Development of a Quantitative and Sensitive Enzyme-Linked Immunosorbent Assay for Ochratoxin A Using Antibodies from the Yolk of the Laying Hen

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Antibodies directed against ochratoxin A (OA) were obtained from hen egg yolk using an optimized purification procedure and applied in an enzyme-linked immunosorbent assay (ELISA) for OA in swine finisher diets. The egg yolk antibody could be recovered at levels as high as 70–80% with purities greater than 86–92% using a mixture of aqueous buffer and chloroform for lipid extraction and poly(ethylene glycol) for antibody precipitation. Ochratoxins C, B, and α and the structurally related mycotoxin citrinin were found to cross-react with the antibody 400, 100, 33.3, and 2%, respectively, in an indirect competitive ELISA. Ochratoxin A could be detected in swine finisher diets at levels greater than 50 ppb using a simplified sample preparation procedure and a indirect competitive ELISA. Recoveries of OA from the diets were validated by conventional HPLC analysis using a proven sample extraction protocol. ELISA-determined OA values correlated highly with those obtained using HPLC analysis ($r = 0.98$). Assay sensitivity was found to be dependent on background absorbance. The mixed anhydride (MA) coupling chemistry used to prepare the immunogens promoted high background absorbances in the quantitative ELISA. The background was overcome by using *N*-hydroxysuccinimide activated ester coupling chemistry for the preparation of plate coating antigen and or incubation of the antibody with bovine serum albumin that had been subjected to the MA reaction. This study demonstrates that antibodies from hen egg yolk can be readily obtained in good yield and purity and used to develop a highly sensitive ELISA for OA.

INTRODUCTION

Ochratoxin A (OA), a secondary fungal metabolite belonging to the group of molecules known as mycotoxins, is known to be hepatotoxic, nephrotoxic, teratogenic, and mutagenic to a wide variety of animals (Roschenthaler et al., 1984; Steyn, 1984; Marquardt et al., 1990). The mycotoxin's widespread natural occurrence in animal feeds and tissues and its potential hazard to humans have led to much research in its detection. Techniques such as TLC, HPLC, and, more recently, ELISAs have been described for OA (van Egmond, 1991). Conventional chromatographic techniques often lack sensitivity, as is the case for TLC, and usually require extensive sample cleanup prior to analysis. Alternatively, ELISAs offer several potential advantages, a lowered requirement for sample cleanup, the possibility of large sample throughput, and improved sensitivity and specificity (Pestka, 1989; Chu, 1990). Mycotoxin-specific antibodies are primarily obtained from rabbits or mice, but, interestingly, there has been no report of the use of laying hen egg yolk polyclonal antibodies, IgY. Although the chicken has not been widely used as a source of polyclonal antibodies, it offers several important advantages over conventional mammalian antisera. Collection of the eggs is convenient and noninvasive, as compared to the repeated bleeding of mammals, and there appears to be a quantitative advantage in the production of antigen-specific antibody (Gassman et al., 1990). Several IgY purification protocols exist and report high yields and purities. Techniques of purification have included simple precipitation with reagents such as poly(ethylene glycol) (PEG) (Polson and von Wechmar, 1980), organic solvent extraction of the lipid component (Auliso and Shelokov, 1976; Bade and Stegemann, 1984), and advanced column chromatographic protocols (McCannel and Nakai, 1990; Hassl and Aspock, 1988). The procedure of Polson (1990) was selected for anti-OA IgY purification as the technique proved to be relatively simple with the potential for further yield improvement and the yields were reported as better than those of earlier published protocols. This paper reports methodology for the production and purification of OA-specific polyclonal antibodies from the yolk of a laying hen and the development of a sensitive and quantitative ELISA for OA in swine finisher diets.

