Immunization of Swine for Production of Antibody Against Zearalenone

J. J. PESTKA 1*, M-T LIU 1, B. K. KNUDSON 2 and M. G. HOGBERG 2

Department of Food Science and Human Nutrition and Department of Animal Science, Michigan State University, East Lansing, Michigan 48824-1224

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ABSTRACT

Production of antisera specific for zearalenone was investigated in swine for potential use in prophylaxis against zearalenone hyperestrogenism. Swine were immunized with zearalenone-6'-carboxymethyloxime bovine serum albumin conjugate by four different protocols. For detection of antigen-zearalenone antibody, a simple indirect enzyme-linked immunosorbent assay (ELISA) was devised whereby porcine antisera was incubated over a zearalenone-6'-carboxymethyloxime poly-L-lysine solid phase and total bound antibodies were detected with peroxidase-labeled anti-swine serum. The optimal immunization protocol consisted of an initial injection of 5 mg of conjugate followed by a 2-mg boost at 4 wk and was sufficient to obtain anti-zearalenone titers of 1:5120 in 8 wk. Competitive indirect ELISA for zearalenone using this antiserum had an assay detection limit of 10 ng/ml for the toxin. Cross-reactivity of the antiserum with α-zearalenol, β-zearalenol, α-zearalanol, and β-zearalanol were 33, 25, 6, and 10%, respectively.

Zearalenone is a naturally occurring β-resorcylic lactone that is produced by members of the genus Fusarium (14). Although it has a low order of toxicity, zearalenone exerts extensive biologic effects on the mammalian reproductive system and has been designated a true estrogen (15). Zearalenone has been detected as a contaminant in corn, wheat, sorghum, barley and mixed animal rations (1), and is often associated with feed causing hyperestrogenic syndrome in swine (12). When purified zearalenone is fed to female swine, it induces enlargement of the uterus and nipples, vulvar swelling, vaginal prolapse, infertility, and reduced litter size. Young male swine exposed to zearalenone exhibit testicular atrophy and enlarged mammary glands. Hence, the effects of zearalenone can have large-scale economic effects on the swine industry.

The conventional means of treating zearalenone toxicoses in swine is by detection of the toxin and subsequent removal of the contaminated feed from the affected animals’ diet. However, because ingestion of zearalenone can be inadvertent and unpreventable, there exists a need for alternate preventative and therapeutic measures directed against zearalenone hyperestrogenism. One possible approach is by the addition of dietary protein supplements, dehydrated alfalfa meal, or anion exchange resins to feed (19). Another means of prevention and therapy might be via active and passive immunization, where in vivo binding of specific antibody to the mycotoxin could hypothetically neutralize the biological effects of the toxin (1). Although immunization has been successfully used to treat the effects of animal, insect, plant, and bacterial toxins, no systematic attempts have been made to test this approach for prevention and treatment of mycotoxicoses.

A number of methods have been reported for production of antibodies to low molecular weight mycotoxins, including aflatoxins, ochratoxins and T-2 toxin in rabbits (3-5), and for use of those antisera in immunoassays of these toxins, in food, feed and clinical samples (6,7,17). Recently, zearalenone antibodies were produced in specific pathogen-free pigs and the antibodies were detected by radioimmunoassay (RIA; 21). We have investigated further the application of this latter approach for immunization of lot-fed swine against zearalenone. The purpose of the following paper is to: (a) describe an indirect enzyme-linked immunoassay (ELISA) for porcine antiserum specific for zearalenone; (b) compare four immunization protocols for eliciting zearalenone antibodies in feedlot swine; and (c) characterize the specificity of porcine anti-zearalenone antiserum toward zearalenone and zearalenone analogs in a competitive indirect ELISA.

