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A 50 kDa Maize γ-Zein Has Marked Cross-Reactivity with the Almond Major Protein

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Cross-reactivity of antibodies against almond major protein (AMP, a legumin), the major almond allergen, with cereal proteins may cause problems in detecting almond contaminants in cereal products when antibody-based assays are used. Rabbit polyclonal IgG antiserum produced against AMP was used to test cross-reactivity with protein extracts from maize, a cereal commonly found in breakfast and snack foods. Gradient SDS-PAGE followed by Western blotting was performed, and two cross-reactive proteins were detected by chemiluminescence. A fraction of maize proteins purified by elution from an IgG anti-AMP affinity column followed by electrophoreseis and immunoblotting showed a high degree of cross-reactivity with a minor 50 kDa protein of maize, as well as low cross-reactivity with the 27 kDa γ-zein. The 50 kDa cross-reactive protein was identified as the 50 kDa γ-zein by immunoreaction with anti-50 kDa γ-zein antiserum. Notably, the 50 kDa maize γ-zein also reacted with IgE from pooled human sera from patients with self-reported severe almond allergies. The high immunoreactivity of the 50 kDa γ-zein should be considered in maize quality improvement programs, and such notable cross-reactivity is of relevance in the design of antibody-based assays for almond allergen detection.

KEYWORDS: **Maize; γ-zein; almond major protein; allergen; cross-reactivity; immunoreaction**

INTRODUCTION

Almonds are widely used as a snack food and as an ingredient in breakfast cereals, baked goods, and snack food manufacture. Although almonds are enjoyed by most people, a small but significant number of people suffer from almond allergies (*1*). The terms "amandin" and "almond major protein" (AMP) refer to a protein fraction of almond that has been shown to contain key reactive polypeptides that are recognized by IgE from almond-allergic patients. Amandin is a legumin storage protein that consists of at least two major types of polypeptides, 20−22 and 42−46 kDa (*2*). Two almond legumin precursors have been cloned and sequenced, named prunin 1 and prunin 2 (*3*). Another almond allergen, a 2S albumin, has also been described (*4*). Almond allergy is considered to be serious because allergy to tree nuts is usually life-long and sometimes life-threatening (*1*). Accurate detection of trace amounts of almonds in foods is therefore important to protect sensitive individuals from accidental exposure. Methods used for the detection of trace quantities of almonds should be not only sensitive but also specific. Breakfast cereal and snack manufacturers are two of the major almond users. Any method designed to detect trace quantities of almonds in cereal products should, therefore, not recognize cereal proteins. The potential for cross-reactivity between maize proteins and almond AMP by rabbit anti-AMP antibodies was investigated in this study following indications observed in a preliminary screening of cereal proteins.

Major proteins in maize endosperm are composed of 2S albumins, 7S and 11S globulins in the cytoplasm, and aqueous alcohol-soluble prolamins (zeins) found in storage protein bodies (*5*). Zein is characterized by a relatively high amount of hydrophobic amino acids, that is, proline, alanine, and leucine, when compared with albumins and globulins. Therefore, zein has moisture barrier properties that permit its use as a food protective coating material for such foods as candies, nuts, and dried fruits and as a coating for pharmaceutical tablets. Zein is composed of polypeptides that are fractionated into four subclasses—α-, β-, γ-, and δ-zeins—and each subclass of zein possesses different characteristics regarding solubility, molecular weight, and primary structure (*6*−*8*). α-Zein (M_r 23800−26700) accounts for 80−85% of the total zein, whereas β-zein (M_r 17000), which contains a methionine-rich polypeptide, and δ-zein (M_r 10000) are minor fractions, together comprising 10%

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of the total zein. γ-Zein, a high-cysteine-containing group, can be further divided into three subtypes according to M_r and uniqueness of their amino acid sequences. The major M_r 27000 and minor 16000 γ-zeins together make up 10−15% of the total zein (*9*). More recently, a third distinct γ-zein (M_r 50000) was identified and characterized as the least abundant among the zeins (*10*). The M_r 27000 γ-zein contains tandemly repeated hexapeptide sequences (Pro-Pro-Pro-Val-His-Leu), whereas the M_r 50000 γ-zein has much larger polyglutamine blocks at the N terminus than the other γ-zeins. Unlike the others, the amino acid sequence of the M_r 16000 protein shows an absence of tandem repeat motifs, despite an otherwise strong sequence homology with M_r 27000. Esen (*9*) therefore proposed the M_r 16000 protein to be a truncated form of M_r 27000.

