

A Cherry Seed-Derived Spice, Mahleb, is Recognized by Anti-Almond Antibodies Including Almond-Allergic Patient IgE

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Abstract: There are a number of examples of immunologic cross-reactivity elicited by pollens, fruits, seeds, and nuts of closely related plant species. Such cross-reactivity is of particular concern for patients with food allergies. In this report, we investigated a spice (mahleb) that is prepared from the kernel of the St. Lucie cherry, *Prunus mahaleb*, for cross-reactivity with almond (*Prunus dulcis*), using enzyme-linked immunosorbent assay (ELISA) and Western blot. Almond and mahleb are members of the same genus. Cross-reactivity between the mahleb and almond was demonstrated by reaction of cherry and almond kernel protein extracts with antibodies raised against almond proteins. Almond-specific murine monoclonal IgG, rabbit polyclonal IgG, and almond-allergic serum IgE each exhibited cross-reactivity with cherry kernel protein. Because of the demonstrated cross-reactivity between almond and mahleb, these findings should be of special concern to almond-allergic patients and attending medical personnel.

Keywords: almond, cherry seed, cross-reactivity, food allergy, mahleb

Practical Application: Mahleb is cross-reactive with almond-allergic patient IgE and is likely to elicit an allergic response in sensitive individuals. This information is important for the protection of almond-allergic patients.

Introduction

Tree nuts are a popular and beneficial food source due to their contents of fiber, protein, unsaturated fats and phenolics (Venkatachalam and Sathe 2006). However, as with a variety of other foods, tree nuts present potential health risks to those experiencing allergic reactions upon consumption (Sicherer and others 2010). Allergy to tree nuts has grown to affect significant percentages of populations, particularly in the United States and Europe. Type I allergic reactions are mediated by IgE antibodies found in the serum and mucosal tissues of sensitized individuals (Sicherer and Sampson 2014). These antibodies recognize specific epitopes on allergen proteins found within the sensitizing agent. While antibody recognition is usually a highly specific interaction, individuals with allergies to certain tree nuts can also have similar responses induced by different, though typically related, species of tree nuts. Documented examples include cross-sensitivity between cashew and pistachio and between walnut and pecan (Sicherer 2001; Garcia and Lizaso 2011). In these cases, the tree nuts capable of eliciting an allergic reaction are members of the same botanical family: *Anacardiaceae* for cashew and pistachio (Goetz and others 2005; Willison and others 2008) and *Juglandaceae* for walnut and pecan (Goetz and others 2005). These close genetic relationships are reflected in amino acid sequence homologies, which, in turn, can yield identical or antigenically similar, and therefore cross-reactive, epitopes. The same effect would therefore be expected from species with a

similar or even closer genetic relationship. In this report, we investigated the potential for cross-reactivity between almond and the spice, mahleb, prepared from seed kernels of the cherry species, *Prunus mahaleb*. Both are members of the same genus. This work was precipitated by the March 2015 recall of putative almond-containing ground cumin spice by the Canadian Food Inspection Agency (CFIA, <http://www.inspection.gc.ca>). The recall was rescinded a month later after it became apparent that it was mahleb that was detected by the anti-almond ELISA test due to cross-reactivity. Significantly, it was stated in the rescinding press release that "... there is no evidence that these products could pose a risk to individuals allergic to almond." It was also stated that "... additional testing has confirmed that the original laboratory results were false positives." (<http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recall-warnings/complete-listing/2015-04-30/eng/1430432363404/1430432364107>).

