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Maize 27 kDa γ-Zein Is a Potential Allergen for Early Weaned Pigs

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Soybean and maize are extensively used in animal feed, primarily in poultry, swine, and cattle diets. Soybean meal can affect pig performance in the first few weeks following weaning and elicit specific antibodies in weaned piglets. Though maize is a major component of pig feed, it is not known if any of the maize proteins can elicit immunological response in young pigs. In this study, we have identified a prominent 27 kDa protein from maize as an immunodominant protein in young pigs. This protein, like some known allergens, exhibited resistance to pepsin digestion in vitro. Several lines of evidence identify the immunodominant 27 kDa protein as a γ-zein, a maize seed storage protein. First, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis of different solubility classes of maize seed proteins revealed the presence of an abundant 27 kDa protein in the prolamin (zein) fraction. Antibodies raised against the purified maize 27 kDa γ-zein also reacted against the same protein recognized by the young pig serum. Additionally, matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis of the peptides generated by trypsin digestion of the immunodominant 27 kDa protein showed significant homology to the maize 27 kDa γ-zein. Since eliminating the allergenic protein will have a great impact on the nutritive value of the maize meal and expand its use in the livestock industry, it will be highly desirable to develop maize cultivars completely lacking the 27 kDa allergenic protein.

KEYWORDS: Allergen; γ-zein; maize; pigs

INTRODUCTION

Optimal growth of livestock can be achieved only with feeds containing the correct levels of nutrients, high quality ingredients, and extensive management. Livestock producers use maize and soy as a base for their animal feed because these provide high quality protein and energy. Livestock consume 47% of the soy and 60% of the maize produced in the US (http://www.soystats.com/ ; http://www.ncga.com/livestock). On a global basis, soybean meal (SBM) accounts for approximately 63% of all protein sources used in animal feeds. Maize is an excellent source of energy, high in digestible carbohydrates and low in fiber, which renders it an ideal ingredient for animal feeds (http://www.ncga.com/livestock).

Maize and SBM are extensively used in swine diets. However, these grain-based diets are not widely utilized during the weaning of pigs. Weaning is a stressful time in a pig's life because of many challenges that it has to face (*1*). One of the major challenges encountered at weaning is the change from liquid sow's milk to a dry starter diet (*2*). During this transition period, a lag in performance manifested in the form of depressed weight gain and feed intake associated with increased disease and mortality is often encountered (*2*). This is especially true where pigs are weaned at younger ages. The main protein source for a young nursery pig is dried skim milk. This expensive ingredient is an excellent source of easily digestible high quality proteins and contains high amounts of several essential minerals and vitamins. However, due to its high cost the use of dried skim milk is limited to the first one or two weeks after weaning (*2*). Attempts to replace dried skim milk with soybean meal have not been successful. Pathological and immunological responses of soybean feeding on pig performance have been documented by several researchers (*3*−*12*). Early weaned pigs develop a transient hypersensitivity response to soybean proteins resulting in diarrhea and reduced growth rate (*4*−*6*). On account of this, it is recommended that only limited amounts of soybean meal be included in diets fed to pigs weaned between 14 and 21 days.

Recently, we had identified the soybean β-conglycinin α-subunit as being a potential allergen for young piglets (*13*). Epitope mapping showed three peptides spanning amino acids S185-R231 were critical for the allergenicity. Computer generated three-dimensional structure model of the α-subunit of β-conglycinin indicated that the antigenic epitopes were located on the

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surface of the protein (*13*). It was proposed that eliminating the allergenic protein from soybean would greatly improve the nutritive value of the soybean meal and expand its use in the livestock industry. In contrast to soybean proteins, very little is known about the ability of maize proteins to elicit allergic response in livestock. Maize proteins have been reported to cause allergies in sensitive individuals (*14*, *15*). A nonspecific 9 kDa lipid transfer protein and a 16 kDa trypsin inhibitor have been identified as major and minor maize allergens (*14*). A less abundant 50 kDa protein, a component of γ-zein, has been recently identified as a potential allergen (*16*). Since swine diets contain a higher amount of maize than SBM and are extensively used in animal diets, we wanted to examine if any of the maize proteins could induce allergic response in pigs. The results of our study demonstrate that a 27 kD γ-zein is a dominant immunogenic protein to young pigs.