Some maize proteins have been reported to show clinical allergenicity (*11*, *12*) as well as in vitro cross-reactivity with serum of patients who were allergic to wheat/barley seed protein or grass pollen (*13*). It should be noted that in vitro cross-reactivity does not, in itself, imply that the individuals from whom the sera were procured would react clinically upon ingestion of the cross-reactive protein (*14*). Using sera from patients with clinical reactivity to maize, several IgE-binding proteins have been found in maize. Pastorello et al. (*11*) identified a nonspecific lipid transfer protein as a major allergen (9 kDa), as well as a trypsin inhibitor (16 kDa) as a minor allergen, in a population of patients with self-reported systemic reactions to maize in which most were also peach allergic and had known allergy to peach lipid transfer protein. These allergens were soluble in saline solution (i.e., albumin-globulin proteins). Pasini et al. (*12*) described a reduced soluble 50 kDa allergenic maize protein that was resistant to pepsin or pepsin plus trypsin and stable to heating (cooking), although the protein itself was not identified. All six patients with challenge-positive maize allergy showed IgE reactivity to the 50 kDa protein. A comprehensive understanding and examination of this latter study suggests the hypothesis that a possible candidate maize allergenic protein may be the γ-zein identified by Woo et al. (*10*), as it is known that these proteins are somewhat hard to digest in vitro, which is a common characteristic of food protein allergens (*15*). This protein also contains a sequence motif (Gln-Gln-Gln-Pro-Gln) similar to the IgE-binding epitope (Gln-Gln-Gln-Pro-Pro) of wheat glutenin (*16*).

In recent years, enzyme-linked immunosorbent assay (ELISA) methods (*17*, *18*) have been developed for the detection of almonds. However, critical assessment of the possible reactivity of cereal proteins, including analysis of complete protein extracts, with anti-AMP antibodies remains unexplored. In this study, we show in vitro cross-reactivity mainly between a 50 kDa maize protein and AMP as assessed by reactivity with both rabbit polyclonal anti-AMP IgG and human polyclonal IgE from almond-allergic patients.

MATERIALS AND METHODS

Extraction of Proteins from Cereal Flours. Almond and cereal flours (100 mg, maize, sorghum, wheat, barley, rice, and oat) were extracted in a microcentrifuge tube with 1 mL of 0.0125 M sodium borate (pH 10), 1% SDS, and 2% (v/v) 2-mercaptoethanol according to the method of Wallace et al. (*19*). After shaking on a rotator at room temperature for 30 min, samples were centrifuged for 20 min at 13000 rpm using a microcentrifuge (Kendro, Newtown, CT). Total zein proteins extracted were partitioned by adding ethanol (final concentration of 70% v/v) to precipitate non-zein proteins, followed by centrifugation for 20 min at 13000 rpm and collection of the zein-containing supernatant.

Electrophoresis and IgG Immunoblotting. Maize protein extracts were loaded and electrophoresed on a 10−18% polyacrylamide gradient gel, and proteins were transferred to a 0.2 μm polyvinylidine fluoride (PVDF) membrane (Bio-Rad, Hercules, CA) using a Mini-Protean II gel electrophoresis system (Bio-Rad). The membrane was blocked with nonfat dry milk and incubated with anti-AMP rabbit polyclonal IgG antiserum (diluted 1:10^5), prepared at Florida State University, in Tris-buffered saline−Tween (TBS-T) (0.02 M Tris, 0.5 M NaCl, pH 7.5, and 0.05% Tween 20), and then washed with TBS-T. Finally, the membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham Pharmacia Biotech), as a secondary antibody, and detected by chemiluminescence using the ECL plus system (Amersham Pharmacia Biotech).