Almonds are a well characterized source of tree nut allergy with many people in the United States and Europe having severe IgE-mediated reactions upon consumption (Sicherer and others 2010; Nwaru and others 2014). Almond IgE-reactive proteins include lipid transfer protein (Pru du 3; Buhler and others 2015), profilin (Pru du 4; Tawde and others 2006), a 60S ribosomal acidic protein (Pru du 5; Abolhassani and Roux 2009), and the 11S legumin-like protein (Pru du 6; Willison and others 2011). Although many other members of *Prunus*, such as apricot, plum, peach, and cherry, are widely consumed and can cause allergic reactions in sensitive individuals, cross-reactivity (that is, allergic reactivity) with almond in fruit-sensitized individuals is not common (Fernández and others 1997; Rodriguez and others 2000). This is likely due to substantial differences between the fruit pulp and/or peel proteins responsible for fruit allergy and those of the seed kernel responsible for almond allergy. Allergy to fruit of the Rosaceae family is primarily mediated by PR-10, profilins, and lipid transfer proteins (Scheurer and others 1997; Scheurer and

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Table 1—Clinical characteristics of almond allergic subjects.

Patient no.	Almond ImmunoCAP (kU/L)
0279	2.10
0284	9.87
0426	8.73
0451	5.72
13779	9.99
Patient No.	Almond RAST ^a
68	Class 5

^aRAST = radioallergosorbent test.

others 2001a, b; Borges and others 2006; Andersen and others 2009).

Prior to the cumin recall, the spice mahleb (a.k.a., mahalab, mahlep, mahaleb), which is prepared from ground seed kernels of the St. Lucie cherry, *P. mahaleb*, had received scant attention. The mahleb spice is widely used in baking and confectionary in various countries in the Mediterranean, the Middle East, and India and imparts a degree of bitterness and a mild cherry aroma (Ozturk and others 2014). Because of the common plant tissue source and the genetic relationship between almond and mahleb, and the cross-reactivity demonstrated by the commercial ELISA kit that sparked the cumin recall, it is reasonable to suspect that mahleb may contain cross-reactive proteins that react with anti-almond IgE in almond-allergic patients. Consequently, severe and potentially fatal reactions to mahleb might be possible for almond-sensitized individuals even if they had never consumed mahleb previously (Bock and others 2001). Adding to the concern is the fact that allergic reaction mediated by seed storage proteins tend to be considerably more severe than those mediated by allergens found in the fruit (Ewan 1996; Flinterman and others 2008).

In this study, we assessed the degree of cross-reactivity between proteins extracted from mahleb spice and almond seeds. The extracts were reacted with a mouse monoclonal antibody (mAb) and rabbit polyclonal IgG raised against almond protein and evaluated by direct binding and inhibition Western blots and ELISAs. In addition, almond-allergic patients' serum IgE was reacted with almond and cherry kernel protein extracts in Western blots. By comparing the similarity and intensity of the signals generated by almond and cherry kernel protein extracts, we demonstrated considerable cross-reactivity between the almond and cherry seed proteins.

Materials and Methods

Materials

Nonpareil almonds were supplied by the Almond Board of California (Modesto, Calif., U.S.A.). Mahleb spice was purchased from a local grocery store. *P. mahaleb* cherry seeds were kindly provided by Dr. Thomas M. Gradziel at Univ. of California, Davis.

Allergic patients' sera

Blood samples from 6 patients testing positive for IgE binding to almond proteins were either purchased from PlasmaLab Intl. (Everett, Wash., U.S.A.) or generously provided by the laboratory of Scott H. Sicherer, MD (Div. of Allergy and Immunology, Mount Sinai School of Medicine, New York, N.Y., U.S.A.). The study was approved by the Institutional Review Boards of Florida State Univ. and Mount Sinai School of Medicine. The presence of almond-reactive IgE was confirmed by Pharmacia ImmunoCAP assay (Pharmacia Diagnostics, Uppsala, Sweden) or radioallergosorbent test (Table 1).

Rabbit anti-almond antiserum

Rabbit antiserum was raised against almond proteins as previously described (Acosta and others 1999). Briefly, 200 µg of almond protein in 1 mL of Freund's complete adjuvant (Sigma Aldrich, St. Louis, Mo., U.S.A.) was administered intradermally to each of 2 New Zealand White rabbits (*Oryctolagus cuniculus*). Beginning 3 wk after immunization, 6 booster doses were administered at 3 to 6 wk intervals. Postimmunization 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.

