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

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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 period. A lag in 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 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.

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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 1500g 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, M07W, MP708, MS153, Gt119, and G3E7 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 bromophenol blue, pH 6.8) in a 30°C shaker for 30 min after centrifugation (15,800g, 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 and boiled for 5 min. Ten-microliter aliquots of the supernatant and boiled for 5 min. Ten-microliter aliquots of the supernatant were subjected to centrifugation at 15,800g for 20 min. The protein pellets were air-dried and solubilized in a small volume of SDS-sample buffer. Total protein 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 trypsination and then trypsinated overnight. The digest solutions recovered from the gel pieces were frozen with liquid nitrogen and then lyophilized overnight. 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.

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 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 stability 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
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.

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 α-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, QQFP- QQQ, QQSPQQQ, and QQSEPQQ. Substitution of alanine at the amino acid positions Q3, P4, Q5, or Q6 in these peptides abolished the binding of human IgE, indicating that these amino acid residues are critical for IgE binding (30). Similar, though
not identical, sequence motifs are also present in the 50 kDa and 27 kDa γ-zeins (QQQPQQPQP, QQQPQPH, 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 (o15) showing significantly lower amounts of the 27 kDa γ-zein, has been reported (32). The o15 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 o15 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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LITERATURE CITED


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