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# Conformational epitope mapping of Pru du 6, a major allergen from almond nut

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### ABSTRACT

Tree nuts are a widely consumed food. Although enjoyed safely by most individuals, allergic reactions to tree nuts, including almond, are not uncommon. Almond prunin (Pru du 6), an 11S globulin (legumin), is an abundant nut seed protein and a major allergen. Conformational epitope mapping studies of prunin have been performed with a murine monoclonal antibody (mAb) 4C10. This mAb reacts with non-reduced but not reduced prunin in immunoblotting assays, indicating the recognition of a conformational epitope. 4C10 competes with patient IgE, as assessed by ELISA, indicating clinical significance of the epitope. To characterize the 4C10 epitope, hydrogen/deuterium exchange (HDX) monitored by 14.5T Fourier transform ion cyclotron resonance mass spectrometry (MS) was performed on the native prunin–4C10 complex and on uncomplexed native prunin. Several epitope candidate peptides that differ in deuterium uptake between the complexed and uncomplexed forms were identified. The epitope was further mapped by analyzing chimeric molecules incorporating segments of the homologous soybean allergen, Gly m 6, in immunoassays. These data indicate that the 4C10 epitope overlaps with a subset of patient IgE binding epitopes on almond prunin and further supports HDX-MS as a valid technique for mapping conformational epitopes.

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## 1. Introduction

Allergic reactions to tree nuts have become a global health concern as the number of affected individuals continues to rise (Sicherer et al., 2010; Zuidmeer et al., 2008). Tree nuts are typically eaten as snacks or are incorporated into food dishes. Almonds are one of the most commonly consumed tree nuts in the United States (King et al., 2008). Per capita consumption in the US from 2010 is reported as almond (1.6 lb), walnut (0.7 lb), pistachio (0.54 lb), pecan (0.54 lb), hazelnut (0.05 lb), and mixed nuts (cashew/macademia/Brazil nut, 1.6 lb) (Plattner and Perez, 2011). Almond ranks third behind walnut and cashew, in the percentage of patients in the US Food Allergy and Anaphylaxis Network Peanut and Tree Nut Registry reporting clinical IgE mediated allergic reactions (Sicherer et al., 2010). A major IgE-reactive allergenic protein in almonds is prunin (Pru du 6), a legumin (Sathe et al., 2002; Willison et al., 2011; Wolf and Sathe, 1998). Almond prunin,

like other 11S globulins, is hexameric in form with each polypeptide comprised of a large 40–42 kDa acidic  $\alpha$ -chain and a small 20 kDa basic  $\beta$ -chain. The two chains are linked by a disulfide bond (Breiteneder and Radauer, 2004; Garcia-Mas et al., 1995; Jin et al., 2009; Sathe et al., 2002; Shewry et al., 2003). Two isoforms of almond prunin have been identified, Pru du 6.01 and Pru du 6.02, which share 64% amino acid sequence identity and 77% similarity (Garcia-Mas et al., 1995; Willison et al., 2011). IgE reactivity to these two isoforms was found to vary significantly, with Pru du 6.01 being recognized by 50% and Pru du 6.02 by 28% of patients in the tested population (Willison et al., 2011).

B cell epitopes are typically categorized as either sequential (linear and continuous) or conformational (discontinuous) (Barlow et al., 1986; Gershoni et al., 2007; Pomés, 2010; Sampson, 2004). Sequential epitopes consist of short contiguous stretches of amino acids and can be readily identified by probing synthetic overlapping peptides with serum from sensitized individuals (Lin and Sampson, 2009). Extensive epitope mapping studies have been performed to identify and compare sequential epitopes on several tree nut allergens (Barre et al., 2007, 2008; Robotham et al., 2002, 2005, 2009; Sharma et al., 2011a,b; Sordet et al., 2009; Venkatachalam et al., 2008; Wang et al., 2002, 2003; Willison et al., 2011). Whereas many sequential nut protein epitopes have been characterized, few studies have focused on conformational epitopes on tree nut

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allergens. The dependence of conformational epitopes upon the tertiary structure wherein one or more peptide segments must be held in a specific orientation for antibody recognition (Barlow et al., 1986; Gershoni et al., 2007; Lin and Sampson, 2009; Sampson, 2004), requires sophisticated techniques such as large scale mutagenesis, X-ray crystallography of immune complexes, nuclear magnetic resonance (NMR), phage display, or the generation of chimeric molecules for identification (Gershoni et al., 2007; Pomés, 2010).

Studies done on tree nut allergens have largely focused on the generation of chimeric molecules as they are easy to create, cost effective, and maintain the overall structure of the allergen (Alcocer et al., 2004; Robotham et al., 2010; Xia et al., 2010). This approach has been successfully applied to the Brazil nut allergen, Ber e 1, where probing of the chimeric molecules by microarray identified a conformational IgE binding epitope located in a helix–loop–helix region (Alcocer et al., 2004). Similarly, a conformational murine IgG binding epitope also targeted by a subset of patient IgE antibodies was identified near the N-terminus of the cashew allergen, Ana o 2, by probing a large set of chimeric and point-mutated molecules in dot-blot and enzyme-linked immunosorbent (ELISA) assays (Robotham et al., 2010; Xia et al., 2010).

To improve our knowledge of conformational epitopes on tree nut allergens, the application of new techniques for epitope identification is critical. Recently, hydrogen/deuterium exchange footprint analysis, monitored by 14.5T Fourier transform ion cyclotron resonance mass spectrometry (HDX-MS), has been applied to conformational epitope mapping studies (Coales et al., 2009; Zhang et al., 2011). HDX-MS can map the surfaces of proteins in their native state based on the solvent accessibility of exposed hydrogens on the amide backbone (Chalmers et al., 2006; Lanman and Prevelige, 2004). The binding of ligands, such as antibodies, followed by comparative mass determination by MS identifies peptides that are likely shielded by the ligand (e.g., epitopes) (Baerga-Ortiz et al., 2002; Chalmers et al., 2006; Coales et al., 2009; Lanman and Prevelige, 2004; Zhang et al., 2011).

To date, limited information is available on conformational epitopes on almond Pru du 6. To investigate these epitopes, we utilized a previously characterized murine monoclonal antibody (mAb), 4C10, that is highly reactive to native prunin and appears to recognize a labile epitope (Kshirsagar et al., 2011). Here we apply HDX-MS to the analysis of native prunin in complex with mAb 4C10 to identify the likely epitope. Analysis of chimeric molecules containing the homologous soybean allergen, Gly m 6, and alanine mutants in conjunction with atomic models further localized the conformational epitope to three discontinuous strands. We also show that 4C10 binding can compete with patient IgE and IgG binding to Pru du 6, suggesting overlap between human and murine epitopes. The results support the utility of HDX-MS for the identification of human allergy relevant conformational epitopes on targeted allergens.

## 2. Methods

### 2.1. Human sera

Blood samples were obtained with informed consent from patients with convincing histories of repeated clinical reactions to almond in the absence of other tree nut exposures or were purchased from PlasmaLab International (Everett, WA). The study was approved by the Institutional Review Boards of the University of California at Davis, and Florida State University. Sera were frozen at  $-80^{\circ}\text{C}$ . The presence of almond-reactive IgE was confirmed by means of Pharmacia ImmunoCAP assay (Pharmacia Diagnostics, Uppsala, Sweden) or by Western immunoblotting as described

below. Control serum was obtained from a patient with a history of asthma and dust mite allergy but who was not food-allergic. The almond-allergic patients chosen for study were those that reacted with native prunin and recombinant (r)Pru du 6.01 in immunodot blot assays (Willison et al., 2011). Patient #14 is a 38-year-old female with allergic responsiveness to almonds from age 5 with an ImmunoCAP<sup>®</sup> score of 1.9 kU/L. She is also clinically allergic to other tree nuts (cashew, Brazil nut, pistachio and walnut). Patient #55 is a 39-year-old male with allergic responsiveness to almond from age 10 with an almond-specific ImmunoCAP<sup>®</sup> score of 9.0 kU/L. He is also clinically allergic to hazelnuts, Brazil nuts, and peanuts. Patient #57 is a 50-year-old male with allergic responsiveness to almonds from age 10 with an ImmunoCAP<sup>®</sup> score of 9.99 kU/L.