Preparation of Anti-AMP IgG and Purification of Cross-Reactive Maize Proteins by Affinity Chromatography. AMP preparation (*2*) and production of rabbit polyclonal anti-AMP antibodies (*17*) have been described earlier. Total IgG from 2 mL of serum from an AMP-immunized rabbit was purified using the Immunopure (protein A) IgG purification kit (Pierce Biotech, Rockford, IL) according to the instructions provided. The resultant IgG was applied to an AminoLink Plus Coupling Gel using the AminoLink Plus immobilization kit (Pierce Biotech). The IgG-coupled gel was finally washed with 15 mL of 0.05% NaN_3 and stored at 4 °C. Maize protein extracted with 0.0125 M sodium borate (pH 10), 1% SDS, and 2% 2-mercaptoethanol was dialyzed against phosphate-buffered saline (PBS) (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2) and applied to the anti-AMP IgG affinity column following equilibration of the affinity column with PBS. The column was then washed with PBS and eluted using the Immunopure IgG elution buffer (Pierce Biotech). The eluted fractions were collected and immediately neutralized by adding 100 μL of 1 M Tris-HCl (pH 7.5) to each 1 mL fraction. The fractions were pooled and applied to a microCentricon concentrator (Millipore, Billerica, MA) to exchange the buffer with PBS.

Identification of the Proteins Purified by the Anti-AMP IgG Affinity Column. The maize protein fraction eluted from the anti-AMP affinity column was concentrated to 0.5 mL using a microCentricon concentrator (Millipore, Bedford, MA), applied to a SDS-PAGE gel, electrophoresed, and transferred to a 0.2 μm PVDF membrane and stained with Gelcode Blue (Pierce Biotech). The result showed that two polypeptides had affinity to anti-AMP IgG. These two protein bands at approximately M_r 50000 and 27000 were cut from the PVDF membrane and analyzed for N-terminal sequencing by an Applied Biosystems Procise 492 (Foster City, CA) dual column instrument based on the Edman degradation reaction. Resulting phenylthiohydantoin (PTH) reactive amino acids were loaded on a reverse-phase C-18 column and detected by the retention time at 280 nm. The identity of the higher molecular mass protein of ∼50 kDa was additionally verified by immunoblotting using anti-50000 M_r γ-zein polyclonal antiserum (diluted 1:10^6) kindly donated by Drs. Brian Larkins and Rudolph Jung of the University of Arizona and Pioneer Hi-Bred International, Inc., USA, respectively.

Anti-AMP IgE Immunoblotting of Maize Proteins. Blood samples were drawn after informed consent from patients with self-reported life-threatening systemic reactions to almond and the sera frozen at −70 °C until use. The study was approved by the institutional review board of the University of California at Davis. After electrophoresis and Western transfers of protein samples to nitrocellulose membranes, membranes were incubated with 1:5 (v/v, in TBS-T) pooled human serum for 16 h at 4 °C. Membranes were rinsed thrice, 5 min each, with TBS-T followed by a final 15 min wash with PBS (pH 7.2) containing 0.01% Triton X-100. The nitrocellulose blots were incubated overnight with 1:5 diluted equine [^{125}I]-anti-human-IgE (Hycor, Biomedical Inc., Garden Grove, CA) in 5% (w/v) nonfat dry milk in PBS, washed with PBS containing 0.01% Triton X-100, and suitably developed using X-ray film (Kodak) as per manufacturers' instructions. Appropriate sample and sera controls were included.

