Detection and Stability of the Major Almond Allergen in Foods

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Almond major protein (AMP or amandin), the primary storage protein in almonds, is the major allergen recognized by almond-allergic patients. A rabbit antibody-based inhibition ELISA assay for detecting and quantifying AMP in commercial foods has been developed, and this assay, in conjunction with Western blotting analyses, has been applied to the investigation of the antigenic stability of AMP to harsh food-processing conditions. The ELISA assay detects purified AMP at levels as low as 87 ±16 ng/mL and can detect almond at between 5 and 37 ppm in the tested foods. The assay was used to quantify AMP in aqueous extracts of various foods that were defatted and spiked with known amounts of purified AMP or almond flour. In addition, AMP was quantified in commercially prepared and processed almond-containing foods. Neither blanching, roasting, nor autoclaving of almonds markedly decreased the detectability of AMP in subsequent aqueous extracts of almonds. Western blots using both rabbit antisera and sera from human almond-allergic patients confirm a general stability of the various peptides that comprise this complex molecule and show that the rabbit antibody-based assay recognizes substantially the same set of peptides as does the IgE in sera from almond-allergic patients.

Keywords: *Almond; AMP; amandin; food allergy; allergen; immunoassay; Prunus dulcis*

INTRODUCTION

On a global basis, almonds rank first in tree nut production (Almond Board of California, 1999 data). A single water soluble storage protein, termed amandin or almond major protein (AMP), accounts for ∼65% of total aqueous extractable almond protein (*1*). AMP is a complex molecule composed of at least 28 peptides (*2*).

According to a recent epidemiologic survey, tree nut allergy affects 0.5% of the population (*3*). Unlike certain food allergies that are temporary and tend to affect primarily young children, allergies to fish, crustaceans, peanuts, and tree nuts often persist throughout life and are sometimes life-threatening (*4*, *5*). Consequently, it is important for food processors and regulatory agencies to be able to ensure accurate labeling of foods to protect the safety of the public and to avoid expensive recalls. We have developed an inhibition enzyme-linked immunosorbent assay (ELISA) for detecting almond in foods that is based on quantification of AMP in aqueous food extracts. In addition, we have assessed the stability of this marker protein to harsh food-processing treatments and correlated the reactivity of the rabbit antibodies used in the assay to those of human almond-allergic patients.

MATERIALS AND METHODS

Chemicals and Supplies. Sources of electrophoresis chemicals have been reported earlier (*6*). 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 (NC, 0.45 μm) were from Schleicher and Shuell Inc. (Keene, NH). Acepromazine, bovine serum albumin (minimum purity of 98% by electrophoresis, suitable for ELISA applications, catalog no. A 7030), fentanyl, droperidol, complete Freund's adjuvant, incomplete Freund's adjuvant, alkaline phosphatase-labeled goat anti-rabbit IgG, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), *N*,*N*-dimethylformamide (DMF), Ponceau S, and phosphatase substrate [*p*-nitrophenyl phosphate, disodium (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).

Preparation of AMP, Whole Almond, and Processed Food Protein Extracts. Reference AMP was purified from defatted almond flour (Nonpareil variety) using anion exchange (DEAE DE-53) and gel filtration (Sephacryl S300) column chromatographic procedures, as previously described (*7*). Briefly, defatted almond flour was extracted with 10 volumes of 0.02 M Tris-HCl (pH 8.1 buffer) with stirring (1 h) followed by centrifugation (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 and then eluted with a 0−0.5 M NaCl gradient in the equilibrium buffer. Fractions containing the AMP (as assessed by SDS-PAGE) were pooled, concentrated, and size-purified over a Sephacryl S300 column in equilibrium buffer containing 0.1 M NaCl. Fractions containing the AMP were pooled, dialyzed against distilled deionized water, and lyophilized. All purification steps were at 4 °C. The lyophilized protein preparations were stored at −20 °C.