### 2.2. Almond protein extract

Almond protein extracts were obtained from defatted almond flour by extraction with buffered saline borate (BSB) pH 8.2 (0.1 M  $\text{H}_3\text{BO}_3$ , 0.025 M  $\text{Na}_2\text{B}_4\text{O}_7$ , 0.075 M NaCl) at room temperature (RT) for 1 h and stored at  $-20^{\circ}\text{C}$  for later analysis as previously described (Sathe et al., 1997).

### 2.3. Purification of native prunin

Native prunin was purified from aqueous almond extract by column chromatography as previously described (Sathe et al., 2002). To obtain a hexameric fraction of native prunin, gel filtration was performed with a Sephacryl S200 HR column equilibrated with BSB. The column flow rate was maintained at 0.4 mL/min, and fractions were collected at 1 min intervals. Protein elution was monitored by UV absorbance at 280 nm. Fractions containing high molecular weight proteins corresponding to the hexameric fraction (~360 kDa) were concentrated with Amicon Ultra 30,000 MWCO (Millipore Corp., Bedford, MA).

### 2.4. Polyacrylamide gel electrophoresis and protein transfer

Almond extract (12–14  $\mu\text{g}$  per 4 mm well width) or native prunin (0.5 mg per 4 mm well width) was subjected to SDS-PAGE. Proteins were mixed with suitable volumes of reducing sample buffer (70 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.05% bromophenol blue and 5%  $\beta$ -mercaptoethanol) or non-reducing sample buffer (70 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.05% bromophenol blue), heated for 5 min in a boiling water bath, then cooled to RT. Samples were electrophoresed on a 12% acrylamide separating gel with a 4% acrylamide stacking gel for 1.5 h at 130 V. Gels were either stained with Coomassie Brilliant Blue R (Sigma-Aldrich, St. Louis, MO) or transferred to nitrocellulose (NC) membranes as previously described (Sze-Tao et al., 2001).

### 2.5. Monoclonal anti-almond antibodies

MAbs against almond extract were raised in the Hybridoma Facility at Florida State University by standard techniques (McCullough and Spier, 1990) and screened in direct-binding ELISA assays as previously described (Willison et al., 2011). The 4C10 mAb was chosen for epitope characterization as this mAb was highly reactive to native prunin and appeared to recognize a labile epitope (Kshirsagar et al., 2011). Additional mAbs (4F10, 2A3, 2B4, 5D1, 2G4) that appeared to recognize sequential epitopes on prunin were selected to serve as controls.

### 2.6. Immunodot blotting

Recombinant Pru du 6.01, Pru du 6.02 fusion proteins, native prunin, MBP, almond protein extract, alanine mutants, and

chimeric (Pru du 6.01/Gly m 6) molecules were applied to NC membranes with a 96-well Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories) as previously described (Tawde et al., 2006). Briefly, recombinant proteins (1 µg per 2 mm dot), native prunin, and almond extract (0.5 µg per 2 mm dot), and MBP (1 µg per 2 mm dot) were applied to NC strips and blocked overnight (o/n) at 4 °C with phosphate buffered saline-Tween 20 (PBS-T) (140 NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4, 0.05% Tween-20)/5% (v/w) non-fat dry milk. Dots were incubated with the almond-specific mAbs, diluted 1:200, 1:400, 1:500, or 1:2000 in PBS-T, at RT for 1 h. Strips were washed 3 × with PBS-T for 20 min each wash and then incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse reagent (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) at a 1:5000 (v/v) dilution in PBS-T for 1 h at RT, and washed as above. Amersham ECL (GE Healthcare, Piscataway, NJ) was used to detect reactivity upon exposure of dot strips to Kodak XAR film (Kodak Molecular Imaging, New Haven, CT).

## 2.7. Immunodot blot testing for monoclonal antibody IgG epitope conformational sensitivity

Native prunin (1 µg) was either boiled for 5 min in reducing sample buffer (70 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.05% bromophenol blue, and 5% β-mercaptoethanol) or was diluted in 100 µl Tris buffered saline (TBS) (20 mM Tris, 137 mM NaCl, pH 7.6) containing either 10% SDS, 6 M urea, or 1.0 M β-mercaptoethanol, and maintained at RT for 10 min. Samples were then dotted onto NC and probed in immunodot blotting assays as described in Section 2.6.

## 2.8. Immunoblotting

NC strips from gel transfers containing 15 µg of almond nut seed protein extract and native prunin were probed as described above. Briefly, strips were blocked o/n then incubated with 4C10 diluted 1:500 (v/v) in PBS-T at RT for 1 h. Strips were washed 3 × with PBS-T for 20 min per wash and then incubated with HRP-labeled goat anti-mouse reagent (Jackson ImmunoResearch Laboratories, Inc.) at a 1:5000 (v/v) dilution in PBS-T for 1 h at RT, and washed as above. Amersham ECL was used to detect reactivity upon exposure of dot strips to Kodak XAR film.

## 2.9. Hydrogen deuterium exchange (HDX)

The entire HDX experiment is automated with the HTC Pal autosampler (Eksigent Technologies, Dublin, CA) and performed as previously described (Zhang et al., 2011). HDX was performed with purified native hexameric prunin. Briefly, a 5 µL stock solution of native prunin (~10 µM concentration) or prunin-4C10 complex (~10 µM for native prunin) was mixed with 45 µL of PBS in D<sub>2</sub>O, pH 7.4, to initiate each H/D (hydrogen/deuterium) exchange period. For the blank control, the initial dilution was made in H<sub>2</sub>O PBS. The HDX reaction periods were 0.5, 1, 2, 4, 8, 15, 30, 60, 120, 240, and 480 min, each followed by simultaneous quench and proteolysis. Each 50 µL total volume was quenched by rapid mixing with 25 µL of 200 mM tris(2-carboxyethyl)phosphine (TCEP), 8 M urea solution in 1.0% formic acid and 25 µL five-fold dilution of saturated protease type XIII in 1.0% formic acid (final pH ~2.3). The protease digestion was performed for 2 min followed by injection for liquid chromatography-mass spectrometry (LC-MS) analysis. Each HDX reaction and assay was performed in triplicate.

## 2.10. On-line liquid chromatography/electrospray ionization/Fourier transform ion cyclotron resonance mass spectrometry

Liquid chromatography/electrospray ionization/Fourier transform ion cyclotron resonance mass spectrometry (LC ESI FTICR MS) was performed as previously described (Zhang et al., 2011). Briefly, after proteolysis, prunin (with or without mAb) peptides were separated and desalted with a Jasco high-performance liquid chromatography/supercritical fluid chromatography (HPLC/SFC) instrument (Jasco, Easton, MD). For LC, 45 µL of the protein digest was injected from a 50 µL loop to a Pro-Zap Expedite MS C<sub>18</sub> column (Grace Davidson, Deerfield, IL), 1.5 µm particle size, 500 Å pore size, 2.1 × 10 mm<sup>2</sup>. A rapid gradient from 2% B to 95% B in 2 min (A: acetonitrile/H<sub>2</sub>O/formic acid, 5/94.5/0.5; B: acetonitrile/H<sub>2</sub>O/formic acid, 95/4.5/0.5) was performed for eluting peptides at a flow rate of 0.3 mL/min. The LC eluent flow rate was reduced by ~1/1000 by a postcolumn splitter for efficient electro spray ionization (ESI). The ionized LC eluent was directed to a custom-built hybrid linear trap quadrupole 14.5 T Fourier transform-ion cyclotron resonance (LTQ 14.5 T FT-ICR) mass spectrometer (ThermoFisher, San Jose, CA). Mass spectra were collected from 380 < m/z < 1300 at high mass resolving power ( $m/\Delta m_{50\%} = 100,000$  at m/z 400, in which  $\Delta m_{50\%}$  is mass spectral peak full width at half-maximum peak height). The total experimental event sequence for each sample was 6.5 min. External ion accumulation was performed in the linear ion trap with a target ion population of three million charges collected for each FT-ICR measurement. LTQ-accumulated ions were transferred (~1 ms transfer period) through three octopole ion guides (2.2 MHz, 250 V<sub>p-p</sub>) to a capacitive coupled closed cylindrical ICR cell (55 mm i.d.) for analysis. The ion accumulation period was typically less than 100 ms during peptide elution and the ICR time-domain signal acquisition period was 767 ms (i.e., an overall duty cycle of ~1 Hz per acquisition).