Inhibition Immunoblot of Maize Proteins with Human Serum IgE. For inhibition blots, 300 μg of maize extract was electrophoresed in a 12% SDS-PAGE gel under reducing conditions and transferred to nitrocellulose (NC) as above. The NC was cut into 3 mm strips and probed with patient serum alone (diluted 1:5 in TBS-T) or 1:5 patient serum containing 500 μg of maize extract or 300 μg of almond AMP. The adsorbed samples were preincubated overnight at 4 °C prior to

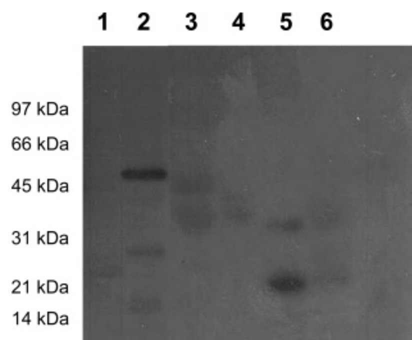

Figure 1. Western blot of several cereal protein extracts with rabbit polyclonal anti-AMP antiserum (IgG): lane 1, sorghum; lane 2, maize; lane 3, wheat; lane 4, barley; lane 5, rice; lane 6, oat.

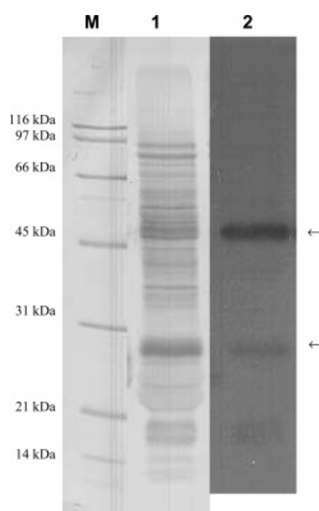

Figure 2. SDS-PAGE and cross-reactivity analysis of maize protein extract probed with rabbit anti-AMP IgG fraction: lane M, molecular mass standards; lane 1, total protein extract of maize stained with Coomassie Blue; lane 2, corresponding immunoblot probed with rabbit anti-AMP IgG.

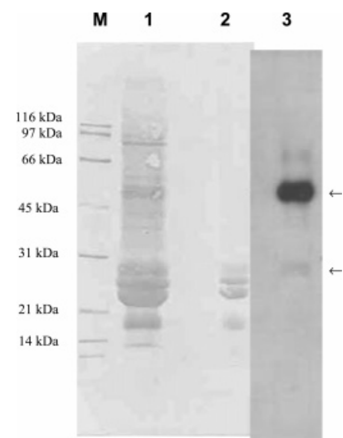

Figure 3. Total zein extraction and reactivity with rabbit anti-AMP antiserum: lane M, molecular mass standards; lane 1, total protein extract of maize; lane 2, total zein extract; lane 3, corresponding immunoblot of purified total zein probed with rabbit anti-AMP IgG.

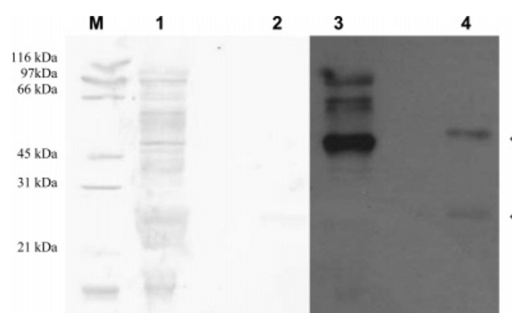

Figure 4. Immunoblot of the affinity-purified proteins with rabbit anti-AMP antiserum: lane M, molecular mass standards; lane 1, total maize protein extract; lane 2, proteins purified by anti-AMP IgG immunoaffinity chromatography (faint band at 27 kDa can be seen); lanes 3 and 4, immunoblots of total protein of maize and the affinity-purified proteins, respectively.

addition to the blotted nitrocellulose. Adsorbed or unadsorbed patient sera were incubated with the NC strips overnight at 4 °C, washed, and incubated for 1 h at room temperature with HRP-labeled mouse anti-human IgE (Zymed, S. San Francisco, CA) diluted 1:10000 in 5% nonfat dry milk in TBS-T. Strips were washed in TBS, incubated each in 800 μL of Pierce SuperSignal West Femto substrate as described by the manufacturer, and exposed to Kodak XAR film.