For denaturation experiments, whole almonds were subjected to heat denaturation in an autoclave at 121 °C/15 psi for the times indicated prior to grinding, defatting, and protein extraction in buffered saline borate (BSB; 0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, 0.075 M NaCl, pH 8.2). Commercially processed dry-roasted, oil-roasted, and blanched almonds were provided by Sam Cunningham, Blue Diamond Co., Sacramento, CA.

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AMP-spiked samples were prepared by adding 20 μL of AMP (containing 1.0 or 10 μg total) to the ground food or flour prior to extraction in 1 mL of BSB. Foods spiked with almond flour were ground and mixed first with a mortar and pestle and then with a Sorvall Omni-Mixer (Ivan Sorvall Inc., Newton, CT) at speed setting "8" for two 10 s bursts with a 5 s interval prior to extraction with BSB.

Rabbit and Human Antisera. Production and characterization of the polyclonal antibodies used in this study have been described previously (*7*). Briefly, rabbits were immunized with purified AMP using Freund's complete adjuvant. After boosting with AMP in incomplete Freund's adjuvant, the rabbits were bled, and the resultant serum was stored at −20 °C until used. Human antisera were from a pool of three almond-allergic patients (no. 38, 42, and 135) selected for the ability to (together) recognize the range of high, intermediate, and low MW amandin peptides typically recognized by almond-allergic patients (unpublished observations).

ELISA. Competitive ELISAs were performed as previously described (*7*) with some modifications. A 96 well polyvinyl microtiter ELISA plate (Serocluster "U" vinal, no. 2797, Costar) was coated with 50 μL/well of the AMP at 10 μg/mL solution in coating buffer (48.5 mM citric acid, 103 mM Na_2HPO_4, pH 5.0) and incubated for 1 h at 37 °C. Wells were washed with BSB, blocked with 1% bovine serum albumin in PBS for 0.5 h at room temperature, and rewashed three times. A dilution of rabbit anti-AMP (1:100000) in 0.1% BSA−BSB previously determined to give 50% of maximum binding in a direct binding assay was added to each well of a second uncoated plate. Soluble inhibitor was serially diluted into the antiserum and incubated for 1 h at 37 °C, whereupon the contents of the plate were transferred to the AMP-coated plate and incubated for an additional hour at 37 °C. This two-step incubation was found to increase assay sensitivity, presumably by ensuring maximal binding of the inhibitor AMP to the antibody prior to the competition phase of the assay. The plates were washed and developed using alkaline phosphatase-labeled goat anti-rabbit IgG (secondary antibody) for 1 h at 37 °C and phosphatase substrate (50 μL of *p*-nitrophenyl phosphate, 1 mg/mL). Reactions were stopped with the addition of 15 μL of 3.0 M NaOH. The amount of AMP in food samples was quantitatively determined by comparison of the relative ability of known amounts of pure AMP (standard curve) and sample extract to inhibit the AMP-anti-AMP reaction.

Electrophoresis and Western Blotting. SDS-PAGE in the presence of β-mercaptoethanol (β-ME) (unless otherwise indicated) was carried out according to the method of Fling and Gregerson (*8*). Proteins from electrophoresis gels were transferred onto 0.45 μm nitrocellulose (NC) paper according to the method described in Towbin et al. (*9*). 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-T)] containing 0.1% (w/v) BSA for 1 h at room temperature with gentle rocking. The NC strips were then incubated with rabbit or patient antiserum in TBS-T at 1:16000 and 1:5 dilution (v/v), respectively, for 1 h at room temperature and overnight at 4 °C, respectively, with rocking. NC sheets were rinsed twice with TBS-T, washed once with rocking for 15 min followed by three 5 min washes with rocking. The blots were then incubated at room temperature for 1 h with horseradish peroxidase-labeled goat anti-rabbit or goat anti-human antibody diluted in TBS-T. The reactive bands were visualized by application of chemiluminescent substrates (ECL Plus, Amersham Pharmacia Biotech, Piscataway, NJ) as described by the manufacturer. Membranes to be reprobed were first stripped with Western Re-Probe (Geno Technology, Inc., St. Louis, MO) as described by the manufacturer.