MATERIALS AND METHODS

Chemicals

All inorganic chemicals and organic solvents were of reagent grade quality or better. Tween 20, hydrogen peroxide, 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS), bovine serum albumin (fatty acid-free) (BSA), dimethylformamide,
N,N'-dicyclohexylcarbodiimide, N-hydroxysuccinimide, poly-L-lysine (MW 57,000), ovalbumin (Grade II) were purchased from Sigma Chemical Co. (St. Louis, MO). Freund’s complete adjuvant (FCA) and incomplete Freund’s adjuvant (FICA) were purchased from Difco Laboratories (Detroit, MI). Trimethylamine and isobutylchloroformate were obtained from Aldrich Chemical Co. (Milwaukee, WI). Peroxidase conjugated rabbit anti-swine IgG was purchased from Cooper Biomedical (Terre Haute, IN).

Immunogen preparation
Zearalenone was first converted to zearalenone-6’-carboxymethylxime (Z-oxime) as described by Thouvenot and Morfin (21). For immunogen preparation, Z-oxime was conjugated to BSA by the mixed anhydride method of Lau et al. (11). Briefly, Z-oxime (15 mg) was dissolved in 5 ml of dry tetrahydrofuran and cooled to -3°C. Trimethylamine (8.0 μl) and isobutylchloroformate (10 μl) were added, and the solution stirred for 20 min. The reaction mixture was then slowly added to a solution consisting of BSA (75 mg) dissolved in water (15 ml) and pyridine (7.5 ml) at 4°C. The mixture was stirred for 30 min at 4°C and then overnight at room temperature. The Z-oxime BSA conjugate was dialyzed against 3 L of distilled water for 3 d (3 changes). An approximate molar ratio of zearalenone:BSA of 20:1 was determined spectrophotometrically for the resulting conjugate (21).

Swine
Male pigs (4 to 5 months old) used in this study were sired by Duroc boars and produced by Yorkshire-Landrace crossbred dams. A 14% protein corn-soybean meal diet was fed ad libitum in self feeders. All other recommended nutrients in the diet met or exceeded NRC requirements. Pigs were housed, (four/pen) in a naturally ventilated building. Pen size was 2.44 by 2.74 m with solid board partition and a solid concrete floor. Straw bedding and free access to water were provided.

Immunization
Four different swine immunization regimens, identified as groups A, B, C and D, were tested in duplicate pigs. Multiple (5 to 6) subcutaneous injections in neck and rear flank were used for initial immunization and booster regimens. Group A pigs were immunized with Z-oxime BSA conjugate (2 mg) dissolved in 4 ml of an emulsion of saline:FCA (1:1) and were then boosted with conjugate (2 mg) in 4 ml of saline:FICA (1:1) at 2, 4 and 6 wk and conjugate (5 mg) in 4 ml of saline:FICA (1:1) at 11 wk. Group B pigs were immunized with conjugate (5 mg) in 4 ml of saline:FICA (1:1) and were then boosted with conjugate (2 mg) at 4 wk and with conjugate (5 mg) at 11 wk, both in 4 ml of saline:FICA (1:1). Group C pigs were immunized with conjugate (9 mg) in 4 ml of saline:FICA (1:1) and were then boosted with conjugate (9 mg) in 4 ml of saline:FICA (1:1) at 4 and 8 wk. Group D pigs were immunized with conjugate (2 mg) in 4 ml of saline:FICA (1:1) and boosted with the same regimen at 4 and 8 wk. Groups A and B were bled at 2, 4, 6, 8, 11 and 12 wk and groups C and D were bled at 8 and 10 wk. Serum was purified by 35% ammonium sulfate precipitation as described by Hebert et al. (8).