RESULTS

Cross-Reactivity of Cereal Proteins with AMP As Detected by Rabbit Anti-AMP Antibodies. A screening of extracts from cereal flours by immunoblot for reactivity with anti-AMP antibodies showed maize protein extract to have the strongest signal of the tested cereal extracts (maize, sorghum, wheat, barley, rice, and oat), showing a single reactive protein at approximately M_r 50000 compared to proteins of the other cereals (**Figure 1**). Maize was then selected for further analysis.

SDS-PAGE and Immunoblotting of Maize Protein Extracts. Figure 2 shows the SDS-PAGE profile of the total maize protein extract and the corresponding immunoblot in which a prominent anti-AMP-reactive protein at M_r 50000 and a weaker reactive protein at M_r 27000 are detected. These two proteins are located at the same positions as the γ-zeins, 50 and 27 kDa, respectively, which led to the hypothesis that anti-AMP antibody cross-reacts with γ-zeins of maize. Total maize protein was therefore subjected to further partitioning with 70% ethanol, to obtain total zein protein, and then SDS-PAGE analysis followed by immunoblotting with anti-AMP antibodies to confirm that the reactive proteins could be classified as zein (**Figure 3**). A comparison of **Figures 2** and **3** confirms the weak reactivity of the anti-AMP antibody with the more prominent M_r 27000 protein and the strong reactivity with the less prominent M_r 50000 protein.

Affinity Purification of Anti-AMP-reactive Maize Proteins. IgG containing the anti-AMP antibody was isolated from rabbit polyclonal anti-AMP antiserum using protein A covalently coupled to agarose beads. This IgG fraction was reacted with total maize protein extract and the bound fraction eluted. The eluate, when subjected to SDS-PAGE analysis, revealed the two major cross-reactive bands of M_r 50000 and 27000 in maize. The band at M_r 50000 was considerably more immunoreactive on the immunoblot than the one at M_r 27000, considering the lesser amount of the upper band in the affinity-purified preparation (**Figure 4**).

Protein Sequencing of Cross-Reactive Proteins. The alignment between the N-terminal sequences of the maize proteins that are cross-reactive with AMP and the two known γ-zein cDNA-derived protein sequences is shown in **Figure 5**. N-Terminal sequence analysis of the lower molecular mass protein band gave a complete match with the 27 kDa γ-zein, whereas that of the higher molecular mass protein gave a positive, but partial match, with the 50 kDa γ-zein. We speculated that the lack of complete sequence match of the latter might be caused by simultaneous sequencing of several related proteins of similar

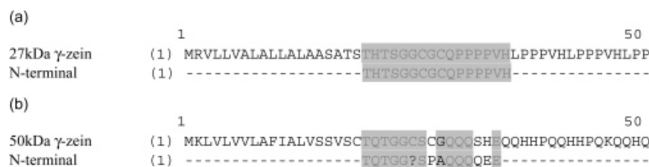

Figure 5. Amino acid sequence alignment of N-terminal amino acid sequence of the purified proteins with a 27 kDa (**a**) (Genbank Accession No. AF371261) and a 50 kDa (**b**) (AF371263) γ-zein. Identical or similar residues are shaded, and a question mark represents an undecided residue in the sequencing result.