## 2.11. HDX data analysis

Data were collected with Xcalibur software (Thermo-Fisher) and analyzed by an in-house analysis package as previously described (Kazacic et al., 2010; Zhang et al., 2011).

## 2.12. PCR amplification and DNA sequencing of recombinant genes

Gene specific primers were designed for prunin 1 and prunin 2 based on the available NCBI sequences (accession numbers X78119 and X78120). The full length prunin cDNAs and the free acidic and basic subunits were amplified, TA cloned (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA), and sequenced on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) as previously described (Willison et al., 2011).

## 2.13. Cloning, expression and purification of cDNA-encoded proteins

The prunin cDNA coding sequences, representing the Pru du 6.01 and Pru du 6.02 isoforms, were ligated into the fusion expression vector pMAL-c4X (New England BioLabs Inc., Beverly, MA) and transformed into *Escherichia coli* BL21(DE3) or Rosetta gamiB(DE3)pLysS cells (EMD Millipore Corporation, Billerica, MA). The cloning, expression, and purification of mannose-binding protein (MBP)-rPru du 6.01 and MBP-rPru du 6.02 fusion proteins were carried out as previously described (Willison et al., 2011).

**Table 1**

Primers used for the amplification and subcloning of almond Pru du 6.01 and soybean Gly m 6 11S globulin genes, constituent subunits, and chimeras.

| Gene        | Primer name: primer sequence (5'–3')                                                     | Amplicon                          |
|-------------|------------------------------------------------------------------------------------------|-----------------------------------|
| Pru du 6.01 | PLG-F: AAATCTAGAGCACGCCAGTCCAGT<br>PSM-R: CGCAAGCTTTTATACAACGCGCTC                       | Full length Pru du 6.01 (PLG–PSM) |
| Pru du 6.01 | PLG-F: AAATCTAGAGCACGCCAGTCCAGT<br>PLG-R: CGCAAGCTTTTAGTTGGCCATCAGTTGTTCTCC              | Pru du 6.01 large subunit (PLG)   |
| Pru du 6.01 | PSM-F: AAATCTAGAAACGGTCTTGAGGAGACCTTCTGC<br>PSM-R: CGCAAGCTTTTATACAACGCGCTC              | Pru du 6.01 small subunit (PSM)   |
| Gly m 6     | GLG-F: AGATCTAGATCCAGAGAGCAGCTCAGC<br>GSM-R: CCCCAAGCTTTCTAAGCCACAGCTCTCTCTG             | Full length Gly m 6 (GLG–GSM)     |
| Pru du 6    | PLG-F: AAATCTAGAGCACGCCAGTCCAGT<br>P*-PLG-R: GTTGCCATCAGTTGTTCTCC                        | P*-PLG                            |
| Gly m 6     | P*-GSM-F: GGCATTGACGAGACCATATGC<br>GSM-R: CCCCAAGCTTTCTAAGCCACAGCTCTCTCTG                | P*-GSM                            |
| Gly m 6     | GLG-F: AGATCTAGATCCAGAGAGCAGCTCAGC<br>P*-GLG-R: CTAATTTCTTCTGCTTTGCTTTGGC                | P*-GLG                            |
| Pru du 6    | P*-PSM-F: GGTCTTGAGGAGACCTTCTGC<br>PSM-R: CGCAAGCTTTTATACAACGCGCTC                       | P*-PSM                            |
| Pru du 6    | PLG-F: AAATCTAGAGCACGCCAGTCCAGT<br>P*-P 74 aa-R: GGTGATTCGAGAGGCGGC                      | P*-Pru du 6-21–74 aa              |
| Gly m 6     | P*-G 73aa-F: CGCAACGCCCTTCGTAGACCTTC<br>P*-G 310aa-R: ATTCTCTGCTTTTGCTTTGGCTTCTCTG       | P*-Gly m 6-73–310 aa              |
| Gly m 6     | GLG-F: AGATCTAGATCCAGAGAGCAGCTCAGC<br>P*-G72aa-R: GTTGAGGTGACGAGAGAGGGC                  | P*-Gly m 6-22–72 aa               |
| Pru du 6    | P*-P 75aa-F: ATTCAGCGCAACGGCTA<br>PSM-R: CGCAAGCTTTTATACAACGCGCTC                        | P*-Pru du 6-75–551 aa             |
| Pru du 6    | PLG-F: AAATCTAGAGCACGCCAGTCCAGT<br>P*-P465 aa-R: CTGCTGCACCTCTGGTCAAGAATTGC              | P*-Pru du 6-21–465 aa             |
| Gly m 6     | P*-G 409aa-F: GGACGGGTGCTGATCGTGCCA<br>GSM-R: CCCCAAGCTTTCTAAGCCACAGCTCTCTCTG            | P*-Gly m 6-409–495 aa             |
| Pru du 6    | PLG-F: AAATCTAGAGCACGCCAGTCCAGT<br>P*-P 195 aa-R: GCGGGTCTTCTGTTGGCGGTCC                 | P*-Pru du 6- 21–195 aa            |
| Gly m 6     | P*-G 135 aa-F: AACTTCAGAGAGGGTGATTTGATCGC<br>P*-G 310 aa-R: ATTCTCTGCTTTTGCTTTGGCTTCTCTG | P*-Gly m 6-135–310 aa             |
| Pru du 6    | PLG-F: AAATCTAGAGCACGCCAGTCCAGT<br>P*-P 166aa-R: TCTTCCTTCTTCTGTTGCTGCTCTCC              | P*-Pru du 6.01-21–166 aa          |
| Gly m 6     | P*-G 115aa-F: CAACAACCTCAACAAGAGGACAAAGCAGC<br>P*-G 213aa-R: TTCTTGCTGATGCTTCTTCTGCG     | P*-Gly m 6-115–213 aa             |
| Pru du 6    | P*-P284aa F: CAACAAGGAAGCGCAACAACGCTTCTCTCC<br>PSM-R: CGCAAGCTTTTATACAACGCGCTC           | P*-Pru du 6.01-284–551 aa         |

#### 2.14. Production of individual subunits and chimeric molecules

The recombinant pMAL-c4X vector containing the full-length Pru du 6.01 gene was used to produce rPru du 6 and served as a template for PCR-based cloning and expression of the large and small subunit genes. The target coding sequences were modified by the addition of restriction sites at the 5' and 3' ends during the PCR reactions with PfuTurbo DNA polymerase (Stratagene, Cedar Creek, TX) and the appropriate chimeric/subunit primers (Table 1). This same amplification strategy was employed for the soybean Gly m 6 11S globulin gene (GenBank accession #X15121) to generate full-length rGly m 6 protein as well as its independent large and small subunits. Stop codons were introduced into both the Pru du 6.01 and Gly m 6 large subunit reverse primers (Table 1). The various Pru du 6/Gly m 6 chimeric constructs were generated by blunt-end ligation of Platinum Pfx DNA polymerase (Invitrogen)-amplified PCR products. These products then served as full-length templates for Pfu Turbo DNA polymerase amplification in subsequent PCR reactions. All PCR products were digested by XbaI and HindIII according to the manufacturer's instructions (Invitrogen). Digested products were purified with a QIAquick Gel Extraction Kit (Qiagen

Inc., Valencia, CA) and ligated with T4 DNA Ligase H.C. (Invitrogen) to their respective sites in the pMAL-c4X expression vector. Sequences were checked for identity to their respective templates by the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The expression and purification of these recombinant molecules was performed as described in Section 2.13.

#### 2.15. Production of Pru du 6.01 alanine mutants

Residues in the HDX-MS identified peptides were selected for mutation to alanine based on their solvent accessibility in the prunin crystal structure (281Q, 282Q, 317Q, 318N, 320N, 326R, 328R, 461Q, 462E, 464Q, 465Q) with the primers listed in Table 2. Plasmids containing Pru du 6.01 cDNA served as the template and mutants were made with the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA) following the manufacturer's instructions. Plasmids were sequenced, transformed into *E. coli* Rosetta gamiB(De3)pLysS cells and purified as described above. These recombinant proteins were concentrated and either stored (briefly) at 4 °C or frozen at –80 °C until use in immunodot blotting assays.