Anti-almond mAb production

The anti-almond 11S legumin mAb 4C10 was produced in the hybridoma facility at Florida State Univ. by standard techniques (McCullough and Spier 1990) in accordance with Natl. Inst. of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Detailed procedure of antibody production has been reported (Su and others 2013). The resultant clones were screened as previously described by Willison and others (2011). Media supernatant was diluted to 1:50000 (v/v) in phosphate buffered saline-Tween (PBS-T; 14 mM phosphate buffer pH 7.2, 0.0005% Tween 20) and used in Western blot assays.

Flour preparation

Nonpareil almond seeds, *P. mahaleb* cherry seeds, and the mahleb spice were finely ground in a blender. The flours were defatted using a Soxhlet apparatus with petroleum ether as the solvent for 8 h. After overnight drying in a fume hood, defatted flours were passed through a 40-mesh sieve for homogeneity and stored at -20 °C until further use.

Protein extracts

Defatted almond, cherry, and mahleb flours were extracted with borate saline buffer (BSB, 0.1 M H₃BO₄, 0.025 M Na₂B₄O₇, 0.075 M NaCl, pH 8.2) at room temperature for 1 h with gentle rotation followed by centrifugation at 14000 × g for 10 min (Sathe and others 1997). Protein concentrations of the extracted samples were determined by measuring the absorbance at 280 nm of a 2 µL sample using a Nanodrop 2000c instrument (ThermoFisher Scientific, Wilmington, Del., U.S.A.). Soluble protein concentrations were 19 mg/mL for almond extract and 8.9 mg/mL for cherry extract. Initial analysis of protein quantification, SDS-PAGE and Western blot were run using flour prepared from whole *P. mahaleb* cherry kernels. These data mirrored that of the purchased spice (data not shown). Extracts of the commercial spice were used thereafter.

ELISA

Whole almond and cherry protein extracts were prepared at 0.01 mg/mL in coating buffer (0.0515 M sodium phosphate, 0.0485 M citric acid, pH 5.0) and coated onto wells of a 96 well polystyrene plate (Corning, New York, N.Y., U.S.A.) for 1 h at 37 °C. The wells were washed with PBS-T and blocked with 5% nonfat dry milk in PBS-T. Coated and blocked plates were then reacted with rabbit anti-almond serum at 1:100000 (v/v) dilution and developed with a goat anti-rabbit HRP conjugated antibody (Sigma Aldrich) at 1:50000 (v/v) dilution and o-phenylenediamine dihydrochloride (OPD) substrate tablets (Invitrogen, Carlsbad, Calif., U.S.A.). Signal intensity was determined by measuring the absorbance of each sample well at 495 nm using