MATERIALS AND METHODS

Animals and Diets. Animal procedures were approved by the University of Missouri-Columbia ACUC. For the detection of antibodies (IgG) specific for corn proteins, blood samples were taken from piglets (17.78 ± 0.56 kg body weight) that were fed corn/SBM diets for 9 days after weaning. The corn/SBM diet was formulated to meet or exceed NRC requirements (*17*) and contained 40% corn, 20% SBM, and 40% supplement (protein, mineral, vitamins, amino acids, and fatty acids, etc.) on a dry matter basis. Blood samples were subjected to centrifugation at 1500*g* for 15 min at 4 °C, and the resulting supernatant was collected and stored at −20 °C until analysis. Plasma from 40 piglets was individually assayed for IgG-binding response to maize proteins.

Protein Separation (SDS−PAGE). Seeds of maize inbred lines B73, NC366, W23a1, W22, MO7W, MP708, MS153, Gt119, and GE37 were kindly provided by Dr. Sherry Flint-Garcia, USDA-ARS, Columbia, MO. Total seed proteins from these maize inbred lines were obtained by extracting 30 mg of seed powder with 1 mL of 1× SDS-sample treatment buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, and 30 mM bromphenol blue, pH 6.8) in a 30 °C shaker for 30 min. After centrifugation (15,800*g*, 10 min), 5% (v/v) β-mercaptoethanol (β-ME) was added to the supernatant and boiled for 5 min. Ten-microliter aliquots of the supernatant were loaded onto a 13.5% acrylamide gel and separated by SDS−PAGE (*18*). Gels were stained overnight with Coomassie Blue G-250. All buffers and reagents were prepared according to Amersham Pharmacia Biotech protocols.

Immunoblot Analysis. Proteins separated by SDS−PAGE were electrophoretically transferred to nitrocellulose membranes (Protran, Schleicher & Schuell Inc., Keene, NH). Membranes were blocked with 5% milk in Tris-buffered saline (TBS, pH 7.3) for 1 h, incubated in 1:500 dilution of plasma overnight at room temperature with gentle rocking. After washing four times with TBS containing 0.05% Tween-20 (TBST) for 10 min each wash, the membrane was incubated for 2 h in 1:5000 dilution of rabbit antipig IgG-horseradish peroxidase conjugate antibody (Sigma, St. Louis, MO). Immunoreactive polypeptides were detected with an enhanced chemiluminescent substrate (Super Signal West Pico Kit; Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol.

Simulated Gastric Fluid (SGF) Digestion Stability of Maize Proteins. Maize seed powder (200 mg) was extracted with 1 mL of solution containing 62 mM Tris-HCl, pH 6.8, and 2% (v/v) β-ME in a 30 °C shaker for 30 min. The slurry was subjected to centrifugation (15,800*g*, 10 min), and the resulting clear supernatant was used for protease digestion. About 100 μg of maize protein was digested with pepsin (Sigma, St. Louis, MO) for various time periods at 37 °C in SGF (100 mM HCl, pH 1.5) as described by Lee and Hamaker (*19*). Briefly, 100 μL aliquots of SGF containing 0.03 mg/mL of pepsin in microcentrifuge tubes were placed in a 37 °C incubation water bath. About 100 μg of the maize protein was added to each of the microcentrifuge tubes containing prewarmed SGF to initiate pepsin digestion. The reactions were terminated at 0, 5, 15, 30, and 60 min after incubation by the addition of 10 μL of 1 N NaOH and 20 μL of 6× SDS-sample treatment buffer (350 mM Tris-HCl, 10% SDS, 30% glycerol, and 175 mM bromophenol blue, pH 6.8). To each tube, 5 μL of β-ME was added, mixed, and boiled for 5 min. Proteolytic fragments generated due to pepsin digestion were examined by SDS−PAGE and Western blot analysis.