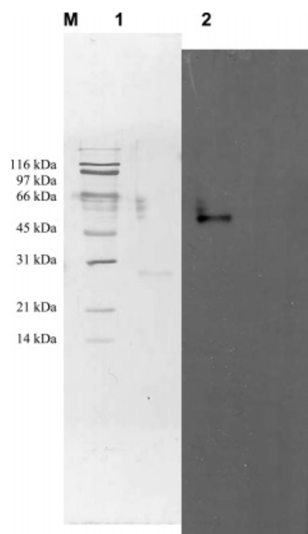

Figure 6. Verification of the identity of the purified 50 kDa protein using rabbit anti-50 kDa γ-zein antiserum: lane M, molecular mass standards; lane 1, proteins purified by anti-AMP IgG immunoaffinity chromatography; lane 2, immunoblot of the purified 50 kDa protein.

mobility. Consequently, the upper protein band of 50 kDa was subjected to further analysis.

Identification of the Higher Molecular Mass Protein. In addition to the sequence analysis based assay, anti-50 kDa γ-zein polyclonal antiserum, which is monospecific to the unique large block of polyglutamine repeats of a 50 kDa γ-zein (*10*), was used for immunoblotting. As shown in **Figure 6**, the upper protein band was clearly recognized by anti-50 kDa γ-zein antiserum, even though it was not visibly detected by Coomassie Blue staining.

Detection of IgE Reactivity toward the 50 kDa Maize Protein Using Sera from Patients with Almond Allergy. Total maize proteins were tested for IgE reactivity using immunoblotting with pooled human sera from patients with self-reported, life-threatening allergies to almonds but no allergy to corn (**Figure 7**). The 50 kDa maize protein was clearly recognized by pooled human serum IgE when incubated for 2 and 5 days. As expected, the reactivity of the IgE with AMP was far greater than with the 50 kDa maize protein.

Inhibition Blot of Maize Proteins. Cross-reactivity between AMP and maize protein was confirmed through IgE inhibition by maize and almond proteins by preincubation with the patient's serum (**Figure 8**). Reactivity of maize zein proteins was fully inhibited, whereas the uninhibited maize protein clearly cross-reacted (see arrow).

DISCUSSION

The analysis of cross-reactivity between AMP and maize protein as detected by rabbit IgG antibodies raised against AMP

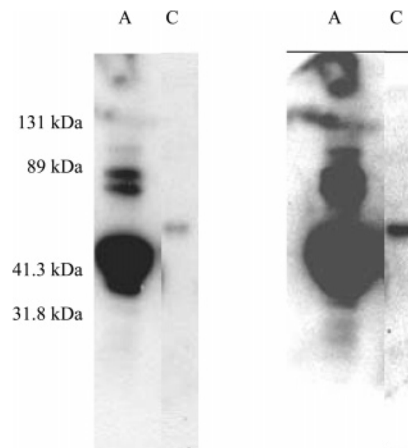

Figure 7. IgE immunoblot of almond (lane A) and maize (lane C) protein extract using pooled sera from patients allergic to almonds. Left and right set of blots are 2 and 5 days exposure, respectively.

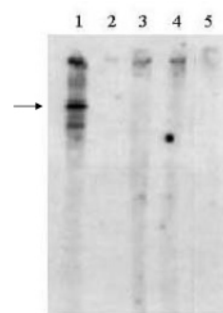

Figure 8. Maize inhibition blot probed with human serum demonstrating cross-reactivity with AMP: lane 1, with no inhibitor (arrow indicates cross-reactive zein protein); lane 2, secondary antibody only (no human serum); lane 3, human serum preincubated with maize protein extract; lane 4, human serum preincubated with AMP; lane 5, nonallergic serum control.