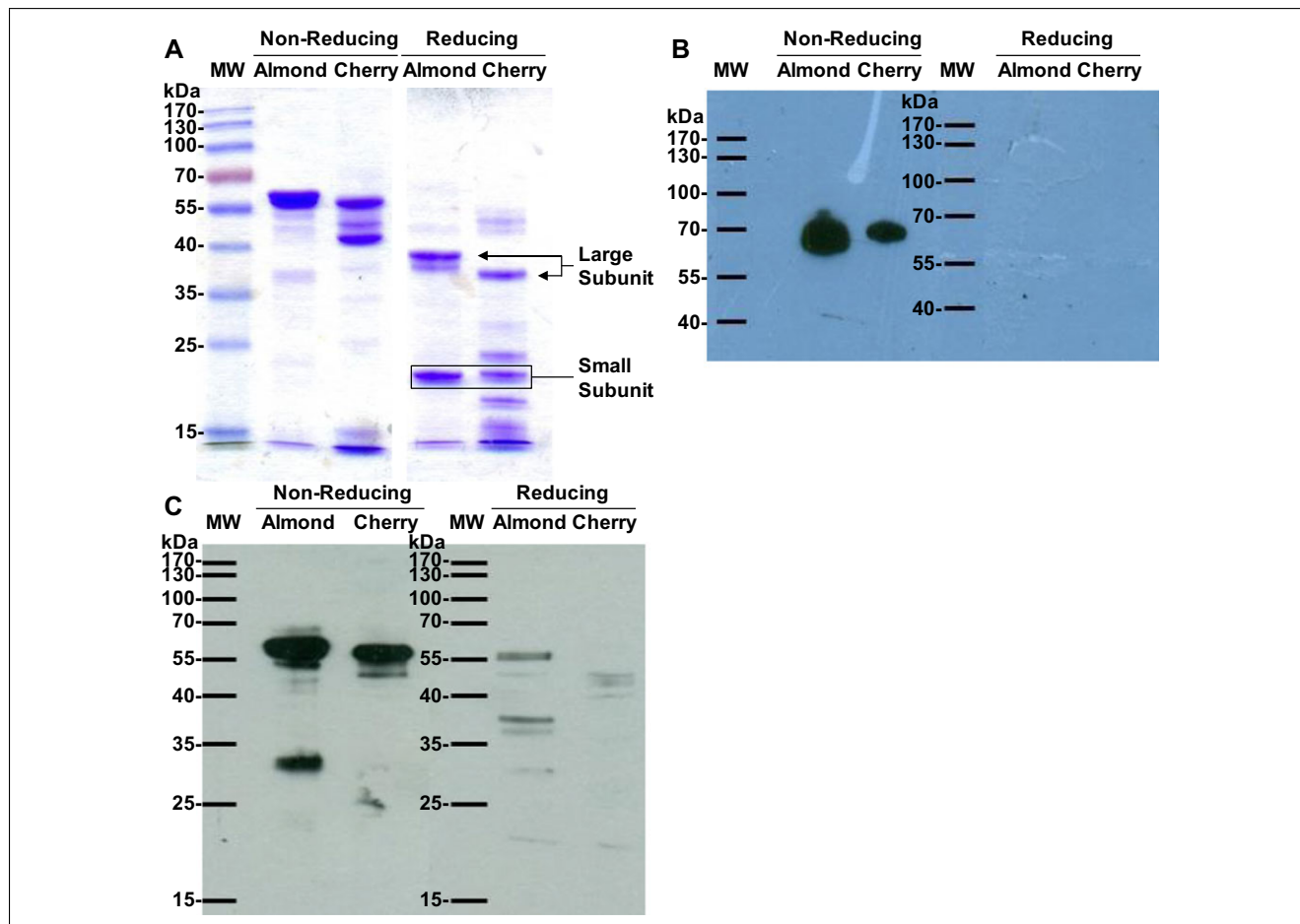


Figure 1—(A) SDS-PAGE of almond and mahleb protein extracts (5 μ g each/lane) under nonreducing and reducing conditions. The large and small subunits of 11S globulin are indicated. (B) Western blot of almond and mahleb protein extracts under non-reducing and reducing conditions developed with anti-almond 11S globulin mAb, 4C10. C. Western blot of almond and mahleb protein extracts under nonreducing and reducing conditions reacted with rabbit anti-almond protein antiserum. MW, molecular weight standard.

a Gemini EM microplate reader (Molecular Devices, Sunnyvale, Calif., U.S.A.).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Whole almond and cherry protein extracts were boiled for 5 min in nonreducing (1% SDS, 50 mM Tris-HCl, pH 6.8, 0.2% bromophenol blue) or reducing buffer (1% SDS, 0.6 M β -mercaptoethanol, 50 mM Tris-HCl, pH 6.8, 0.2% bromophenol blue), loaded onto 12% acrylamide gel and run in a vertical Tetra blotting module (Bio-Rad, Hercules, Calif., U.S.A.) at 135 V for approximately 1.5 h. Almond and cherry protein load was 5 μ g/lane.

Western blot

Following SDS-PAGE, almond and cherry proteins were transferred to nitrocellulose membranes (Millipore, Bellerica, Mass., U.S.A.) at 80 V for 3 h using a vertical Tetra blotting module (Bio-Rad). Membranes were cut into 3 mm wide strips, blocked overnight in 5% nonfat dry milk in PBS-T before incubation with mAb or antisera diluted in PBS-T.