MATERIALS AND METHODS

Materials. Pure OA was obtained via surface liquid fermentation with *Aspergillus alutaceus* (*Aspergillus ochraceus* Whilhelm, NRRL 3174) as previously described (Sreemannarayana et al., 1988). Ochratoxin α (Oα) was prepared by hydrolysis in strong acid according to the procedure of Doster and Sinnhuber (1972). Ochratoxin B (OB) was isolated in the same manner as that reported by Madhyastha et al. (1990). Citrinin, L-phenylalanine, bovine serum albumin (BSA), dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), complete Freund's adjuvant, and polyethylene glycol 8000 (PEG) were obtained from Sigma Chemical Co., St. Louis, MO. Microtiter plates (Falcon 3911, Microtest III) were obtained from Becton Dickinson Labware, Oxnard, CA. All remaining solvents and reagents were of reagent grade quality or better.

Immunization of the Laying Hens. White Leghorn laying hens (Shavers SX 288) that were approximately 20 weeks of age were immunized and used for egg production in accordance with standard animal care regulations. The immunogen consisted of 2 mg of BSA–OA conjugate prepared according to the mixed

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anhydride (MA) procedure of Chu et al. (1982). The immunogen was dissolved in 0.5 mL of 0.1 M saline and emulsified with an equal volume of complete Freund's adjuvant prior to injection. One milliliter of the mixture was injected intramuscularly (pectoral muscles) at two different sites on the hen. Two booster injections using the same preparation were given subsequently at days 21 and 70. Eggs were collected every day following the final boost for two months, pooled, purified, and stored at 4 °C.

IgY Activity Recovery ELISA Used for Optimization of IgY Purification and Comparison Analysis. The amount of IgY recovered during an extraction was quantified indirectly on the basis of the final recoverable anti-ochratoxin A IgY (anti-OA) activity in a manner similar to that previously described (Kühlmann et al., 1988). In this procedure, the total amount of IgY in the sample as determined by the indirect noncompetitive ELISA was estimated on the basis of the dilution of the unknown sample that was required to produce the same absorbance as a standard anti-OA IgY preparation. A reference absorbance was selected at 25% of the maximal absorbance and was found to yield acceptable accuracy and reproducibility when compared to the originally proposed value of 10%.

Ochratoxin A was conjugated to ovalbumin using the mixed anhydride reaction previously described (Chu et al., 1982). The ELISA plates were coated with 100 μL of 0.1 μg/mL ovalbumin–OA conjugate (OV–OA) in 0.05 M carbonate buffer (pH 9.6) overnight at 4 °C. After the plates were twice washed with 0.1 M (pH 7.2) phosphate buffer containing 0.1 M saline (PBS), the plates were "blocked" using PBS containing 2% instant skim milk powder. Following the "blocking" step the plates were washed twice with PBS and 50 μL of the whole yolk or partially purified IgY possessing anti-OA activity that was diluted initially 1:100 and then subsequently diluted by doubling serial dilutions. The plates were again incubated at 37 °C for 1 h. Following 1 h of incubation, the plates were washed twice again with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Fifty microliters of the alkaline phosphatase labeled rabbit anti-chicken IgG diluted 1:2500 in PBS-T containing 0.2% skim milk powder was applied, and the plate was incubated for 1 h at 37 °C. Plates at this last step were washed extensively using PBS-T (at least six washing cycles) and then developed by the addition of 50 μL of the substrate solution [1 mg/mL *p*-nitrophenyl phosphate, diethanolamine buffer (pH 9.8)]. The plates were incubated at room temperature for 1 h, after which time, the absorbency was measured using a microplate reader (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada, Model 450) fitted with a 405-nm filter. The reference yolk sample was found to be stable for a period of at least 3 months at 4 °C. Recoveries of anti-OA IgY activity and total protein were determined for the procedures of Polson et al. (Polson and von Wechmar, 1980; Polson et al., 1985; Polson, 1990). The total proteins following the extraction were adjusted to the same concentration and the activity recoveries determined for comparison purposes.