**Table 2**

Primers used for site directed mutagenesis of selected residues to alanine in almond Pru du 6.01.

| Residue | Primer name: primer sequence (5'–3')                                                    |
|---------|-----------------------------------------------------------------------------------------|
| Q281A   | 281F: caaccattcggacgtcccagagcacaagaacaaga<br>281R: tcctgtgttcttctgtcctgggacgtccgaatggtg |
| Q282A   | 282F: cattcggacgtcccagacaagcagaacaagaagcg<br>282R: cgcttcctgtgttctgtcctgggacgtccgaatg   |
| Q317A   | 317F: caggaaacctcaggagcggaacgacaacaggaac<br>317R: gttcctgtgtcgttcctcctggaggttctg        |
| N318A   | 318F: ggaacctccaggagcggcagacaacaggaacaaa<br>318R: ttgggttctgtgtcggcctgtcctggaggttcc     |
| N320A   | 320F: ctccaggagacagacgacccaggaacaaatcatccg<br>320R: cggatgatttggttctcgtcgtcgttctcctggag |
| R326A   | 326F: caggaaacaaatcatcgggttaaggggcaacctc<br>326R: gaggttgcccttaccgcatgatttggttctg       |
| R328A   | 328F: aggaacaaatcatcgggttagcgggcaacctcgac<br>328R: gtcgaggttgccctaccgcatgatttggttctc    |
| Q461A   | 461F: gagatgcaattcttgacgagggtgacgagggac<br>461R: gtcctgtcgtcactccgctcaagaattgcatctc     |
| E462A   | 462F: caattcttgaccaggcggtgacgagggaca<br>462R: tgcctcgtcgtcaccgctggtcaagaattg            |
| Q464A   | 464F: ctgaccaggaggtgagcagggacagctgtt<br>464R: aacagctgtcctcgccactcctgtgtaag             |
| Q465A   | 465F: gaccaggaggtgacgagggagacagctgttcat<br>465R: atgaacagctgtcctcgtcgtcgtcctcgtg        |
| C32A    | 32F: gtccgcagaaccaggccagctcaaccagc<br>32R: gctggttgagctgggctgttctcggac                  |
| C65A    | 65F: ggaggacttcaggctccggggtcgcc<br>65R: ggcgaccccgagcctggaagtctctc                      |
| C108A   | 108F: ggcagtgttctcgtggggccctgagactttcga<br>108R: ttcgaaagtctcaggggcccgagaaactctgc       |
| C374A   | 374F: gtcttgaggagacttcgacagcttgaggtgaagg<br>374R: ccttcaactcaagctggcgaaggtctcctcaagac   |

### 2.16. Molecular modeling

Molecular modeling was performed with chimera 1.6.1 (<http://www.cgl.ucsf.edu/chimera/>) using the prunin (PDB# 3FZ3) (Jin et al., 2009) and soybean glycinin homohexamer (PDB# 1OD5) A3B4 (Adachi et al., 2003) crystal structures. The solvent accessible surface area (areaSAS) and solvent excluded surface area (areaSES) was calculated for each residue with the structure analysis tool in chimera.

### 2.17. Separation of total IgE and IgG from pooled patients' sera

Total IgG was purified from Pru du 6.01 reactive pooled patients' sera (#14, 55, 57) with a protein G affinity column. Pooled serum was passed over the column and bound IgG was eluted with 0.2 M glycine sulfate, pH 2.3. The eluate was immediately neutralized with 1.0 M Tris. Total IgE from pooled patients remained in the effluent. The purified IgG and IgE fractions were then tested by ELISA to verify separation of these antibody populations (described below). For detection of bound antibodies, HRP-conjugated mouse anti-human IgE (Zymed Laboratories, Inc.) at a dilution of 1:1000 or HRP-conjugated goat anti-human IgG (Sigma–Aldrich) diluted to 1:80,000 in PBS-T was used. All samples were incubated with both secondary antibodies to verify the depletion of IgG from the effluent, its presence in the eluate, and the depletion of IgE in the eluate and its presence in the effluent.

### 2.18. ELISA and inhibition

The ELISA assay was performed as previously described (Willison et al., 2011). Briefly, native prunin (40 µg/mL) diluted in coating buffer (0.1 mol/L carbonate–bicarbonate buffer, pH 9.6) was coated (100 µL/well) onto the wells of 96-microwell flat bottom polystyrene microtiter ELISA plates (Costar, Cambridge, MA) at 37 °C for 1 h. Plates were washed 3 times between steps with PBS-T. Blocking was performed with PBS-T/5% (w/v) nonfat dry milk at 4 °C o/n. Total IgE or IgG from pooled patients (#14, 55, 57) was diluted 1:2 (v/v) in PBS-T/5% (w/v) nonfat dry milk, then added to the coated wells (100 µL/well), and incubated at 37 °C for 3 h. After washing, 4C10 was diluted to 1:4000 in PBS-T and then added to wells (100 µL/well) for 30 min at 37 °C. Bound 4C10 IgG was reacted with HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) at a dilution of 1:1000 and incubated for 1 h at 37 °C. IgG reactivity was detected by a colorimetric reaction with o-phenylenediamine (Zymed Laboratories Inc.) and H<sub>2</sub>O<sub>2</sub> as substrate. Reactions were quenched with H<sub>2</sub>SO<sub>4</sub> and OD was measured in a KC4 v2.5 ELISA reader (Bio-Tek Instruments, Inc., Winooski, VT) at 495 nm. All assays were performed in triplicate. Controls included wells exposed to 4C10 without any inhibitor and wells exposed to IgE and IgG from an atopic individual without a history of tree nut allergies.

OD values obtained for 4C10 binding with no inhibitor were normalized to 100% binding. The percentage of human IgE and IgG inhibition on 4C10 binding was calculated as follows: (M+, mAb 4C10 with inhibitor; M-, mAb 4C10 without inhibitor; OD, optical density).

$$\text{Inhibition (\%)} = \frac{\text{OD(M-)} - \text{OD(M+)}}{\text{OD(M-)}} \times 100$$

## 3. Results

### 3.1. Conformational sensitivity of the 4C10 epitope

The mAb 4C10 was chosen for epitope characterization as this mAb was highly reactive to native prunin and appeared to recognize a conformational epitope (Kshirsagar et al., 2011). Immunoblot assays of whole almond extract and native prunin indicated that 4C10 strongly reacted with non-reduced but not reduced samples indicating the recognition of a labile epitope (Fig. 1A). To further assess the stability of this epitope, native prunin was subjected to various denaturing conditions prior to immunodot blotting and probing with 4C10 (Fig. 1B). The results confirmed that treatment with β-ME and reducing buffer plus boiling eliminated 4C10 reactivity, indicating the recognition of a conformational epitope dependant on the intramolecular disulfide bond within the large subunit and/or the intermolecular bond association of the large and small subunits. Additional mAbs (4F10, 2A3, 2B4, 5D1) directed against sequential epitopes on prunin were used as controls (Fig. 1B). To further assess the role of disulfide bonding in 4C10 epitope expression, rPru du 6 was expressed in two bacterial strains: the traditional *E. coli* BL21(DE3) cells, which do not favor disulfide bond formation, and Rosetta gamiB(DE3)pLysS cells which facilitate disulfide bond formation. Immunodot blot assays demonstrated that only expression of recombinant Pru du 6.01 in the cell strain that allows formation of disulfide bonds were reactive with 4C10 (Fig. 1C). Consequently, all further epitope mapping studies were performed with recombinant proteins synthesized by Rosetta gamiB(DE3)pLysS cells.