Protein Determination. Soluble protein was determined according to the method of Lowry et al. (*10*). 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 for each assay.

Statistics. All reactions were done in duplicate, and data are reported as mean ± standard deviation.

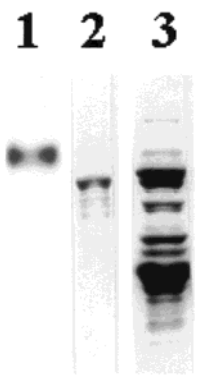

Figure 1. Gel electrophoresis patterns of AMP stained with Coomassie Brilliant Blue R: (lane 1) NDND-PAGE (3−30% linear acrylamide gradient), 20 μg; (lane 2) SDS-PAGE (8−25% linear acrylamide gradient), 25 μg with no β-ME; (lane 3) SDS-PAGE (8−25% linear acrylamide acrylamide gradient) with 2% β-ME, 50 μg.

RESULTS

Purification and Biochemical Characterization of AMP. We have previously identified AMP as the major almond storage protein and demonstrated that it is a major allergen in almond allergic patients (*11*). As seen in Figure 1, the single major AMP band observed in non-denaturing non-dissociating polyacrylamide gel electrophoresis (NDND-PAGE) yields numerous peptide species on a reducing SDS-PAGE gel. Previous analyses have shown that there are two major types of subunits with estimated molecular masses of 63 and 65 kDa (lane 2, Figure 1) (*6*). These subunits, in turn, are each composed of two major types of polypeptides with estimated molecular masses of 20−22 and 38−42 kDa linked via disulfide bond(s). Several minor polypeptides are also evident on reducing SDS-PAGE gels.

Immunological Characterization of AMP Using Rabbit Polyclonal Antibody. Two rabbits were immunized with purified AMP and their antisera compared in preliminary Western blots and ELISAs. No differences were detected, and one antiserum (no. 6235) was selected for use in this study. The sensitivity (IC_{50}) of the inhibition assay was found to be 87 ± 16 ng (n = 13) with pure AMP in the current study. We have previously characterized the specificity of this reagent by inhibition of ELISA with a variety of nut and seed extracts. The degree of cross-reactivity ranged from 2.7×10^{-3} for cashew globulin to $<5.6 \times 10^{-5}$ for rice and Inca peanut albumins and soybean β-conglutinin and glycinin (*7*). In addition, we have shown that AMP is the primary allergen in almonds (*11*). Comparative Western blot assays of AMP (Figure 2) reveal a close correspondence between the peptides recognized by human almond-specific IgE and those recognized by the rabbit anti-AMP antisera.

Initially, we wished to determine if the cultivar sources of the almond varieties differed substantially in their AMP contents. Eight almond samples representing five marketing varieties (Carmel, Mission, Neplus, Nonpareil, and Peerless) typically planted in commercial groves were tested. Aqueous extracts of each were found to be similarly inhibitory in our assay, suggesting that AMP content is fairly constant among these varieties with the AMP representing, on average, 74 ± 14% of the aqueous buffer-extractable seed proteins (data not shown).

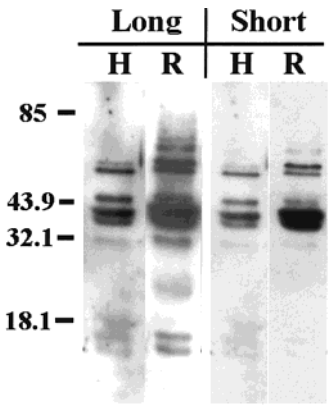

Figure 2. Western blot showing similar reactivities of a human almond-allergic patient's serum IgE and rabbit anti-AMP. The same lane of unprocessed almond extract from a nitrocellulose transfer (shown in full in Figure 3) was first probed with pooled human almond-allergic antiserum (H), stripped, and then reprobed with rabbit anti-AMP antisera (R). The lanes are shown here side-by-side for comparison. Long and short exposures of the same strips are shown.

