturation (Seideman et al., 1963). We observed that the immunoreactivity of protein extracts prepared from blanched almonds, roasted almonds, and almond paste was respectively reduced by 50.0%, 56.6%, and 68.4% (noncompetitive ELISA, Figure 7) when compared with the immunoreactivity of the untreated AMP. Similarly, Tukur et al. (1996) have reported decreased reactivity of rabbit antisera produced against two thermally processed major soybean proteins, glycinin (11S) and β-conglycinin (7S). These investigators found that the reactivity of glycinin and β-conglycinin was reduced by 68% and 60%, respectively, when toasted soy flour was compared with defatted soy flour (the control). Exposing porcine meat proteins to heat at 65 (30 min) and 120 °C (20 min) has been shown to decrease the immunoreactivity by 11% and 86%, respectively (Morales et al., 1994). The loss of immunoreactivity due to thermal processing of the proteins may be due to destruction of structure-based epitopes, changes in protein folding, or interactions with other biochemicals that may mask the reactive epitopes.

Reactivity of AMP with PA was only slightly influenced in the pH range 4−11.5 but decreased significantly (Figure 9, *n* = 11, noncompetitive ELISA) when
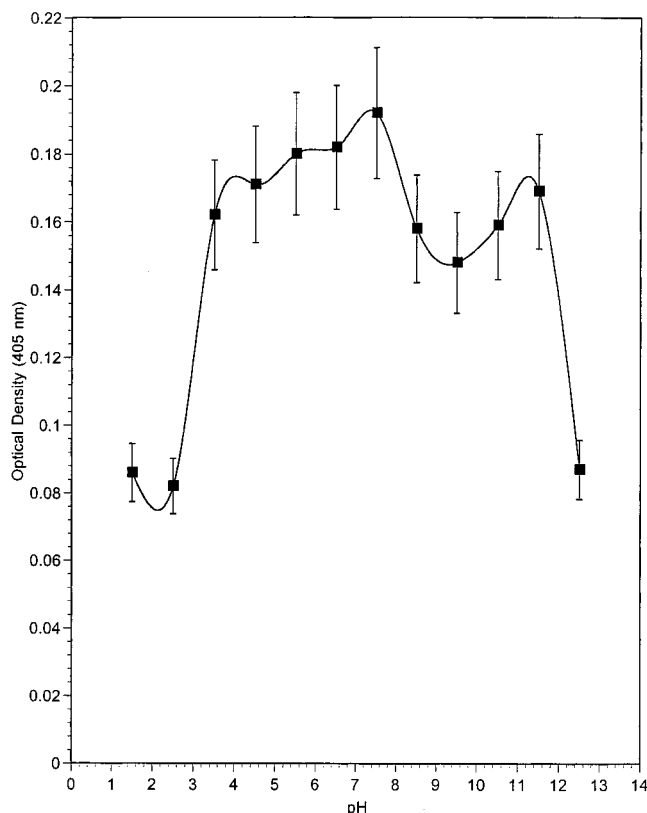
the AMP was preexposed to pH extremes (≤3 and 12.5) probably due to protein denaturation. Since the antigen−antibody binding is mainly due to multiple weak interactions, such binding can be strongly influenced by ionic conditions in the reaction medium. The stability of AMP immunoreactivity that we observed in the pH range 4−11.5 is consistent with similar stability (pH range 5.1−9.1) reported for actin, myosin, and tubulin (Labrousse et al., 1994) and bovine β-lactoglobulin in the pH range 4−8 (Kamata et al., 1996). The bimodal nature of the data in Figure 9 also suggests that there may be at least two groups of epitopes (one in the pH 5−7 range and the other in the pH 8−11 range) on the AMP that have different binding affinities toward the antibodies. Alternatively, there may two different populations of antibodies that recognize AMP and bind with AMP with different affinities depending on the pH to which AMP is exposed.

CONCLUSIONS

PA could detect AMP on plates coated with as little as 1−10 ng/mL (noncompetitive ELISA) and could detect soluble AMP at 300 ng/mL (competitive inhibition ELISA). PA reacted with protein extracts from all U.S. major marketing cultivars of almonds (Mission, Neplus, Peerless, Carmel, and Nonpareil). The immunoreactivity of the protein extracts prepared from blanched almonds, roasted almonds, and almond paste was respectively reduced by 50.0%, 56.6%, and 68.4% when compared to the immunoreactivity of the AMP. Moist heat (121 °C, 15 min) pretreatment of AMP reduced the PA reactivity by 87%. Exposing AMP to pH 12.5 and pH 1.5−2.5 caused 53% and 57% reduction in PA reactivity, respectively. PA displayed low levels of cross-

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reactivity with cashew globulin and tepary and Great Northern bean phaseolins but not with other seed and nut proteins. Competitive inhibition ELISA assays were able to detect the presence of almonds in a commercially processed breakfast cereal that is known to contain almonds. These results demonstrate the feasibility of developing an immunobased assay to detect the presence of small quantities of almond in food products. Because AMP has been demonstrated to be the most abundant almond protein as well as a major allergen in almond-sensitive individuals, we believe an AMP detection assay, once optimized, will be of value to the food industry and allergic individuals for the detection of almond contamination in foods.

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