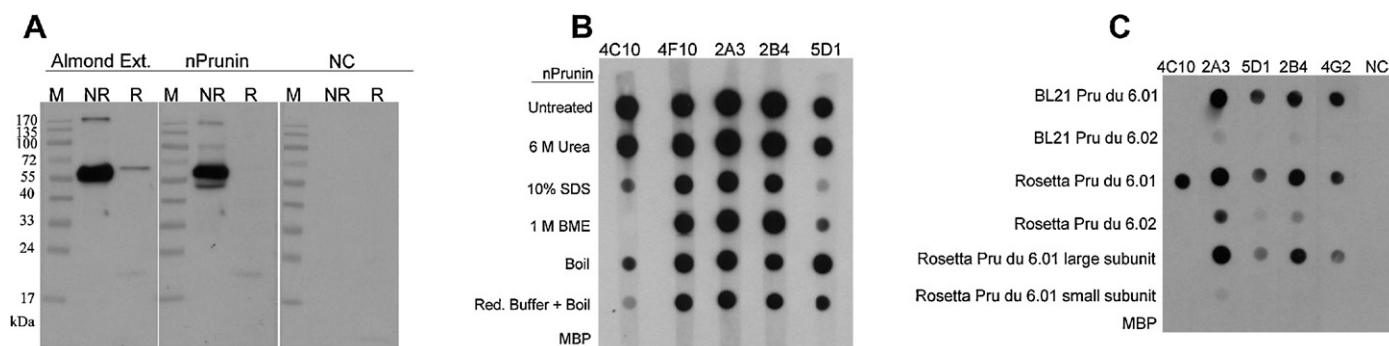


**Fig. 1.** Immunoblot and immunodot blots. (A) Immunoblot of 4C10 showing reactivity with non-reduced, but not reduced, almond extract and native prunin. (B) Immunodot blot of native prunin treated with various denaturants followed by probing with five anti-almond mAbs. (C) Immunodot blot demonstrating anti-almond mAb reactivity with recombinant Pru du 6 expressed in different bacteria cell strains. NR = non-reducing buffer conditions, R = reducing buffer conditions, M = size marker, MBP = maltose binding protein, NC = negative control.

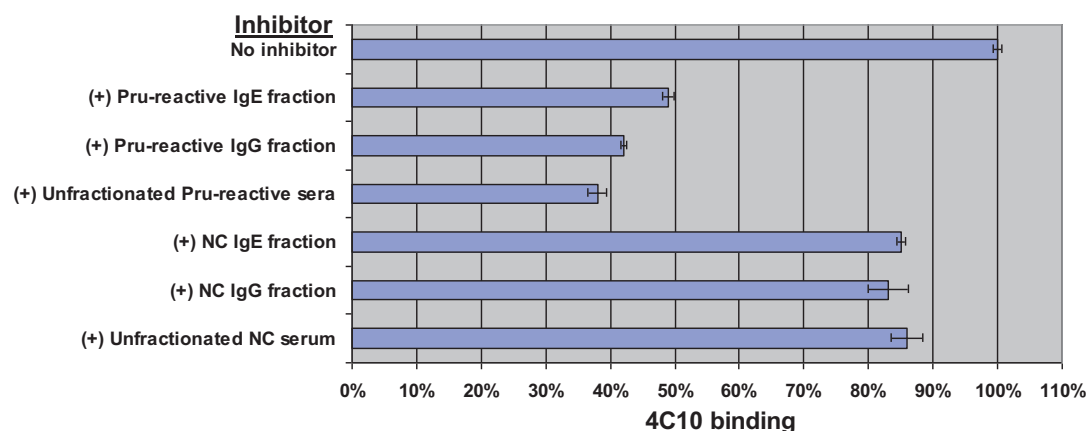


**Fig. 2.** Inhibition ELISA demonstrating the ability of Pru du 6.01-reactive patient antibodies to inhibit 4C10 binding to native prunin expressed as a percent of uninhibited control. NC = atopic serum negative control.

### 3.2. 4C10 recognizes an epitope targeted by prunin-reactive patients' sera

To determine if the epitope region targeted by 4C10 is also recognized by Pru du 6-reactive patients, an inhibition ELISA was performed. Pooled Pru du 6-reactive patients' sera (#14, 55, 57) was separated into total IgG and IgE fractions and tested for the ability to inhibit 4C10 binding to prunin. Both fractions were found to block 4C10 binding by varying degrees when pre-incubated with prunin-coated ELISA plates prior to probing with 4C10 (Fig. 2). Incubation with total IgE resulted in a 51% inhibition of 4C10 binding and total IgG inhibited binding by 58% as compared to the uninhibited control (Fig. 2). No significant inhibition was observed when total IgG and IgE fractions from an atopic control patient (NC) served as inhibitors. Overall, it appears that both human IgG and IgE binding epitopes (sequential and/or conformational) may overlap or sterically hinder mAb 4C10 binding to prunin.

### 3.3. HDX identification of peptides masked by 4C10 binding

MAb 4C10 was found to be reactive with native prunin purified from almond extract and rPru du 6.01 (Fig. 1). Reactivity with 4C10 was eliminated when prunin was denatured and reduced indicating the recognition of a conformational epitope. To identify peptides involved in the 4C10 conformational epitope, H/D exchange was performed with free prunin and prunin–4C10 complex. 4C10 was incubated with prunin in the presence of D<sub>2</sub>O to generate a soluble Ag–mAb complex, digested, and the resultant peptides analyzed

for deuterium uptake by MS. Both Pru du 6 isoforms are present in native prunin and peptides from each were recovered (Fig. 3). The segments covered 72% of Pru du 6.01 and 60% of Pru du 6.02. All segments were identified in at least two of the three replicates for every H/D exchange period. Only peptides corresponding to the sequence of Pru du 6.01 showed a deuterium uptake difference. These results were consistent with those of the immunodot blot assays in which 4C10 bound exclusively to Pru du 6.01 (Figs. 1C and Fig 3). The binding of mAb 4C10 to prunin affords significant protection from HDX for Pru du 6.01 aa segments 184–190, 200–203, 281–288, 314–328, 395–400, 401–405, 450–474, and 496–507 (Fig. 3). These peptides are likely to be involved in 4C10 conformational epitope or are otherwise blocked upon mAb binding to prunin.

### 3.4. Molecular modeling of HDX-identified peptides

A molecular model of native prunin was obtained from the protein data base (PDB# 3FZ3) (Jin et al., 2009). Native prunin is biosynthesized as a large proprotein that is posttranslationally cleaved into large (367 aa) and small (184 aa) subunits that remain associated via disulfide bonds (Garcia-Mas et al., 1995; Jin et al., 2009; Shewry et al., 2003; Staswick et al., 1984). The uncleaved proprotein normally forms trimers which, upon cleavage to the mature native form, dimerize via association of the hydrophobic faces (IE face) to form hexamers (Adachi et al., 2003; Jin et al., 2009; Shewry et al., 2003). Peptides demonstrating a difference in deuterium uptake are highlighted on the prunin model (Fig. 4).

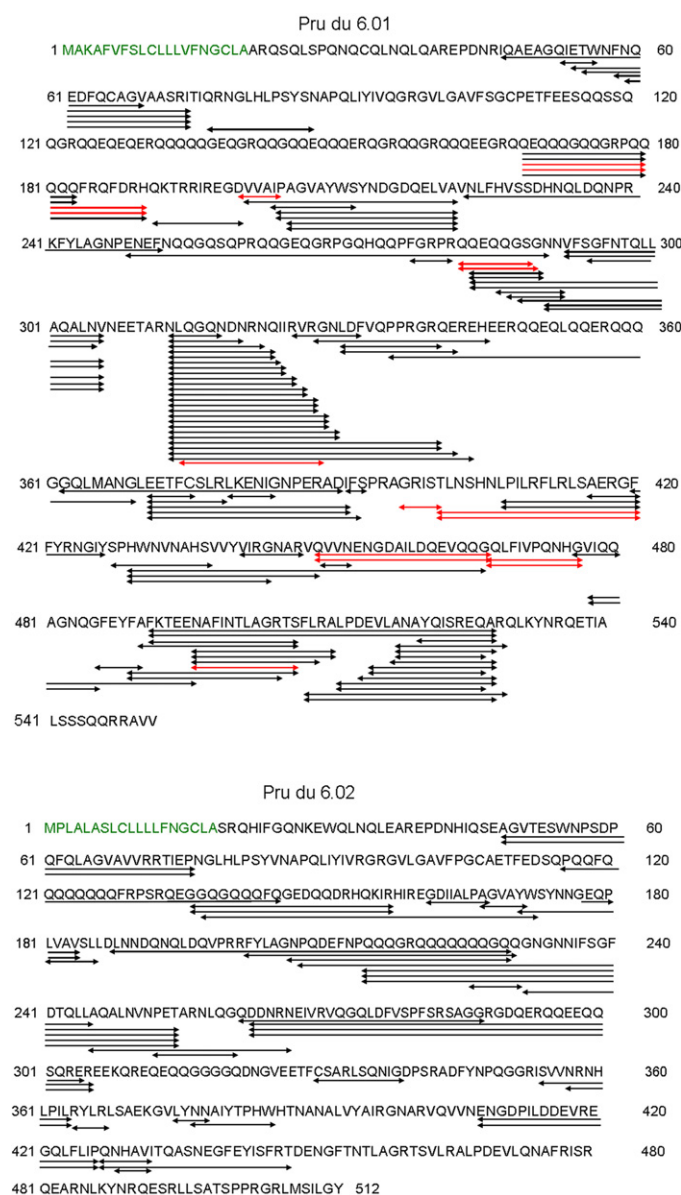


**Fig. 3.** Sequence coverage for the analyzed native prunin–4C10 complex. Peptides indicated in red were found to have deuterium uptake difference between the free prunin and prunin–4C10 complex of greater than 30%. Underlined amino acids correspond to the signal sequence which is not found on the mature protein. Asterisks indicate the large–small subunit cleavage site (aa 367/368) in the mature protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