Table 1. Detection of AMP in 100 mg Samples of AMP-Spiked Foods

spiked food	added AMP (μg)	detected AMP (μg)
raisin bran cereal	10	12.5 ± 0.10
	1	1.2 ± 0.28
rolled oats	10	11.4 ± 0.01
	1	1.3 ± 0.13
chocolate filled cookie	10	5.4 ± 0.10
	1	0.5 ± 0.01
milk chocolate candy	10	13.7 ± 0.0
	1	3.7 ± 0.21
rice cereal	10	24.4 ± 1.54
	1	2.9 ± 0.19

Recovery of AMP from Food Matrices. To determine if different food matrices interfere with the reaction between AMP and anti-AMP, we initially spiked several ground and defatted food samples with known amounts of purified AMP. Each of five 100 mg food samples was spiked with a known amount of AMP dissolved in 20 μL of BSB and the mixture subsequently extracted with 1 mL of BSB. The extracts were then used to inhibit the binding of anti-AMP to AMP-coated plates. The foods tested were rolled oats, a rice cereal, a raisin bran breakfast cereal, a chocolate filled cookie, and a chocolate candy. As shown in Table 1, in those samples spiked with 10 μg of added soluble AMP/100 mg of food (i.e., 100 ppm), the calculated IC_{50} values ranged from 5.4 to 24.4 μg, which is equivalent to 54−244 ppm of AMP in the food. When 1.0 μg of AMP was added to these same foods (10 ppm), the detection values ranged from 0.5 to 3.7 μg (5−37 ppm). Most of the spiked foods that produced values above or below the actual amount of added AMP did so for samples at both the 10 and 1.0 μg spiking level. For example, the rice cereal and the milk chocolate candy showed the greatest overestimation and the chocolate filled cookie the greatest underestimation. These variances for the expected values probably reflect either low levels of cross-reactivity or nonimmunological interference in the assay by components of the food.

The assay was further tested for robustness by spiking a variety of foods with known amounts of almond flour prior to aqueous extraction (Table 2). At a 1000:1 food/almond flour ratio (w/w), the theoretical yield of AMP in the sample is ~26−30 μg, on the basis of our observation that ~40% of the weight of almond flour is extractable protein and ~65−75% of the extractable protein is AMP. The range of values observed was 7.4−60 μg for the various foods tested. Rolled oats yielded about twice the predicted values, and whole wheat flour and rice cereal gave slightly higher than expected value. The chocolate filled cookie gave below predicted values and, in this assay, unlike that in Table 1, chocolate candy also gave low values. Additional testing showed that mixing chocolate candy or cookie with various foods in the presence of almond flour gave AMP values that were not markedly influenced by presence of the chocolate in the mixture (Table 2).

Table 2. Detection of AMP in Foods Spiked with Almond Flour

host food plus almond flour (at 1000:1 dry weight)	detected[a] AMP (μg of AMP/100 mg of food)
whole wheat flour	39.6 ± 4.5
raisin bran cereal	23.0 ± 6.6
rice cereal	38.0 ± 0.9
rolled oats	60.0 ± 4.8
chocolate filled cookie	7.4 ± 0.3
milk chocolate candy	7.4 ± 0.5
rice cereal and chocolate filled cookie, 1:1	23.9 ± 1.3
rolled oats and chocolate filled cookie, 1:1	49.7 ± 2.6
rice cereal and milk chocolate candy, 1:1	34.6 ± 3.4
rolled oats and milk chocolate candy, 1:1	53.4 ± 5.4

[a] The theoretical yield of AMP is assumed to be 26−30% of the amount of almond flour added (~40% of the almond flour is water soluble and ~65−75% of the extract is AMP). Thus, a 1:1000 mix would have 26−30 μg (26−30% of 100 μg).