Optimal Conditions for IgY Purification. Egg yolk antibody, IgY, was purified as described by Polson (1990) with the following yield-optimizing modifications. In general, 1 volume of yolk was diluted with 4 volumes of 0.1 M (pH 7.2) phosphate-buffered saline (PBS) and 1 volume of chloroform. The mixture was shaken well and centrifuged for 30 min at 10000*g*. The top aqueous layer was recovered, and IgY was precipitated twice with 14% (w/w) polyethylene glycol 8000. The IgY precipitate was reconstituted in PBS containing sodium azide (0.005%) and 5% glycerol and was stored frozen at −20 °C. IgY preparations for routine usage were kept at 4 °C after washing and concentration of the IgY preparation by ultrafiltration (Amicon, Centriprep 10 concentrator, Beverly, MA) to approximately 5–10 mg mL^{-1}. This preparation appeared to be stable for more than 6 months, even though some precipitation was observed.

Total Protein Determination. The protein content of the extracted yolk samples was determined using the bicinchoninic acid method for determining total protein concentration (Smith et al., 1985). Bovine serum albumin was selected as a standard. The final sample absorbency was measured on a 96-well microtiter plate using a microplate reader (Bio-Rad Laboratories, Model 450) fitted with a 570-nm filter.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a Mini-Protein II electrophoresis apparatus (Bio-Rad Laboratories) under reducing conditions was performed according to the method of Laemmli (1970). A gradient polyacrylamide gel with a concentration range of 4–20% (Bio-Rad Laboratories) was selected for separating protein bands. The resulting protein bands were stained according to the manufacturer's instructions (Bio-Rad Laboratories) using Coomassie brilliant blue R250. Purity of the final protein preparation was estimated using a Beckman DU-8 spectrophotometer fitted with a gel scanning apparatus set to read protein peaks at 590 nm.

Preparation of Indirect Competitive ELISA Plate Coating Antigen. Ochratoxin A was conjugated to ovalbumin (OA–OV) using the MA reaction described by Chu et al. (1982). The alternate plate coating antigen was prepared in a manner similar to that previously reported by Gendloff et al. (1986). Ten milligrams of pure OA, 40 mg of NHS, and 40 mg of DCC were added to 1 mL of dry tetrahydrofuran. The mixture was allowed to react in the dark for at least 30 min at 25 °C with constant stirring. The white precipitate (oxidized DCC) that was formed during the reaction was removed by centrifuging at 10000*g* for 15 min. Residual reactant was removed from the precipitate by several re-extractions with tetrahydrofuran followed by its filtration through a Pasteur pipet plugged with a glass wool frit. Supernatant and washings were pooled and dried under a stream of nitrogen gas. The dried product was reconstituted in 2 mL, final volume, of *N*,*N*-dimethylformamide. Two milliliters of the activated OA–NHS ester was added slowly and dropwise to a continuously stirred solution containing ovalbumin (100 mg in 50 mL of 0.1 M sodium bicarbonate). This mixture was allowed to react for 30 min at 25 °C with constant stirring in the dark and was subsequently dialyzed against 6 L of distilled water (at least three changes). The conjugate following dialysis was collected, diluted with PBS to a concentration of approximately 1 mg mL^{-1}, and stored at 4 °C until coated onto ELISA plates.

Preparation of Mixed Anhydride Modified Bovine Serum Albumin. A bovine serum albumin conjugate, termed "mock conjugate", that contained only MA determinants was prepared by a modified version of the MA reaction of Chu et al. (1982). In this procedure OA was removed from the reaction and substituted with a mole equivalent of L-glycine, while BSA was selected as protein for conjugation. L-Glycine was selected as a substitute carboxyl-containing molecule. The conjugate denoted BSA–MA was extensively dialyzed against double-distilled water to remove all organic solvents and unreacted L-glycine. The protein was subsequently diluted with PBS (1–5 mg mL^{-1}) and stored at 4 °C with sodium azide (0.005%). Storage periods of greater than 3 months did not affect its ability to counteract the nonspecific background binding.

Preparation of OA Contaminated Swine Finisher Diet. Crushed and moistened (32% water) soybeans were inoculated under sterile conditions with a preparation of *A. alutaceus* (*A. ochraceus* Whilhelm, NRRL 3174) spores and then incubated at 28 °C for 30 days. Inoculated soybean samples were analyzed for OA production by HPLC (data not shown) and were added to an OA-free swine finisher diet. The diet samples were blended and reground to give the desired concentration of OA. The mixed diets were composed of 89% barley, 7.8% soybean meal, 2.5% vitamin pre-mix, and an additional 0.7% of OA-contaminated crushed soybeans.