Fractionation of Maize Seed Proteins. The different solubility fractions of maize seed proteins were extracted following the procedures described previously (*20*, *21*) with some modifications. Briefly, 100 mg of dry seed powder was sequentially extracted in a 30 °C shaker for 30 min each with 1 mL of 50 mM Tris-HCl, pH 6.8, and 1 mM EDTA (albumin), then 50 mM Tris-HCl, pH 6.8, 1 mM EDTA, and 0.5 M NaCl (globulin), then 50% isopropanol (α, β zeins), and then 50% isopropanol and 5% β-ME (γ zeins). Each extraction was followed by centrifugation for 10 min at 15,800*g* in a microcentrifuge, the supernatant removed, and the remaining pellet re-extracted with the next solution. Three volumes of ice-cold acetone was added to the supernatants from each centrifugation step and stored at −20 °C overnight. Precipitated proteins were recovered by centrifugation at 15,800*g* for 20 min. The protein pellets were air-dried and solubilized in a small volume of SDS-sample buffer. Total zein fraction was also obtained by extracting 100 mg of seed powder separately with 1 mL of 50% isopropanol and 5% β-ME as described above.

MALDI-TOF Mass Spectrometry Analysis of 27 kDa Maize Protein. A partially purified γ-zein fraction was fractionated on a 13.5% SDS−PAGE and stained briefly with Coomassie Blue G-250. A 27 kDa protein which reacted with young pig serum was excised from the gel and processed (destained, reduced with dithiothreitol, and alkylated with iodoacetamide) for trypsinization and then trypsinized overnight. The digest solutions recovered from the gel pieces were frozen with liquid nitrogen and then lyophilized dry. The dried digest for each sample was reconstituted with 5 μL of 990/10 (v/v) water/88% formic acid and desalted on a C18 micro-Ziptip. The final sample of peptides was eluted from the Ziptip in 3 μL of 700/290/10 (v/v/v) acetonitrile/water/88% formic acid. A 1-μL aliquot of each desalted sample was combined with an equal volume of α-cyano-4-hydroxycinnamic acid (CHCA) matrix, and a portion of the mixture was analyzed in the positive ion mode on an Applied Biosystems Inc. 4700 MALDI TOF/TOF mass spectrometer. MALDI TOF/TOF MS/MS were acquired for the 8 most intense ions in the MS spectrum of each digest sample (common trypsin autolysis peptides and contaminant ions were excluded from selection). The digest of 27 kDa maize protein was also analyzed by MALDI TOF MS over the mass range 8000−26000 Da in the linear mode with Sinapinic Acid matrix and external mass calibration (Applied Biosystems Inc. Voyager DEPro mass spectrometer). This analysis was performed in search of the missing large N-terminal tryptic peptides expected for the identified proteins. MALDI TOF/TOF spectra were processed/formatted in the combined MS and MS/MS mode with Applied Biosystems GPS Explorer software (vers. 3.6) and submitted to an in-house copy of Matrix Science's Mascot program (www.matrixscience.com) for database searches against the NCBInr Viridiplantae protein database.

RESULTS

Pigs Fed with Maize/Soy Show Immunogenic Reaction against the 27 kDa Protein. Previous results demonstrated that some soybean seed proteins are potential allergens in young pigs (*8*, *12*, *13*). To examine if any of the maize proteins could also elicit immunogenic response, we obtained blood samples from piglets fed maize/SBM diets for nine days after weaning. Plasma from 40 piglets was individually assayed by immunoblot analysis for IgG-binding response to maize proteins (**Figure 1**). As shown, the plasma from the young pigs revealed immunogenic reaction against a 50, 27, 21, and 16 kDa protein from maize. The immunogenic reaction against the 50, 21, and 16 kDa maize proteins were variable among individual pigs. In contrast, the reaction against the 27 kDa maize protein was strikingly elevated in all of the pigs examined (**Figure 1**). On the basis of this observation, we concluded that the 27 kDa protein in maize is the dominant protein causing problems to pigs and focused our attention on the identification and characterization of this protein. To examine whether the 27 kDa immunodominant maize protein is widely distributed in different genetic backgrounds, we performed immunoblot analysis using total seed proteins form

maize inbred lines B73, NC366, W23a1, W22, MO7W, MP708, MS153, Gt119, and GE37. This analysis revealed that the 27 kDa protein was the most immunodominant protein in all maize inbred lines examined (**Figure 2**).