Preparation of Z-oxime poly-L-lysine conjugate
Z-oxime was conjugated to poly-L-lysine, for use as an ELISA solid phase, by the N-hydroxysuccinimide ester method of Kohen et al. (10). Z-oxime (10 mg) was dissolved in dry dimethylformamide (0.2 ml) and equivalent amounts of N,N’-dicyclohexylcarbodiimide (7.1 mg) and N-hydroxysuccinimide (4 mg) were added to the solution. The solution was mixed for 30 min at 25°C, then added slowly to poly-L-lysine (20 mg) dissolved in 0.5 ml of 0.13 M NaHCO3. The reaction mixture was stirred for 30 min at 25°C and then dialyzed against 0.1 M NaHCO3 (4 L) for 1 d and distilled water (4 L) for 2 d at 4°C. The ratio of zearalenone to poly-L-lysine was determined spectrophotometrically to be 19:1.

Antibody titration by indirect ELISA
One hundred microliters of Z-oxime poly-L-lysine (2 μg/ml in 0.1M sodium bicarbonate buffer, pH 9.6) was added to each well of a 96-well microtiter plate (Immunonol Removawell, Dynatech Laboratories, Alexandria, VA) and incubated overnight at 4°C or for 2 h at 37°C. Plates were washed three times with 0.25 ml of 0.1M phosphate buffer in 0.15 M saline (PBS, pH 7.5) containing 0.05% (vol/vol) Tween 20 (PBS-Tween). To block unbound solid phase sites and minimize nonspecific binding, 200 μl of 1% (wt/vol) ovalbumin in PBS was added to each well and incubated for 30 min at 37°C. Plates were washed two more times in PBS-Tween and then incubated with serial dilutions of antisera in 1% ovalbumin in PBS-Tween for 1 h at 37°C. Wells were washed five times and then reacted with rabbit anti-swine peroxidase conjugate, diluted 1:500 in 1% ovalbumin in PBS-Tween for 30 min at 37°C. Plates were washed six more times with PBS-Tween, and bound peroxidase was determined by the procedure described by Pestka et al. (18).

Competitive ELISA
The indirect competitive ELISA was identical to the indirect titration procedure except that 50-μl portions of zearalenone (or zearalenone analog), dissolved in 10% methanol in PBS (vol/vol) were simultaneously incubated with 50 μl of zearalenone antiserum diluted (1:25 to 1:10) in 1% ovalbumin in PBS-Tween for 1 h at 37°C. Bound antibody was then determined as described above.

RESULTS AND DISCUSSION

Titration zearalenone antibody by ELISA
In order to rapidly determine titers of anti-zearalenone antibody, a simple indirect ELISA was devised in which porcine antiserum was incubated over a Z-oxime poly-L-lysine solid phase and total bound antibodies were subsequently detected with peroxidase-labeled anti-swine serum. Results of a typical ELISA titration are shown in Figure 1. The last well in an antiserum dilution series to yield color visually distinct from a preimmune serum control was arbitrarily designated as the titer endpoint (17). Absorbances of titer endpoints at 414 nm were approximately twice that obtained for equivalent dilution of preimmune serum control. Preimmune serum controls, as well as controls run in the absence of porcine anti-zearalenone antibody, and assays run in the absence of peroxidase-labeled anti-swine serum all showed negligible absorbance as
Figure 1. Titration of porcine anti-zearalenone antiserum by indirect ELISA. Symbols are: complete assay with Group B antiserum (Week 8) (●); complete assay with Group B preimmune serum (Week 0) (○); complete assay in minus porcine serum (■); and complete assay with Group B antiserum (Week 8) minus peroxidase-conjugated rabbit anti-swine serum (□). Titer was 1:5120 for this antiserum.