Simplified Swine Finisher Diet OA Extraction Procedure. A "simplified" methanol-based extraction procedure was evaluated and involved the extraction at 25 °C with constant shaking of a 5-g sample of diet with 30 mL of methanol–water (80:20) in a closed capped Nalgene centrifuge tube for 30 min. The mixture was centrifuged for 30 min at 5000*g*, and the cleared supernatant was recovered by decanting. Aliquots of supernatant were removed, diluted with HPLC grade methanol, and filtered through a 0.5-μm filter prior to HPLC and ELISA analysis.

Ochratoxin A Extraction Procedure Used for Comparison of Recoveries. The methanol-based extraction and reversed-phase cartridge cleanup procedure, which was used for comparison, was a slight modification of that originally described by Lee and Chu (1984) for the determination of OA in wheat by ELISA. In brief, 5 g of diet sample was mixed with 15 mL of pure methanol and shaken for 30 min. The mixture was filtered through a Whatman (No. 4) paper filter and the filtrate was

Table I. Percent Recovery of Anti-OA IgY Activity and the Relative Fold Purification of IgY for the Procedure Developed in the Current Study As Compared with Those of Other Procedures

procedure	current study: % anti-OA IgY activity recovered[a]	current study: relative fold purification	current study: yield of IgY[b] (mg/egg)	reported yields of IgY[b] (mg/egg)
Polson and von Wechmar (1980)	61.2	11.5	77.5	80, 65, 83, 50[c]
Polson et al. (1985)	29.6	5.6	37.5	45[d]
Polson (1990)	57.1	10.8	72.3	
current procedure	75.0	14	95	

[a] Values shown are the means of at least four replicates, standard errors (≤ ±5). [b] Calculations of yields were made on the assumption that the volume of an average egg yolk is 15 mL and its total protein content is 160 mg/mL. Recovery assay was the same as that described under Materials and Methods. [c] Values reported by Altschuh et al. (1984), Gassmann et al. (1990), Gottstein and Hemmeler (1985), and Hassl et al. (1987). [d] Value reported by Hassl and Aspöck (1988) and Hassl et al. (1987).

passed through a C_{18} Sep-Pak (Waters Associates, Milford, MA). The samples, following cleanup, were dried using a rotary evaporator (Buchi, Brinkman, Rotavapor R110), reconstituted in a small volume of methanol, and passed through 0.5-μm filters prior to ELISA or HPLC analysis.

High-Performance Liquid Chromatography for OA Recovery Analysis and ELISA Correlation. Analytes were separated on a Beckmann 5-μm Ultrasphere ODS reversed-phase C_{18} 25 cm × 4.6 mm analytical column using an isocratic mobile phase containing 70% methanol and 30% distilled water adjusted to pH 2.1 using H_3PO_4 with a system similar to that described by Sreemannarayana et al. (1988). Ten to 50 μL of diet extract was injected and chromatographed using a solvent flow rate of 1.6 mL min^{-1} at a column temperature of 40 °C. OA peaks were detected using fluorescence emission with a Shimadzu Model RF-535 detector set at 333 nm for excitation and at 450 nm for emission.