Maize 27 kDa Protein Is Able to Withstand Digestion by Pepsin. Several allergens are known to be stable when subjected to pepsin digestion (*22*). To evaluate the risk of the 27 kDa maize protein as a potential feed allergen, we examined the ability of this particular protein, along with others, to withstand digestion by pepsin (**Figure 3A**). α-Zein (22 and 18 kDa), which represents about 70% of the zein fraction, was liable to digestion by pepsin. Under the experimental conditions, the 18 kDa α-zein showed a much faster rate of digestion than the 22 kDa α-zein. The 18 kDa α-zein was completely digested within 5 min after pepsin treatment. In contrast, the 27 kDa protein was resistant to pepsin digestion, and a significant amount of this protein remained undigested even after 60 min of incubation with pepsin. These observations were confirmed by immunoblot analysis (**Figure 3B**). Additionally, this analysis also failed to detect any major peptide fragments resulting from pepsin digestion of the 27 kDa protein.

Maize 27 kDa Protein Is Enriched in the Prolamin Fraction. Seed storage proteins have been traditionally classified as albumins, globulins, prolamins, and glutelins on the basis of their solubility properties (*23*). The prolamins, the predominant protein fraction of maize seed, can be separated by SDS−PAGE into four major groups, α-, *β*-, *γ*-, and *δ*-zeins (*24*). The gamma-zeins (*γ*-zeins) are composed of three proteins with apparent molecular weights of 50, 27, and 16 kDa (*25*). The 50 kDa protein accumulates at lower levels than the 27 and 16 kDa *γ*-zeins and can be barely detectable by SDS−PAGE (*25*). The molecular weight of the pig IgG binding protein was similar to that of the 27 kDa *γ*-zein. In order to test whether the 27 kDa immunodominant protein was indeed a *γ*-zein, we fractionated maize seed proteins into different solubility classes and performed immunoblot analysis (**Figure 4**). An examination of the Coomassie stained gel showed that the 27 kDa protein was enriched in the prolamin fraction (**Figure 4**, left panel). Fractionation of the prolamin further into α-zein, *β*-zein, and *γ*-zein fractions revealed that the 27 kDa protein was preferentially associated with the *γ*-zein fraction (**Figure 4**, left panel). Immunoblot analysis using plasma from young pigs further confirmed this observation (**Figure 4**, right panel).

27 kDa Maize Protein Is Recognized by the *γ*-Zein Antibody. Since the immunodominant maize protein is a major component of the zein fraction and has the same molecular weight as the *γ*-zein, we wanted to confirm whether this protein is indeed a *γ*-zein. To test this possibility, we performed immunoblot analysis using maize total proteins and a prolamin fraction enriched in *γ*-zein. Antibodies specific for *γ*-zein (*26*) reacted strongly to 27 and 16 kDa *γ*-zeins (**Figure 5**, left panel). When the nitrocellulose membrane was incubated with young pig serum, a strong reaction was detected against the 27 kDa maize zein but not against the 16 kDa *γ*-zein (**Figure 5**, middle panel).

Identification of the 27 kD Maize Protein as *γ*-Zein by MALDI-TOF MS. To further confirm the identity of the immunodominant maize protein, the 27 kDa protein was subjected to MALDI-TOF mass spectrometry. This analysis revealed a significant

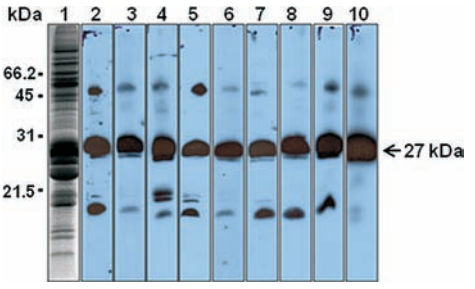

Figure 1. Immunological detection of maize antigenic proteins. B73 maize seed proteins fractionated by SDS−PAGE (lane 1) were transferred to nitrocellulose membranes, incubated with pig plasma collected from different pigs. Immunoreactive proteins were identified using antipig IgG-horseradish peroxidase conjugate antibody followed by chemiluminescent detection.