Inhibition assays

Mouse mAb and rabbit antiserum were 1st diluted at 1:50000 (v/v) and 1:100000 (v/v) in PBS-T, respectively. For rabbit anti-

serum, 1062.5 μ L of diluted serum was mixed with 1 μ L (19 μ g) of almond or 2.13 μ L (19 μ g) whole cherry protein extract. A 5-fold dilution series was then created. Mouse mAb was diluted and mixed in the same fashion to form a 5-fold dilution series. The antibody-antigen mixtures were incubated at 37 $^{\circ}$ C for 1 h before being added to blocked nitrocellulose strips containing almond proteins. Nitrocellulose strips were incubated with inhibited antiserum for 1 h at room temperature then washed with PBS-T and developed with a goat antirabbit HRP conjugated antibody (Sigma Aldrich) at 1:100000 (v/v) dilution or rabbit antimouse HRP (Sigma Aldrich) at 1:50000 (v/v). Both assays were developed with ECL substrate (GE Healthcare, Chicago, Ill., U.S.A.).

Results and Discussion

Initial experiments sought to compare the protein profiles of almond and cherry kernel aqueous extracts. To this end, the extracts were subjected to SDS-PAGE under reducing and nonreducing conditions (Figure 1A). The most pronounced protein bands in the almond extract have been identified and characterized as allergens (Willison and others 2011). The most abundant almond allergen is the 11S legumin, Pru du 6, a $\sim$ 427 kDa hetero-hexamer in which each subunit contains a large and small subunit of roughly 40 and 20 kDa, respectively (Willison and others 2011). These subunits are held together by a disulfide bond and separate under reducing conditions in SDS-PAGE analysis. A protein with