indicates that at least two protein species in maize share cross-reactive epitopes with AMP. Amino acid sequence alignment (**Figure 9**) between prunin, the major component of purified AMP, and γ-zeins supports this idea by showing conserved large polyglutamine blocks in both the prunin and 50 kDa γ-zein proteins and smaller blocks with the 27 kDa γ-zein. The immunoblot analysis of affinity-purified γ-zeins a (**Figure 4**) also leads to speculation regarding a potential relationship between immunoreactivity and the sizes and amounts of glutamine blocks of the γ-zeins. The 50 kDa protein is clearly more immunoreactive than the 27 kDa protein, as the former is barely discernible in the Coomassie-stained gel while eliciting a strong immunoreaction on the blot. We hypothesize that the larger and more numerous polyglutamine blocks of the 50 kDa γ-zein compared to the 27 kDa γ-zein may result in higher cross-reactivity in the former. Similarly, Watanabe et al. (*15*) have reported a low molecular weight (LMW) glutenin allergen with 15 Gln-Gln-Gln-Pro-Pro (QQQPP) motif repeats that comprise the IgE-binding epitope. Murayama et al. (*20*) reported a dramatic reduction in LMW wheat glutenin immunoreactivity with sera of patients allergic to wheat through deamidation of glutamine residues with acid, suggesting a critical role for glutamine residues in IgE recognition in this allergen. It is possible that some of the glutamine residues that are deamidated may be critical for the integrity of certain epitope(s) involved in IgE recognition. These results are consistent with reduction in AMP antigenicity as a result of acid pH exposure (*2*, *17*). Leduc et al. (*21*, *22*), on the other hand, have shown that a

Figure 9. Amino acid sequence alignment of the 50 kDa (**a**) and 27 kDa (**b**) γ-zeins with prunin (prunin 1, CAA55009), a major component of AMP. Identical residues are shaded and designated below as consensus.

wheat "isolate" obtained by deamidation of gluten and used in processed meat products is a neoallergen capable of inducing severe anaphylaxis in sensitized patients who may not also be allergic to wheat.

BLAST (NCBI) comparison of prunin (AMP) using the allergen database allergenonline.com finds predicted cross-reactivities based on sequence homology. Using this method wheat is predicted to be more cross-reactive with prunin than is maize; however, in our study the maize 50 kDa γ-zein was far more reactive with anti-AMP antibodies than were wheat or other cereal proteins (**Figure 1**). Likewise, using a FAO/WHO guideline for predicting cross-reactivity of proteins with allergens (*23*), the maize γ-zein and prunin (AMP) do not have the requisite eight contiguous amino acids (**Figure 9**) to suggest probability of cross-reactivity. However, in the present study among the cereals tested, the anti-AMP antibodies were far more reactive with maize proteins.

Interestingly, Pasini et al. (*12*) demonstrated an allergenic 50 kDa protein from maize flour that was recognized by sera of maize-allergic patients and concluded that the 50 kDa protein is the major allergen for IgE-mediated food allergy to maize. Although Pasini and co-workers did not attempt to identify the allergen through sequence analysis, they did classify the protein as a reduced soluble protein as defined by Wilson et al. (*24*). This grouping of proteins, based on their solubility in a solution of neutral salt with reducing agent, is composed of the γ-zein polypeptides, at 27 and 50 kDa, and a 12 kDa protein (*25*, *26*). Originally, the 50 kDa γ-zein protein was believed to be a dimer of the 27 kDa protein; however, Woo et al. revealed it as a novel γ-zein protein (*10*). These data, taken together, suggest that the 50 kDa maize allergen identified by Pasini et al. (*12*) is possibly the same immunoreactive 50 kDa γ-zein identified in this study. These data suggest that further studies to assess possible clinical relevance of the cross-reactivity between the maize 50 kDa γ-zein and almond AMP may be warranted, but it should be noted that none of our almond allergic patients reports allergy to corn.

This information is potentially important to scientists involved in research to improve maize quality. For example, quality protein maize contains up to 4 times more 27 kDa γ-zein than wild-type maize and may, similarly, contain more of the 50 kDa protein (*19*). Other protein mutants could also affect γ-zein expression levels.

Perhaps even more relevant to the food-processing industry is the possibility that such cross-reactivity could interfere with rapid detection assays for almond protein in maize-containing foods, particularly snack and breakfast cereal foods. Assays that rely on polyclonal antibody-based ELISA assays may consequently be compromised with respect to their specificity (*27*). A careful and thorough evaluation of such ELISA assays must therefore be undertaken.

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