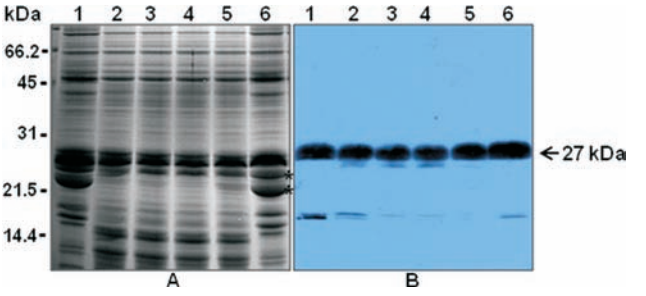

Figure 3. Stability of maize seed proteins subjected to pepsin digestion. Total seed proteins digested with pepsin over different time periods (lane 1, 0 min; lane 2, 5 min; lane 3, 15 min; lane 4, 30 min; lane 5, 60 min) were fractionated by SDS−PAGE on a 13.5% gel and stained with Coomassie Blue (panel **A**) or transferred to a nitrocellulose membrane and probed with pig plasma (panel **B**). Immunoreactive proteins were identified using the antipig IgG-horseradish peroxidase conjugate antibody followed by chemiluminescent detection. Maize seed proteins incubated in the buffer for 60 min in the absence of pepsin served as the control (lane 6). Asterisks in panel **A** point to the α-zeins.

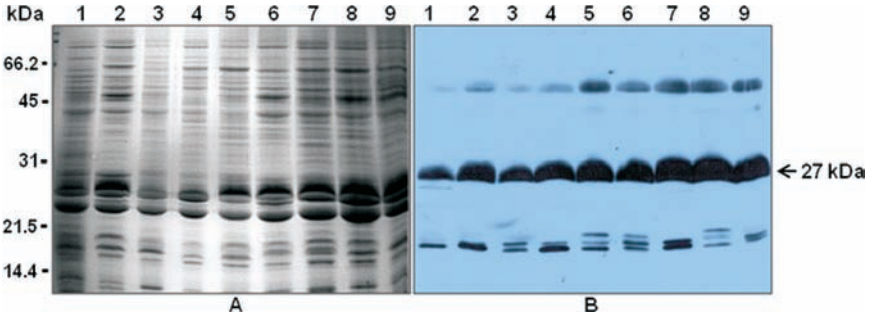

Figure 2. Immunological detection of maize antigenic proteins from different maize inbred lines. Total seed proteins were fractionated by SDS−PAGE on a 13.5% gel and stained with Coomassie Blue (panel **A**). Proteins shown in panel **A** were transferred to a nitrocellulose membrane and probed with pooled pig plasma (panel **B**). Immunoreactive proteins were identified using the antipig IgG-horseradish peroxidase conjugate antibody followed by chemiluminescent detection. Lane 1, B73; lane 2, NC366; lane 3, W23a1; lane 4, W22; lane 5, MO7W; lane 6, MP708; lane 7, MS153; lane 8, Gt119; lane 9, GE37.

sequence homology to the 27 kDa γ-zein (**Table 1**). The statistically significant protein scores for the matches with γ-zein included at least 2 protein matches with MOWSE scores above the 95% confidence level (**Table 1**). The peptide recovery for γ-zein, based on MALDI-TOF/TOF MS over the mass range of 700−4500 Da, was only about 20% of the total protein sequence.

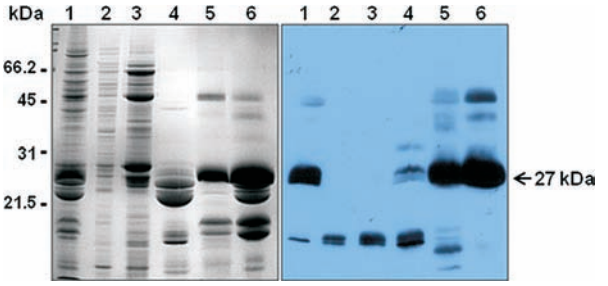

Figure 4. Distribution of maize antigenic proteins in different solubility fractions. Total seed protein (lane 1), albumin (lane 2), globulin (lane 3), α-, β-, and δ-zeins (lane 4), γ-zein (lane 5), and prolamin (lane 6) fractions isolated from maize seeds were fractionated by SDS−PAGE on a 13.5% gel and stained with Coomassie Blue (left panel) or transferred to a nitrocellulose membrane and probed with pig plasma (right panel). Immunoreactive proteins were identified using the antipig IgG-horseradish peroxidase conjugate antibody followed by chemiluminescent detection.