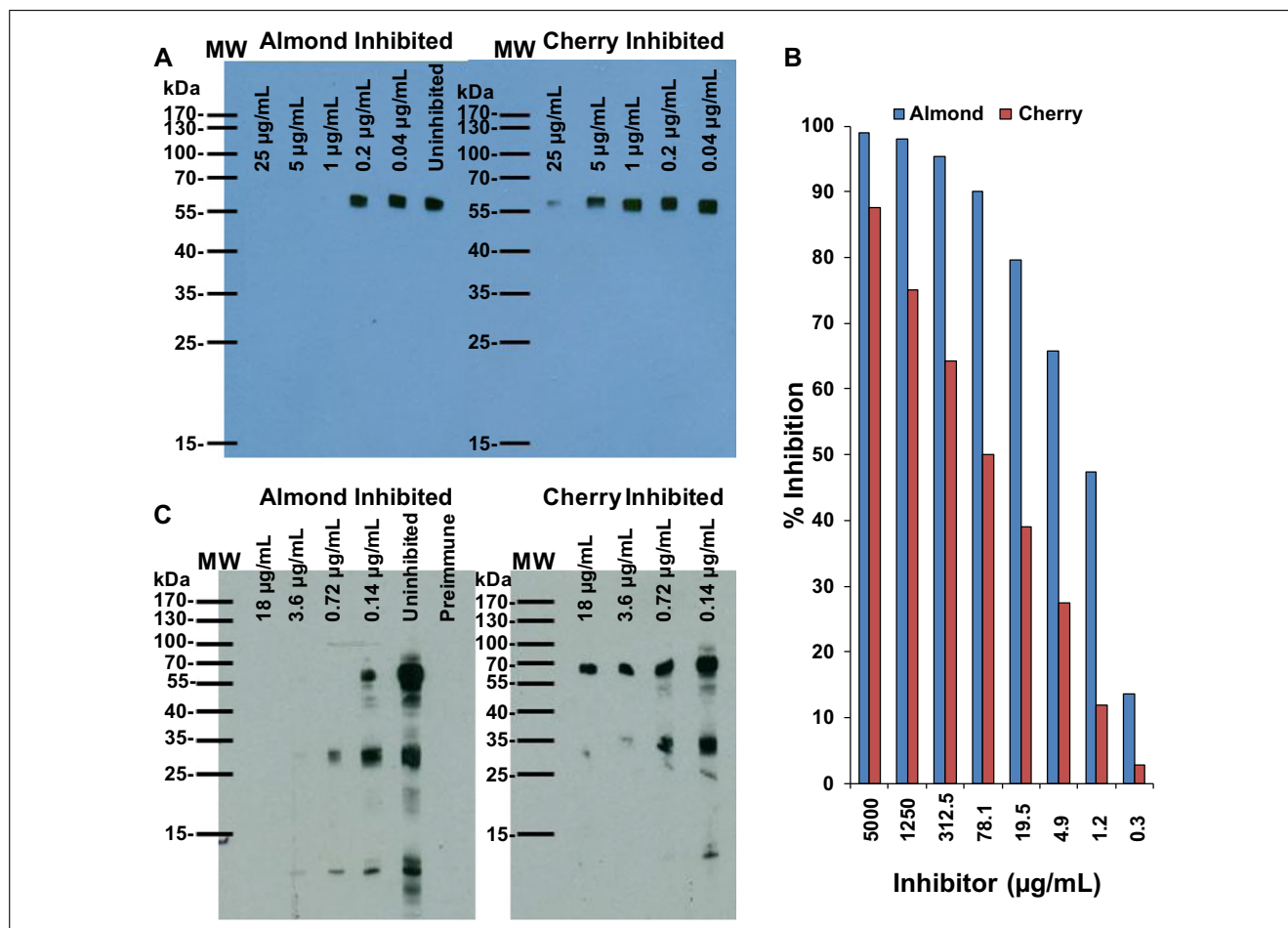


Figure 2—(A) Inhibition Western blot of almond proteins (5 μg each/lane) developed with 4C10 mAb inhibited with almond (left) or cherry (right) protein extracts over a range of concentrations. (B) Inhibition ELISA of almond proteins developed with rabbit anti-almond antiserum inhibited with almond (blue) and cherry (red) protein extracts over a range of concentrations. (C) Inhibition Western blot of almond proteins (5 μg each/lane) developed with rabbit anti-almond antiserum inhibited with almond (left) and cherry (right) protein extracts over a range of concentrations. MW, molecular weight standard. Preimmune, rabbit serum collected before immunization.

homologous characteristics was clearly visible in the cherry kernel extract, although the presumptive large subunit runs slightly faster than that of the almond under reducing conditions. Because Pru du 6 is a major IgE-binding protein in allergic patients' sera, it could be expected that the cherry homologue may serve as a target for cross-reactive antibodies in almond-allergic patients.

The amino acid sequence of the cherry 11S globulin and other protein homologues are not available for comparison to those of almond, however, the degrees of similarity are likely to be high. For example, the 11S globulin of peach, *P. persica*, (accession number: XM007201654), another member of the *Prunus* genus, shares 98% identity and 99% similarity with almond 11S globulin (accession number: GU059260). Consequently, our assessment of the almond and mahleb protein relatedness focused on the relative ability of anti-almond antibodies to cross-react with those of mahleb.

Almond-specific murine monoclonal, rabbit polyclonal IgG, and patients' serum IgE antibodies were assessed. To further substantiate the identity of the cherry kernel homologue of the almond 11S protein observed on SDS-PAGE, a monoclonal antibody (mAb 4C10) raised against the almond 11S legumin, was reacted with almond and mahleb soluble protein extracts in Western blots (Figure 1B). This mAb was previously shown to bind a conformational epitope present in Western blots only under

nonreducing conditions (Willison and others 2013). Interestingly, this mAb has been shown to inhibit the binding of a significant fraction of almond-allergic patients' anti-almond 11S IgE. Thus, the mAb recognizes an epitope, or a region overlapping with an epitope, recognized by almond-allergic patient IgE (Willison and others 2013). Its binding to the cherry kernel protein (Figure 1B) demonstrated not only that the band indicated is the cherry homologue of Pru du 6 but that the almond and cherry legumin proteins share a common or similar epitope and, in both cases, the epitope is destroyed by reduction. Judging by the relative intensity of the corresponding almond and cherry kernel signals on Western blot, mAb 4C10 exhibited a lower affinity for the cherry legumin (Figure 1B). These data demonstrated cross-reactivity between almond and cherry 11S globulins at a single, clinically relevant, epitope. The commercial mahleb spice was analyzed in SDS-PAGE and Western blots. However, the immunoresponse from known genetic *P. mahaleb* cherry seeds has also been tested and similar response has been observed.