Table 3. Quantification of AMP in Commercial Foods Listing Almond as an Ingredient Compared to Similar Foods Not Listing Almond as an Ingredient

food	dilution factor[a]	AMP detected (μg/100 mg of food)
cranberry almond wheat cereal	10^4	304 ± 32
wheat cereal	10	<0.1
oat almond cereal	10^4	35.8 ± 0.1
oat cereal	10	1.3 ± 0.1
cookie with almonds	10^4	1030 ± 5
cookie	10	0.4
rice almond cracker	10^4	454 ± 74
rice cracker	10	<0.1
milk chocolate and almond candy (1)	10^4	1010 ± 237
milk chocolate candy (1)	10	1.4 ± 0.9
milk chocolate and almond candy (2)	10^4	954 ± 15
milk chocolate candy (2)	10	0.3 ± 0.01

[a] The dilution of inhibitor extract used that gave a measurable value based on the standard curve. 1:10 was the minimum dilution tested.

We next tested several foods known to contain almond and compared each to a similar food that does not list almonds as an ingredient (Table 3). Included in this assay were two each of almond-containing cereals, cookies/crackers, and chocolate candies. The results show between 36 and 1030 μg of AMP/100 mg (360 and 10300 ppm, respectively) of defatted food listing almonds. In contrast, values for foods not listing almond as an ingredient gave AMP values of 1.4 μg/100 mg of defatted food (14 ppm) or less, with two foods giving values below the level of detection (0.1 μg, 1 ppm).

Effect of Processing on AMP Detection. As almonds are often subjected to harsh processing conditions prior to, or during, their addition of other foods, we tested the effect of roasting, blanching, and autoclaving (some of the most commonly employed process-

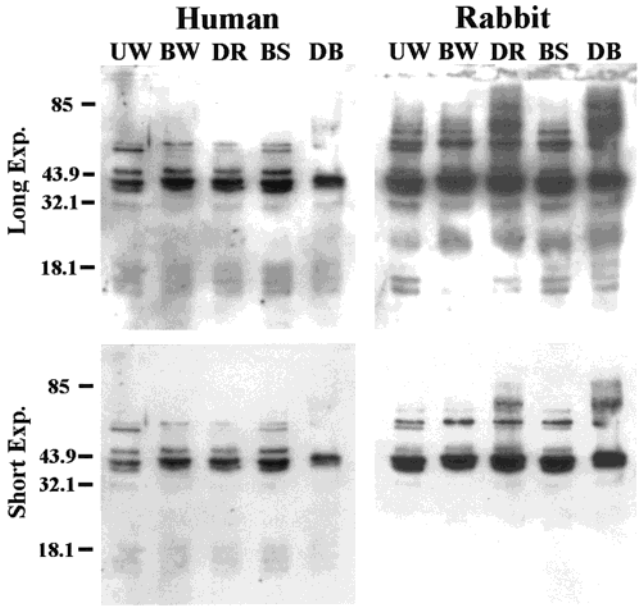

Figure 3. Western blot of reduced (SDS-PAGE with 2% β-ME) native and processed almonds probed with human almond-allergic and rabbit anti-almond antiserum. The blot was first probed with pooled human almond-allergic serum, then stripped, and reprobed with rabbit anti-AMP antisera. UW, unprocessed whole; BW, blanched whole; DR, dry-roasted; BS, blanched sliced; DB, dry-roasted and blanched. Long and short exposures of the same gels are shown for comparison.