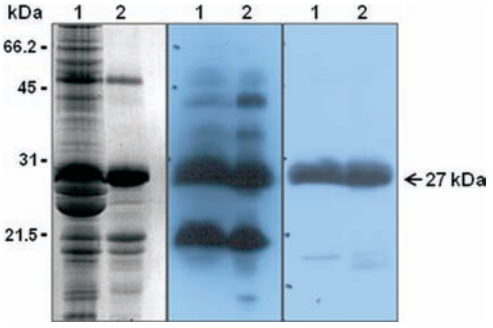

Figure 5. Reactivity of γ-zein antibody with the 27 kDa maize protein. Total proteins (lane 1) and γ-zein fraction (lane 2) were fractionated by SDS−PAGE on a 13.5% gel and stained with Coomassie Blue (left panel). Proteins shown in the left panel were transferred to nitrocellulose membranes and probed with antibodies raised against the purified γ-zein (middle panel) or pig plasma (right panel). Immunoreactive proteins were identified using either the antirabbit IgG-horseradish peroxidase conjugate antibody (middle panel) or the antipig IgG-horseradish peroxidase conjugate antibody (right panel) followed by chemiluminescent detection.

This low recovery stemmed from the fact that the γ-zein contains an ca. 12,000-Da region (near the N-terminus) in which there are no tryptic cleavage sites. MALDI-TOF MS of the digest of total protein in the presence of sinapinic acid matrix enabled detection of several large peptides from the sample proteins. These peptides were assigned on the basis of mass to portions of the large missing N-terminal regions of the γ-zein (**Table 1**). These results confirm that the abundant maize protein eliciting strong immune response in young pigs is indeed the 27 kDa γ-zein.

DISCUSSION

One of the known properties of food allergens is that they exhibit remarkable digestive stability. Poor digestibility enables the proteins to retain allergenicity since the epitopes responsible for the immunogenic reaction are retained intact in the digestive tract of the animals. In addition to our study, a previous study has also shown the maize 27 kDa zein to be highly resistant to proteases (*19*). The maize 27 kDa γ-zein is a cysteine rich protein (7.35 mol %) and contains four disulfide bonds (*27*). These disulfide linkages have been suggested to make the maize 27 kDa γ-zein structurally resistant to proteases (*28*). By individually changing the conserved cysteine residues (C144A, C148A, C155A, and C156A), it was demonstrated that one of the substitutions (C155A) resulted in a remarkable increase in digestibility to pepsin, chymotrypsin, and trypsin (*19*). It should be pointed out that elimination of intramolecular disulfide bonds can reduce allergenicity (*29*). Thus, it will be interesting to test whether altering the disulfide linkages of the γ-zein could also eliminate their allergenicity.

Although some maize proteins have been recognized as a food allergen, only limited investigations have been conducted on their clinical significance. A nonspecific 9 kDa lipid transfer protein and 16 kDa trypsin inhibitor have been identified as major and minor maize allergens (*14*). A 50 kDa protein, a component of γ-zein, has been identified as a potential allergen (*16*). Polyclonal antibodies raised in rabbits against the major almond allergen (AMP) showed a high degree of cross-reactivity with the 50 kDa maize γ-zein and lower level of cross-reactivity with the 27 kDa γ-zein (*16*). The 50 kDa γ-zein also strongly reacted with IgE from human sera from patients sensitive to almond proteins (*16*). The ω-5 gliadin has been identified as a major allergen in wheat-dependent exercise-induced anaphylaxis (*30*). The Ig-E binding epitopes of this allergenic protein has been localized to six sequence motifs: QQIPQQQ, QQLPQQQ, QQFPQQQ, QQFPQQQ, QQSPQQQ, and QQSPEQQ. Substitution of alanine at the amino acid positions Q^1, P^4, Q^5, Q^6, or Q^7 in these peptides abolished the binding of human IgE, indicating that these amino acid residues are critical for IgE binding (*30*). Similar, though