To gain a broader sense of the potential for cross-reactivity between almond and cherry kernel proteins, rabbit antiserum raised against almond protein extract was similarly assessed. Western blots clearly demonstrated that many of the almond kernel proteins share epitopes with their cherry kernel protein homologues

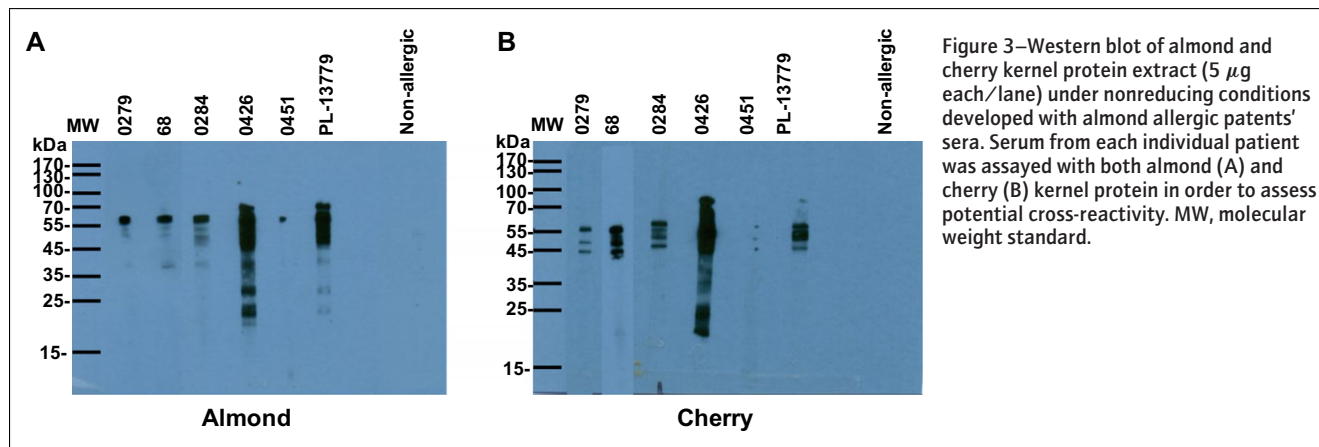


Figure 3—Western blot of almond and cherry kernel protein extract (5 μ g each/lane) under nonreducing conditions developed with almond allergic patients' sera. Serum from each individual patient was assayed with both almond (A) and cherry (B) kernel protein in order to assess potential cross-reactivity. MW, molecular weight standard.

(Figure 1C). In line with these observations are those of a previous report demonstrated significant cross-reactivity between almond and kernels of other members of the Prunoideae subfamily, including peach, plum, apricot, nectarine, and cherry, using 2 commercial ELISA-based almond detection kits (Röder and others 2011). The cherry species assessed in that case was the sweet edible pulp cherry variety, *P. avium*. It should be noted that peach and apricot kernels are used in a confectionary known as persipan. The 2 ELISA kits tested by Röder and others (2011) are described by the manufacturers as cross-reacting with apricot kernel protein. In addition, Su and others (2015) reported cross-reactivity of the anti-almond 11S legumin mAb 4C10 with *P. persica*, *P. mira*, *P. webbii*, *P. argentea*, *P. fenizliana*, *P. bucharia*, and *P. tangutica* seed kernel proteins in ELISA, Western blot, and dot blot.

Our Western blots using the rabbit anti-almond antiserum demonstrated reactivity with a similar array of proteins in the almond and cherry kernel extracts (Figure 1C). As with the mouse mAb, the rabbit antibodies gave stronger signals with almond as compared to mahleb. For both extracts, most of the signal intensity was attributed to the 11S legumin proteins under both non-reducing and reducing conditions. These data also exhibited that many of the shared epitopes are conformational and are denatured by the reducing conditions in both almond and cherry kernel proteins.