As the hexameric form of prunin was used in the HDX assay, those differentially labeled peptides localized to the solvent-exposed IA face are most likely to be interacting with 4C10 directly, whereas those labeled peptides located on the occluded IE face are likely the result of conformational shifts due to mAb binding (Fig. 4A). Judging from the atomic model, significant portions of peptides aa 281–288, 314–326, and 450–474 are solvent-exposed on the IA face and peptides aa 184–190, 200–203, 395–400, 401–405, and 496–507 are either buried within the monomer or are predominantly located on the occluded IE face (Fig. 4B). Peptides on the IA face were found to be clustered in two distinct regions on the prunin monomer (Fig. 4B). Due to the spatial separation of the identified regions in a given monomeric subunit, it is unlikely that a single 4C10 binding site is directly interacting with both. However, in the context of the hexamer, the labeled peptides on adjacent

large and small subunits do lie in close proximity (Fig. 4C). These data suggest that 4C10 may be directly interacting with peptides on one subunit and covering or blocking solvent access to a region on the adjacent prunin monomer. The specific amino acids corresponding to the HDX-defined peptide segments on the IA face are highlighted on the prunin model in Fig. 4.

### 3.5. Immunodot blot screening of Pru du 6.01 alanine mutants

To further fine map the 4C10 epitope, solvent accessible residues in the HDX-identified peptides were substituted with alanine and tested for mAb reactivity. The results show that of the 11 residues screened, alanine substitutions completely abolished 4C10-reactivity for two (R328A and E462A), greatly reduced binding for four (N320A, R326A, Q461A, and Q464A), and had little to no effect for five (Q281, Q281A, Q317, N318A and Q465) (Fig. 5). The specific residues identified by mutagenesis likely represent the core of the 4C10 epitope, but it is possible that additional contact or otherwise structurally important residues may lie in adjacent regions or on peptide segments not recovered in HDX. To investigate this possibility, chimeric molecules in which orthologous segments of Pru du 6 and the soybean 11S globulin homolog, Gly m 6, were exchanged and probed with 4C10 (described below).

### 3.6. Assessment of the role of disulfide bonds in 4C10 epitope expression

The loss of reactivity of Pru du 6 with mAb 4C10 following reduction suggested the importance of the disulfide bonds in stabilizing the structure of the epitope (Fig. 1C). To further substantiate this, alanine substitutions were made in cysteine residues involved in the disulfide bond located within the large subunit (residues 32 and 65) and, independently, the disulfide bond linking the large and small subunits (residues 108 and 374). Mutation of intrasubunit residues 32 and 64 to alanine completely eliminated 4C10 reactivity indicating that this bond plays a critical role in fostering the 4C10 conformational epitope (Fig. 5A). In contrast, mutation of intersubunit residues 108 and 374 had little effect on 4C10 reactivity, suggesting that this bond does not contribute structurally to the epitope. The specific cysteine residues involved in the disulfide bonds are indicated by arrows on the IA face of the prunin model in Fig. 5B.

### 3.7. Immunodot blot screening of Pru du 6/Gly m 6 chimeric constructs and isolated subunits

To further localize the 4C10 conformational epitope, chimeric molecules were generated by substituting Pru du 6.01 segments with orthologous segments from the 11S globulin soybean allergen, Gly m 6. Alignment of Pru du 6 (PDB# 3FZ3) and Gly m 6 (PDB# 1od5) crystal structures demonstrated a high degree of structural similarity, indicating that Gly m 6 is a suitable stand-in for segments of Pru du 6 (data not shown). A variety of chimeric molecules were constructed in an attempt to define the 4C10-reactive segment(s) identified by HDX and to determine if regions that were not recovered in HDX are also involved in the epitope (Fig. 6A). These chimeric molecules were screened by immunodot blot for maintenance or loss of 4C10 reactivity (Fig. 6B). The results demonstrated that the chimeras could remain reactive upon Gly m 6 substitutions for Pru du 6 segments within the middle of the large subunit but not for substitutions at the N- or C-termini of the large or small subunit (#7, 8, 9, 10) (Fig. 6). A construct containing Pru du 6 at the N-terminal (aa 21–74), Gly m 6 in the large subunit (aa 75–310) and the Pru du 6 small subunit (#6) was also not reactive. Additionally, expression of free large and small Pru du 6 subunits and substitution of entire Pru du 6 subunits with Gly m 6 resulted in

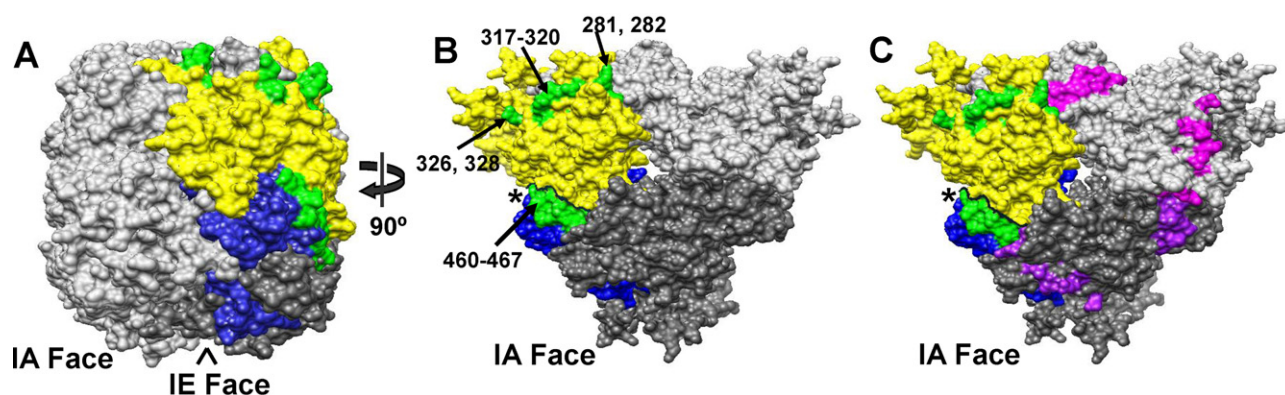


**Fig. 4.** Structural models of prunin (PDB# 3FZ3). (A) Side view of the prunin hexamer showing the lateral IA faces and the IE face-to-face association of two trimers. One of the prunin monomers is color coded (large subunit, yellow; small subunit, blue) and the additional monomers are represented in light gray and dark gray. Peptides identified by HDX are shown in green on one monomer. (B) Top view of the prunin trimer. The asterisk indicates the black line separating the large and small subunit in the color coded monomer. The peptides identified by HDX are shown in green. Surface exposed residues on the HDX-identified peptides are indicated by arrows. (C) HDX-identified peptides are shown on each of three monomer in green, purple, and magenta, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