Table 1. Identification of Immunogenic Maize Protein as 27 kDa γ-Zein by MALDI-TOF Mass Spectral Analysis

protein identified	accession number NCBInr (locus)	MOWSE (50 ppm)	sequence coverage	theoretical MW (Da)	experimental MW (Da)	peptides matched
27 kDa storage protein, zein (1−181 amino acids)	gil22550 (CAA41175)	218	82%	19,476	27,000	R-HQCSPTATPYCSPQCQALR-Q (134−152) R-QQCCQQLR-Q (153−160) R-QVEPQHR-Y (161−167) THTSGGCGCQPPPPVHLPPPVHLPPPVHLPPPVHLPPPVHL-PPPVHLPPPVHLPPPVHLPPPPCHYPTQPRPQPHPQPHP-CPCQQPHPSPCQLQGTCGVGSTPILGQVCEFLR (20−133)[a]
γ-zein (1−209 amino acids)	gil168695 (AAA33537)	83	16%	22,225	27,000	R-HQCSPTATPYCSPQCQSLR-Q (120−138) R-QQCCQQLR-Q (139−146) R-QVEPQHR-Y (147−153)

[a] The digest of zein total protein was also analyzed by matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF) over the mass range 8000−26000 Da in the linear mode with sinapinic acid matrix on an Applied Biosystems Voyager DEPro mass spectrometer. This analysis was performed in search of the missing large N-terminal peptides not containing internal trypsin cleavage sites that were unable to be acquired in MS/MS over the mass range 700−4500 Da on the Applied Biosystems 4700 MALDI TOF/TOF mass spectrometer.

not identical, sequence motifs are also present in the 50 kDa and 27 kDa γ-zeins (QQQPQQP, QQQPQQH, and QQQPQSG). However, we do not know if these sequence motifs also serve as the IgE-binding epitopes. Our attempts to localize the binding epitopes of the 27 kDa γ-zein utilizing planar cellulose support technology (SPOT) were not successful.

In this study, we demonstrated that young pigs consuming maize generate antibodies (IgG) directed against the 27 kDa γ-zein. This observation along with the ability of this protein to withstand pepsin digestion indicates that the 27 kDa γ-zein could be a potential allergen to young pigs. However, the unequivocal identification of this protein as an allergen awaits the demonstration of IgE binding. Recently, rabbit antipig IgE has been developed by two groups from the serum of pigs sensitized with *Ascaris suum*-specific protein and peanut proteins (*31*). However, antipig IgE is not commercially available. The development and commercialization of antipig IgE would greatly facilitate studies aimed at the identification of pig allergens and elucidation of pig immune response.

Even though our studies suggest that the 27 kDa γ-zein may have a negative effect on a pig's performance, conclusive evidence awaits feeding trials using maize seeds that lack the 27 kDa γ-zein. A survey of the literature revealed the presence of several mutants with reduced synthesis of maize storage proteins (*24*). However, mutants lacking the 27 kDa γ-zein have not been reported. Interestingly, an ethylmethanesulfonate induced mutant, opaque-15 (*o*15) showing significantly lower amounts of the 27 kDa γ-zein, has been reported (*32*). The *o*15 mutation causes a 2- to 3-fold reduction in γ-zein mRNA and protein synthesis, and reduces the amount of the 27-kDa γ-zein in the endosperm (*32*). However, the seeds of this mutant do not germinate normally and require nutrient supplements for the embryos to develop. It has been suggested that since γ-zeins are the first to be hydrolyzed during germination, the absence of this protein may interfere with the germination process (*33*). Even though *o*15 mutation is associated with poor germination, it could be utilized as a starting material in breeding programs to develop maize inbred lines lacking the 27 kDa γ-zein. An alternative approach is to generate transgenic maize plants lacking the 27 kDa γ-zein. We are currently employing RNAi technology to lower the accumulation of 27 kDa γ-zeins in maize. This technology has been recently utilized to generate mutants of various classes of zeins to assess their role in influencing the shape and stability of protein bodies (*34*). Development of 27 kDa γ-zein free maize cultivars will greatly improve the nutritive value of maize in animal feed and expand its use in the livestock industry.

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