To further gauge the degree of relative affinity of anti-almond antibodies for almond and cherry kernel proteins, competitive inhibition reactions were performed wherein almond and cherry kernel proteins were pre-incubated with mouse or rabbit antibodies prior to reaction with almond protein in Western blots and, for the rabbit antibodies, ELISAs. For mAb 4C10 inhibition Western blots, approximately 25-fold more cherry kernel than almond protein was needed to inhibit an almond 11S globulin-4C10 interaction (Figure 2A).

The rabbit polyclonal antibody inhibition ELISA data showed that, at most concentrations, the cherry protein extract was a substantially less potent inhibitor, though at the highest concentration, its ability to inhibit approached that of the almond (Figure 2B). About 60-fold more cherry kernel protein than almond protein was needed for 50% inhibition. A similar effect was seen with inhibition Western blots using the polyclonal antibodies (Figure 2C). Together, these results illustrated that, as might have been anticipated, the immunogen, an almond protein extract, is a more complete and efficient inhibitor than the homologous set of proteins in the cherry kernel extract. Significantly, however, it appeared that a substantial proportion ($\geq 87\%$) of the rabbit anti-almond

antibodies did react with cherry protein epitopes, albeit at lower average overall affinity (Figure 2B).

This high degree of cross-reactivity in the rabbit anti-almond antisera raises the possibility that the cherry kernel spice might be allergenic in almond sensitized individuals. To address this possibility, almond and cherry kernel protein extracts were reacted with almond-allergic patients' serum IgE. Sera from 6 patients with a clinically confirmed allergy to almonds were assayed by Western blot under non-reducing conditions. Of the 6 patients, 5 showed strong reaction to both almond and cherry kernel proteins (Figures 3A and B). The sixth patient showed weak reaction to almond and cherry kernel proteins. This minimal reaction might reflect a low titer of serum anti-almond IgE or that the IgE recognizes primarily conformational epitopes which are denatured by SDS-PAGE. By reacting the mahleb spice with almond-allergic patients' sera, we have demonstrated that this spice clearly contains proteins with sufficient homology to those of almond to cross-react with their serum IgE. This observation indicates that mahleb spice may well be capable of eliciting an allergic response in almond-sensitized individuals with the potential to pose a serious health risk.

Conclusions

We sought to determine if mahleb spice, prepared from the kernels of a species of cherry, *P. mahaleb*, was capable of cross-reacting with antibodies raised against almond, including those of almond-allergic patients. The almond and cherry kernel extracts were reacted with antibodies from 3 different sources: a mouse anti-almond 11S globulin mAb, a rabbit anti-almond protein IgG, and almond-allergic patients' serum IgE. In each case, the antibodies recognized proteins in both almond and cherry kernel extract though the apparent affinity of the antibodies for cherry proteins were lower as would be expected given that the immunizing/sensitizing agents were almond proteins.

These results indicate that this spice, often found in Mediterranean and Middle Eastern baked goods and confectionary, is likely to elicit an allergic response in some of the more sensitive almond-allergic individuals and should serve to increase awareness of patients and medical personnel to the potential danger of inadvertent consumption. As the styles of cooking and prepared foods from traditional mahleb-consuming regions are becoming increasingly popular in western countries, the potential for mahleb-induced allergic reactions in almond sensitive individuals will likely increase. The fact that some commercial anti-almond kits cross-react with mahleb (as well as peach kernel proteins in persipan) should not

be viewed as a fault described as a “false positive” but rather as an added element of protection since it is likely that at least some almond allergic individuals would react upon consuming the cross-reactive product. Assays specifically targeting peach or cherry kernel proteins are not available and, because of the limited consumption of these foods in western countries, are unlikely to be marketed in the near future. Consequently, almond allergen test kits should serve as a 1st line of defense against unwelcome exposure to cross-reactive ingredients while the development of analytical methods that specifically target peach and cherry kernel proteins is underway.

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