Comparison of immunization procedures

The indirect ELISA was used to titrate serum obtained from swine immunized by the four procedures. Although all groups showed antibody titers to zearalenone, Groups A and B showed earlier and higher titers than Groups C and D (Fig. 2). Maximal average reciprocal titers at 8 wk were 3200 ± 1920 and 5120 ± 0 for Groups A and B, respectively, whereas average 8-wk reciprocal titers for Groups C and D were 320 ± 0 and 1280 ± 0, respectively. In Group B, a single immunization with 5 mg of Z-oxime BSA was sufficient to obtain a reciprocal titer of 3200 ± 1920 at only 4 wk. Although the use of only two animals per group limits in depth interpretation of these data, on the overall basis of time and reagent economy, the immunization protocol for Group B, i.e., multiple site injection of 5 mg of Z-oxime BSA in saline:FCA followed by a single boost at 4 wk with 2 mg of the same immunogen in saline:FICA, appeared optimal for eliciting an anti-zearalenone antibody response in lot-fed swine. Of the four approaches, this one would be most applicable to studies testing the feasibility of using immunization for prevention and therapy of zearalenone toxicoses. This approach was also simpler and more efficient than biweekly immunization of swine with conjugate (2 mg) described by Thouvenot and Morfin (21), where 14 wk were required to obtain an optimal RIA titer.

Specificity of anit-zearalenone sera

Competitive indirect ELISAs were conducted to determine the specificity of porcine anti-zearalenone antibody towards the zearalenone analogs shown in Figure 4. Using the concentration of analog required to reduce absorbance by 50% as a basis for comparison, we estimated the relative cross-reactivities for zearalenone, α-zearalenol, β-zearalenol, α-zearalanol and β-zearalanol to be 100, 33, 25, 6 and 10%, respectively. This suggests that: (a) the double bond at the C1'-C2' position (Fig. 4) was predominant in determining specificity of the antibody for zearalenone analogs as evidenced by cross-reactivity among zearalenone, α-zearalenol, and β-zearalenol and (b) the antibody showed little ability to discriminate the α-configuration over the β-position. Because α- and β-zearalenol are major metabolites of zearalenone (15,22) and are also estrogenic (9,20), the ability of our sera to bind to these metabolites as well as zearalenone would be advantageous should immunization be utilized for neutralization of zearalenone toxicoses in swine.

PRODUCTION OF ANTIBODY AGAINST ZEARALENONE

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Figure 2. Summary of anti-zearalenone titers obtained for four immunization trials. For the basis of comparison, each bar represents the mean ± standard error of two pigs. Immunization protocols for groups A, B, C and D are summarized in Table I.

Figure 3. Competitive indirect ELISA for zearalenone. Each data point represents mean ± standard error of triplicate wells in a single ELISA plate. A 1:50-dilution of antibody was used. Correlation coefficient of best fit line (power function) was 0.97.

In contrast to our results, Thouvenot and Morfin (21) determined that the relative cross-reactivities for zearalenone, α-zearalenol, β-zearalenol, α-zearalanol and β-zearalanol in RIA were 100, 100, 44, 68 and 44%, respectively. Hence, their antibody showed preferential specificity for the α-configuration but less ability to discern whether the C1'-C2' bond was saturated or unsaturated. These apparent differences might be attributed to two factors. First, we utilized a mixed anhydride conjugation procedure for immunogen preparation which resulted in a 20:1 molar ratio of zearalenone to BSA as compared to the carbodiimide procedure of Thouvenot and Morfin (21) which yielded a 7:1 molar ratio of zearalenone to BSA. Spatial arrangement of this increased number of zearalenone molecules on our immunogen might have favored the specificity observed in our antibody. The higher toxin:carrier ratio may have also enhanced our ability to obtain antibody earlier during the immunization. Secondly, use of free tritiated zearalenone...
in competitive RIA might yield different cross-reactivity data than that obtained using solid phase Z-oxime poly-L-lysine as a marker ligand in the indirect ELISA.

In conclusion, we have described an optimized procedure which is effective for immunizing swine against zearalenone and utilized an indirect enzyme immunoassay for detection of the resulting antibody. This immunization protocol might be applicable to testing active immunization as a method for prevention of zearalenone mycotoxicosis in swine. In addition, antisera collected from immunized swine might be utilized in passive therapy for neutralizing the estrogenic effects in swine already exposed to zearalenone. Further research directed towards testing these hypotheses is ongoing in our laboratory.

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