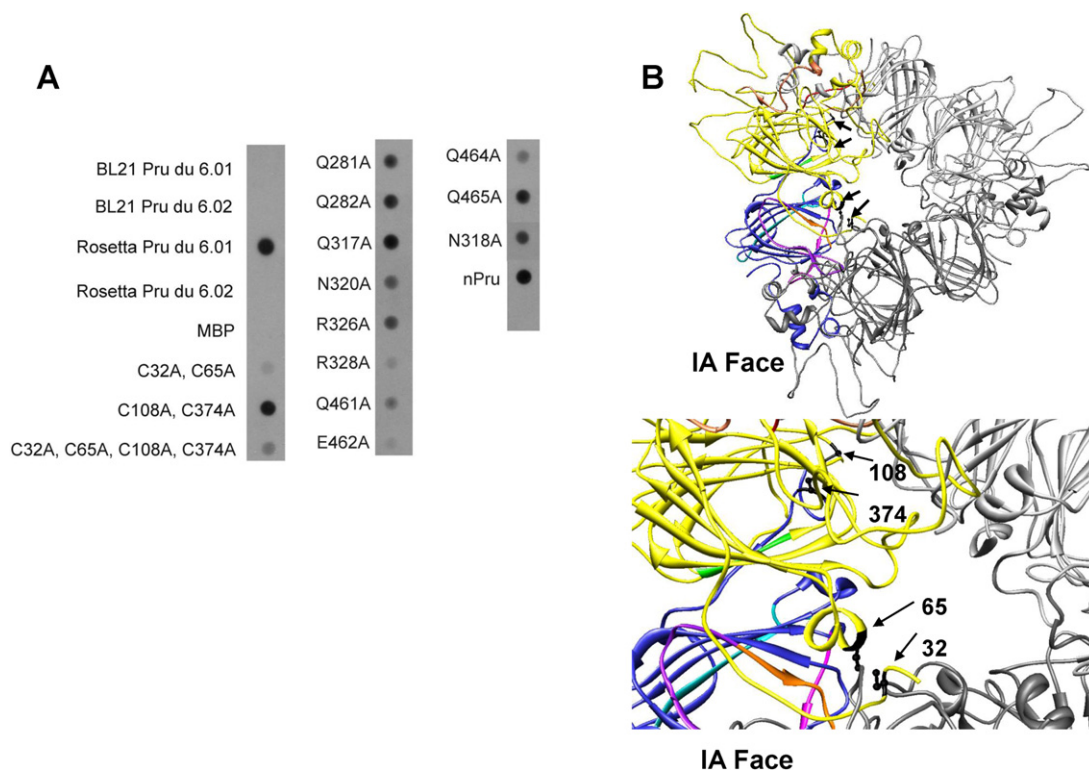


**Fig. 5.** Immunodot blot and structural models of prunin (PDB# 3FZ3). (A) Immunodot blot of Pru du 6.01 alanine mutants probed with 4C10. MBP = maltose binding protein, nPru = native prunin. (B) Top view of the prunin trimer with the cysteine residues involved in disulfide bonds indicated by arrows.

a loss of 4C10 reactivity (#4, 5, 13, and 14) (Figs. 1C and 6). These results indicate that expression of the 4C10 epitope is dependent upon the large and small subunit association.

### 3.8. Residues involved in the 4C10 conformational epitope

HDX assays identified several segments of Pru du 6.01 (aa 184–190, 200–203, 281–288, 314–328, 395–400, 401–405, 450–474, and 496–507) that may be involved in the 4C10 epitope. Analyses of 4C10 reactivity to alanine point mutants and chimeric molecules were performed to verify specific peptides involved in the conformational epitope and to identify key residues. Chimeric molecules containing N-terminal Gly m 6 substitution (aa 21–74,

#7) abrogated 4C10 reactivity (Fig. 6). Peptides corresponding to aa 46–74 were recovered in HDX but did not show a deuterium uptake difference between free prunin and 4C10 bound prunin. Peptides corresponding to aa 21–45 were not recovered in the HDX assay. The overall loss of 4C10 reactivity to the chimeric molecule and analysis of the HDX data indicates that residues aa 21–45 are likely involved in the 4C10 epitope. Molecules in which the middle of the Pru du 6 large subunit was substituted (aa 115–213, #9) with Gly m 6 maintained 4C10 reactivity, whereas substitution at the C-termini of the large subunit (aa 135–310, #8) eliminated 4C10 reactivity (Fig. 6). Analysis of 4C10 reactivity to these molecules indicates that regions at the N and C-termini (aa 21–166 and aa 284–367) of the large subunit are likely to be involved in the conformational

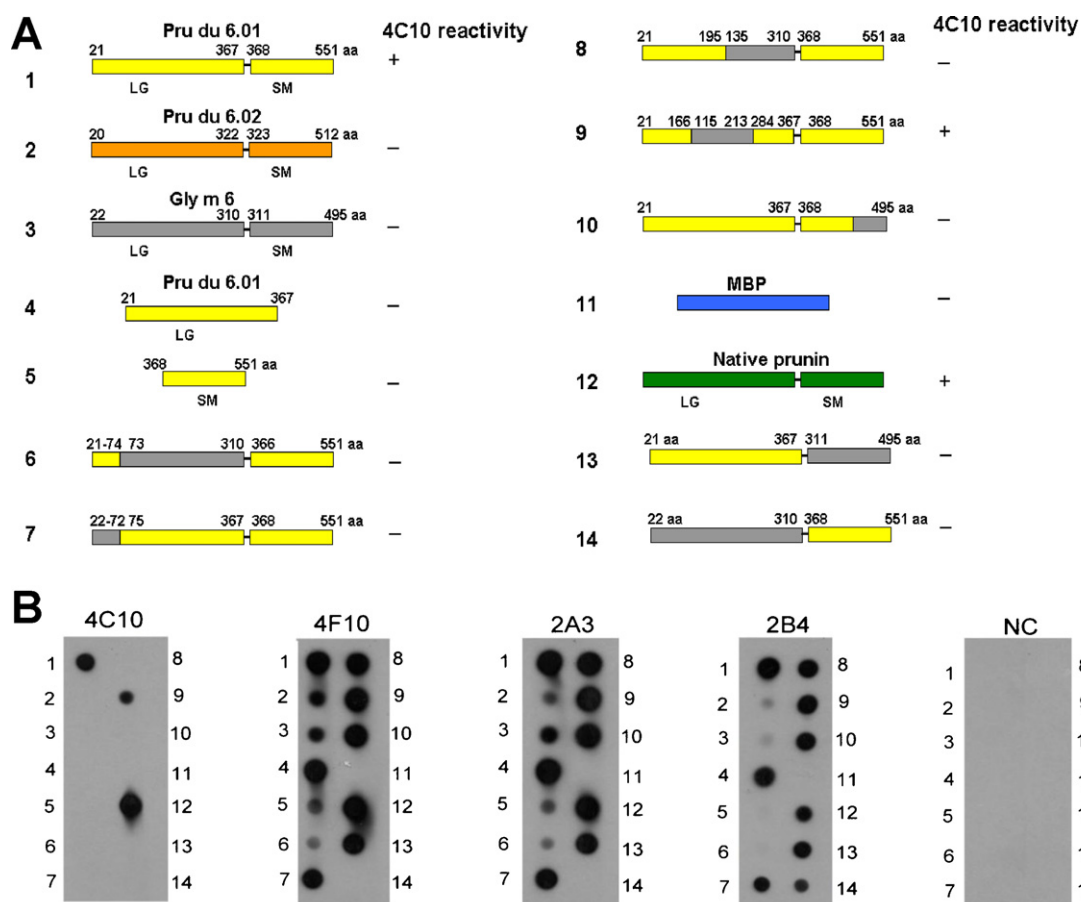


**Fig. 6.** Chimeric molecules and immunodot blot assays. (A) Schematic representation of Pru du 6.01, Gly m 6 and chimeric molecules. Yellow = Pru du 6.01; orange = Pru du 6.02; gray = Gly m 6; green = native prunin; blue = MBP. Note that all molecules have N-terminal-associated MBP, which is not shown. (B) Immunodot blot of chimeric molecules probed with 4C10 and control mAbs (4F10, 2A3, and 2B4) directed against sequential epitopes on Pru du 6. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

epitope. HDX peptides showing a deuterium uptake difference in these regions identified several peptides that were localized to the IA face (aa 281–288, 314–328, and 450–474). Solvent-exposed residues in these peptides were mutated to alanine. Screening of these mutants showed that substitutions N320A, R326A, R328A, E461A, Q462A, and Q464A resulted in a significant reduction or eliminated of 4C10 binding as compared to the unmutated control. In contrast, alanine mutations in peptide aa 281–288 had no effect on 4C10 reactivity (Fig. 5). Comparison of the aa sequence of the two Pru du 6 isoform sequences shows several differences (aa 328, 461 and 464) in the suspect regions which could explain the ability of 4C10 to bind Pru du 6.01 but not 6.02 (Fig. 7A). Collectively, these results indicate that the 4C10 conformational epitope is comprised of three discontinuous strands, aa 21–45, 320–328, and 460–465.