Table 4. Effect of Processing on AMP Detection per Milligram of Protein Extract

cultivar	mg of AMP detected/mg of protein extract	value as % of unprocessed cultivar
Nonpareil		
unprocessed whole	1.06 ± 0.01	100
blanched whole	0.94 ± 0.01	89
dry-roasted whole	0.81 ± 0.01	76
blanched sliced	0.69 ± 0.09	65
dry-roasted, blanched whole	0.66 ± 0.03	62
Carmel		
unprocessed whole	0.81 ± 0.07	100
blanched whole	0.81 ± 0.004	100
dry-roasted whole	0.62 ± 0.05	77
dry-roasted, blanched whole	0.53 ± 0.08	65
oil-roasted whole	0.65 ± 0.03	80

ing methods) on the ability of AMP to be detected by the rabbit and human antisera. Protein extracts of processed and unprocessed Nonpareil and Carmel almonds were first normalized for protein content and then tested by inhibition ELISA. Some diminution of reactivity was noted for most of the samples; however, all retained >60% of their pretreatment reactivity, and both cultivars showed a similar pattern of reactivity (Table 4).

The processed Nonpareil samples were also analyzed by Western blot in which the rabbit IgG anti-AMP was compared to that of pooled human IgE from almond allergic antisera. The data showed that most protein bands were heat stable, although there was some variation in band intensity in the 55−57 kDa band pair (Figure 3). Interestingly, the two samples subjected to dry-roasting showed the staining of apparently new higher molecular weight bands as well as the intensification of other higher molecular weight bands (75−84 kDa). The processing stability of AMP was further investigated by subjecting whole almonds to autoclave temperature and pressure (121 °C/15 psi) for various

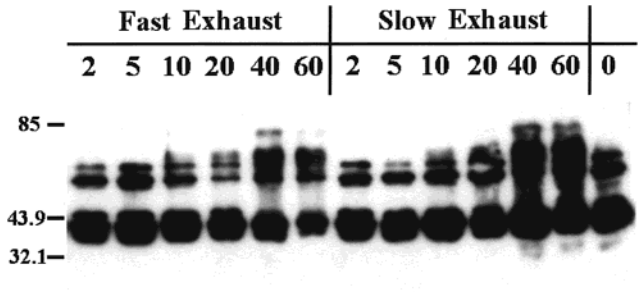

Figure 4. Western blot of protein extracts prepared from almonds subjected to autoclaving for the number of minutes indicated using either the slow or fast exhaust cycle and probed with rabbit anti-AMP antiserum. Also shown is a control lane (0) with protein extract from almonds not subjected to autoclaving.

Table 5. Detection of AMP in Almond Flour Derived from Whole Almonds Subjected to Autoclaving

duration of autoclaving (min)	mg of AMP detected/100 mg of almond flour	value as % of unautoclaved sample
0	27.0 ± 4.9	100
2	15.1 ± 2.5	56
5	15.2 ± 2.1	56
10	13.6 ± 3.8	50
20	12.8 ± 3.6	47
40	11.5 ± 2.0	43
60	9.3 ± 2.9	34

periods up to 1 h. As shown in Table 5, there was an initial loss of about half of the reactivity within the first 2 min with some additional loss over time as detected by ELISA. Western blot analyses, which are much less quantitative than the inhibition ELISA assays, showed a general stability of the individual peptides (Figure 4) as observed for the blanched and roasted almond samples described above. Interestingly, the 40 and 60 min samples displayed a slight brownish cast and yielded more intense signals in the higher molecular mass complex in Western blots, suggesting heat-induced protein aggregation. Both the slow exhaust and fast exhaust autoclave cycles yielded similar results.

DISCUSSION

Both in the United States and on a global basis, almonds lead the per capita consumption of tree nuts (at 0.55 lb/person in the United States; Almond Board of California, 1999 data). Five major cultivars (Carmel, Mission, Neplus, Nonpareil, and Peerless) account for 90% of the U.S. almond market (S. Cunningham, personal communication).