### 3.9. Comparison of 4C10 conformational epitope with sequential IgE binding epitopes

The relative location of the 4C10 conformational epitope was compared to the previously mapped sequential Pru du 6 patient IgE-reactive epitopes (Willison et al., 2011). Of the six sequential epitopes identified on Pru du 6.01, epitopes aa 118–132, 145–159, and 281–295 were in close proximity to the peptides likely involved in the 4C10 epitope (Fig. 7B). Residues involved in the sequential epitope aa 281–295 were found to be sterically occluded on the adjacent prunin monomer upon 4C10 binding in the HDX assay. This suggests that these epitopes define a region of high immunogenicity on prunin. The clustering may also account for the

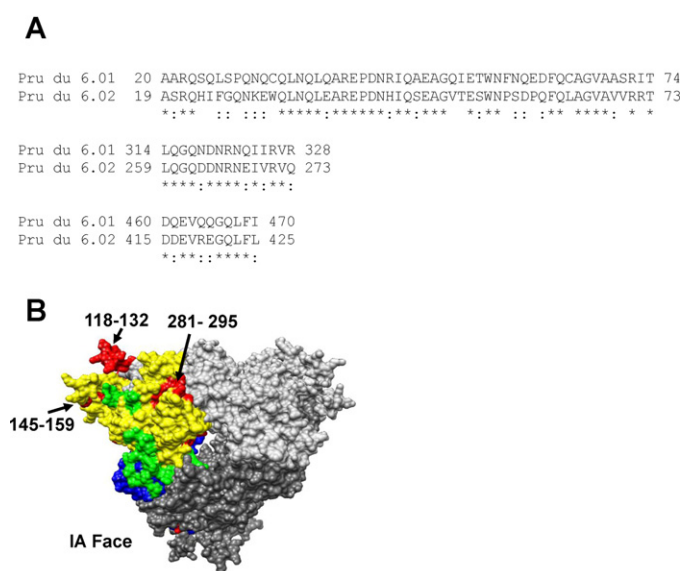


**Fig. 7.** Pru du 6 sequence alignment and structural model. (A) Sequence alignment of Pru du 6 peptides involved in the 4C10 epitope. Asterisk (\*) denotes aa identity, colon (:) indicates aa similarity. (B) Structural model of prunin (PDB# 3FZ3) with peptides involved in the 4C10 epitope highlighted in green. Sequential IgE binding epitopes previously identified on Pru du 6 are highlighted in red (Willison et al., 2011). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

observation that patient IgE can inhibit the binding of mAb 4C10 to this allergen (Fig. 2).

#### 4. Discussion

The identification of epitopes is critical for understanding the allergenic nature of foods, the etiology of food allergy, and the development of immunotherapies for the treatment of food allergies. Extensive research has been performed to identify sequential epitopes on food allergens. Tree nut sequential epitopes have been described for pecan (Car i 1 and 2) (Sharma et al., 2011a,b), cashew (Ana o 1, 2, 3) (Robotham et al., 2005; Wang et al., 2002, 2003), walnut (Jug r 1 and 4) (Robotham et al., 2002; Sordet et al., 2009), hazelnut (Cor a 9) (Robotham et al., 2009), and almond (Pru du 6) (Willison et al., 2011). In contrast to the abundant information on sequential allergen epitopes, few conformational epitopes have been identified, a situation resulting from the technical challenges associated with their characterization. Methods to identify and characterize conformational epitopes include large scale mutagenesis, X-ray crystallography of immune complexes, negative stain immunoelectron microscopic analysis of individual allergen–mAb immune complexes, NMR, phage display to identify allergen mimotopes, and the generation of chimeric molecules (Pali-Schöll and Jensen-Jarolim, 2008; Riemer et al., 2004; Xia et al., 2010; Gershoni et al., 2007; Pomés, 2010).

Recently, hydrogen/deuterium exchange footprint analysis, monitored by 14.5 T Fourier transform ion cyclotron resonance mass spectrometry (HDX-MS), has been applied to conformational epitope mapping studies (Coales et al., 2009; Zhang et al., 2011). Analysis of a mAb directed against human thrombin successfully identified a conformational IgG-binding epitope composed of two adjacent discontinuous surface strands by HDX-MS (Baerga-Ortiz et al., 2002). Comparison of the epitope recognized by a mAb directed against horse cytochrome c by HDX-MS found that it correlated with the residues identified in both X-ray crystallography and NMR studies (Coales et al., 2009). HDX-MS has also been applied in epitope mapping studies of the recombinant cashew allergen, Ana o 2 (Zhang et al., 2011). The IgG epitopes identified by an anti-conformational and an anti-sequential mAbs overlapped with the epitopes previously identified on Ana o 2 in mutagenesis studies and by probing short synthetic overlapping peptides (Zhang et al., 2011). Collectively, these studies demonstrate the utility of HDX-MS for epitope mapping and validate the application of this technique for mapping the more difficult to identify conformational epitopes.

In the present study, a murine mAb antibody, 4C10, was used to assess the ability of HDX-MS to identify an unknown epitope on the almond allergen, Pru du 6. This mAb is of particular interest because it appears to recognize a conformational epitope that is dependent on the association of the large and small subunits and is susceptible to denaturation (Fig. 1). Inhibition ELISA assays showed that both IgG and IgE from Pru du 6-reactive patients could inhibit 4C10 binding to prunin to varying degrees (Fig. 2). These results suggest that the 4C10 epitope, recognized by this murine IgG mAb, overlaps epitopes (sequential and/or conformational) recognized by human patient IgG and, more importantly with respect to almond allergy, IgE epitopes. As such, information gained through its study should shed light on the nature of the epitopes on this allergenic protein that are targeted by the immune system.

HDX-MS assays were performed on immune complexes composed of native prunin and 4C10. Because recombinant Pru du 6 is in the uncleaved precursor form of the protein, naturally cleaved native prunin was utilized in the HDX-MS assay to ensure that the allergen expressed a complete set of epitopes. HDX-MS was able to identify several peptides that demonstrated a deuterium

uptake difference between free prunin and the prunin–4C10 complex (Fig. 3). To help distinguish between peptides likely involved in the 4C10 epitope and those differentially labeled as a result of conformational perturbation upon 4C10 binding, labeled peptides were mapped onto the prunin crystal structure (PDB# 3FZ3) (Fig. 4). Several peptides (aa 184–190, 200–203, 395–400, 401–405, and 496–507) were buried on the IE interface of the prunin hexamer and are thus unlikely to directly contribute to mAb binding. The identification of candidate peptides on the solvent-exposed IA face of the hexamer (aa 281–288, 314–328, and 450–474) allowed the construction of targeted alanine mutants and chimeric molecules, which greatly reduced the time and number of constructs needed to fine map the epitope (Figs. 5 and 6).

Analysis of 4C10 reactivity to these various recombinant molecules indicated that the conformational epitope is comprised of three discontinuous strands, aa 21–45, 320–328, and 460–465, that are held in close proximity due to the secondary and tertiary structure of prunin (Figs. 4–7). Comparison of the location of the peptides involved in the 4C10 epitope with the previously mapped sequential IgE epitopes on Pru du 6 (Willison et al., 2011) did not reveal any direct epitope overlap (Fig. 7B). However, several epitopes (aa 118–132, 145–159, and 281–295) were in close proximity to the 4C10 epitope on the crystal structure of Pru du 6, which could explain the IgE inhibition of 4C10 binding (through steric clashes) observed in ELISA assays (Fig. 2).

Though epitopes tend to be categorized as either sequential or conformational, it should be noted that individual epitopes may have a mixture of both conformational and sequential characteristics as short stretches of continuous residues may be part of more complex conformational epitopes (Aalberse, 2000; Van Regenmortel, 1996; Sivalingam and Shepherd, 2012). Therefore, sequential epitopes identified on Pru du 6 may be part of larger epitopes or could represent only the epitope core with additional contact residues that were undetected in the mapping assays. Further assessment with patient IgE is needed to fully define the presence and nature of conformational epitopes on Pru du 6 and to determine if these more complex epitopes directly overlap with that of 4C10.

In summary, these results indicate that HDX-MS is a technique that can aid in the identification and characterization of conformational epitopes, especially when coupled with atomic modeling, by allowing one to narrow the search to specific regions on the molecule. This approach reduces or eliminates the need for extensive chimeric and mutated molecules, which can speed up the overall epitope identification process.

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