A recent survey suggests that 0.5% of the U.S. population has some degree of hypersensitivity to tree nuts. The same survey indicated 0.6% of the population is allergic to peanuts, and an additional 0.3% report allergies to nuts but do not distinguish between peanut or tree nut allergy (*12*). It is estimated that at least 100 people die per year from food allergy in the United States and that the most frequent culprits are seed proteins (*13*, *14*). Among individuals with life-threatening tree nut sensitivity, almonds have been implicated (*15*−*17*). There is currently no treatment for IgE-mediated food allergy and, therefore, complete avoidance is recommended. Avoidance, however, is not always possible because the implicated allergen may be present in trace amounts in a processed food due to shared

equipment or it may be in a food in which its presence was not expected by the consumer.

It is apparent that inexpensive assays to detect tree nuts in food would be of value to the food industry to help monitor cross-contamination of foods and avoid expensive recalls; this action, in turn, would help improve food safety for the allergic consumer. Recently, Hlywka et al. (*18*) described a sandwich ELISA in which whole almond extract, rather than the major allergen, AMP, was used as the immunogen for sheep (first or capture) antibody and rabbit (second) antibody. Despite the differences in the purity of the immunogen between our assay and that of Hlywka et al., both showed some degree of apparent cross-reactivity with other tree nuts and seeds (*7*, *18*). Efforts to remove cross-reactive antibody by affinity chromatography have thus far proven to be unsuccessful (unpublished observations). A peanut assay with detection limits of 400 ng/g (0.4 ppb) (*19*) has been commercially developed and is already in use in the United Kingdom. However, such ultrasensitive assays raise the very real questions of false positives. An important and distinguishing aspect of our assay is the demonstration that there is extensive overlap between the peptides recognized by allergic patients and those recognized by the rabbit antibody used in the assays. This correlation may even extend to the epitopes recognized because both the rabbit and human show nearly identical patterns of peptide reactivity even when the proteins are subjected to harsh food-processing regimes (e.g., blanching and roasting), which might be expected to differentially affect epitopes on the same peptide. Pasini et al. (*16*) have recently described Western blotting data from a small number ($n = 5$) of patients expressing IgE, which reacts with almond proteins. Interestingly, those patients with demonstrable clinical allergic reactions ($n = 2$) to almond recognize a set of peptides (at ~37 kDa) that differed from those ($n = 3$) with anti-almond IgE but lacking allergic reactions (in the 50−62 kDa range). Verification of this pattern of association will necessitate a larger sample size. Nevertheless, it would appear that our rabbit antisera recognize both of these sets of peptides as do our human antisera. This similarity in recognition should enhance the ability of the assay to determine not only the amount of almond in a given food sample but the relative allergenicity potential as well. The fact that most of the assay-reactive peptides are relatively heat stable helps to explain why anecdotal patient reports indicate that processed almonds remain allergenic. To confirm this pattern of reactivity, double-blind, placebo-controlled patient challenges (including a group with systemic reactions and a group with oral allergy syndrome) with variously processed almonds will need to be performed to determine what role, if any, the heat-labile allergens play in such reactions. Immunoassays based on total almond extract may be skewed in their ability to predict the levels of the major allergens if the antibody reactivity of the allergenic and non-allergenic peptides are differentially affected in a substantial way by food processing.

The data presented support our position that AMP is an excellent marker protein for use in an ELISA to detect trace amounts of almond in food, especially because it accounts for the overwhelming majority (65−75%) of total almond protein and is substantially heat stable. In addition, although other proteins may also be implicated in almond food allergy for a particular patient, AMP appears to include the key IgE-reactive polypeptides in sera from patients with life-threatening almond food allergy (*11*, *16*).

ABBREVIATIONS USED

AMP, almond major protein; BSB, buffered saline borate; ELISA, enzyme-linked immunosorbant assay; NC, nitrocellulose; PBS, phosphate-buffered saline.

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