Indirect Competitive ELISA for Quantitation of OA in Swine Finisher Diets and Determination of Antibody Cross-Reactivity. Coat ELISA plates with 2.5 μg/200 μL/well of the activated ester prepared coating antigen (OA–OV) dissolved in 0.05 M carbonate buffer (pH 9.6) overnight at 37 °C. Wash plates two times with PBS and block plate with PBS containing 1% skim milk (200 μL/well) for 1 h at 37 °C. Coated and blocked plates can be stored empty and dry at 4 °C for several months. Dilute anti-OA antibody and mock conjugate in PBS to achieve dilution of antibody and a final protein concentration of the mock conjugate of 1:1500 and 3 mg/mL, respectively. Incubate the mixture for 1 h at 37 °C prior to adding to ELISA plates. Apply onto the coated and blocked ELISA plates, consecutively, 65 μL of PBS, then 10 μL of the sample which contains either unknown or OA standards in methanol, and then 75 μL of the diluted and preincubated, with the mock conjugate, laying hen anti-OA IgY. Mix wells carefully and incubate for 1 h at 37 °C. Wash plates with PBS-T three times and dry plates. Apply 150 μL of the alkaline phosphatase labeled rabbit anti-chicken IgG diluted at 1:2000 in PBS-T containing 0.2% skim milk powder. Incubate the plate for 1 h at 37 °C. Wash plates six times with PBS-T and dry plates thoroughly. Apply 150 μL of alkaline phosphatase substrate solution and incubate the plate for 30 min at 37 °C or until absorbency of the sample with no free OA is greater than 1.5 OD units. Read plates directly on microplate reader at 405 nm.

RESULTS AND DISCUSSION

Selection and Optimization of a Procedure for the Purification of Anti-OA IgY. Several procedures for the purification of antigen-specific IgY were examined for recovery and purity, and the values obtained were compared with those reported in the literature. The amounts of IgY recovered in the comparison experiment, as determined by the indirect noncompetitive ELISA, compared favorably with those values originally reported in the literature (Table I). The results obtained for the Polson (1990) technique were unexpected, as the original report suggested higher yields than reported by Polson and von Wechmar (1980); yet, in comparison to the other isolation procedures tested, the procedure of Polson (1990) was the most readily improved. The systematic investigation of factors affecting the yield and purity of IgY demonstrated that the most practical ratio of solvents for the extraction of yolk was 1(10 mL):1(10 mL):4(10 mL), respectively, for yolk, chloroform, and PBS (Table II). The anti-OA activity appeared to be quantitatively precipitated with polyethylene glycol 8000 at a concentration of greater than 14% (w/w), with maximal purity being obtained after two successive precipitations, Figure 1 and Table III, respectively. Gradient gel (4–20%) SDS–PAGE carried out according the the method of Laemmli (1970) followed by scanning densitometry demonstrated

Table II. Percent Recovery of Total Protein, Anti-OA IgY Activity, and the Relative Fold Purification of IgY in the Aqueous Supernatant following Lipid Extraction[a]

volume of $CHCl_3$ (mL)	volume of PBS dilutant (mL): 0	10	20	40
Percent Recovery of Total Protein in Supernatant[b]				
0	100.0 ± 3.1	51.8 ± 1.5	59.4 ± 1.9	71.4 ± 2.0
10	10.6 ± 0.6	23.1 ± 1.1	24.9 ± 1.4	32.2 ± 1.8
40	4.8 ± 0.2	13.7 ± 0.8	22.9 ± 1.3	31.0 ± 1.7
Percent Recovery of Anti-OA IgY Activity in Supernatant[b]				
0	100.0 ± 11.1	100.4 ± 9.6	90.0 ± 10.6	95.2 ± 8.5
10	23.7 ± 4.2	69.2 ± 6.8	72.2 ± 5.6	86.0 ± 6.4
40	16.3 ± 2.9	49.4 ± 3.9	67.0 ± 7.4	85.8 ± 8.0
Relative Fold Purification of IgY[b,c]				
0	1.0 ± 0.1	1.9 ± 0.2	1.5 ± 0.2	1.3 ± 0.1
10	2.2 ± 0.5	3.0 ± 0.4	2.9 ± 0.4	2.7 ± 0.3
40	3.4 ± 0.7	3.5 ± 0.5	2.9 ± 0.5	2.8 ± 0.4

[a] The volume of yolk extracted for all tests was 10 mL. The concentration of protein in the egg yolk was 160 mg/mL. [b] Values represent the mean and standard error of at least eight replicas. [c] Relative fold purification (= recovered anti-OA IgY activity in sample/recovered total protein in sample).

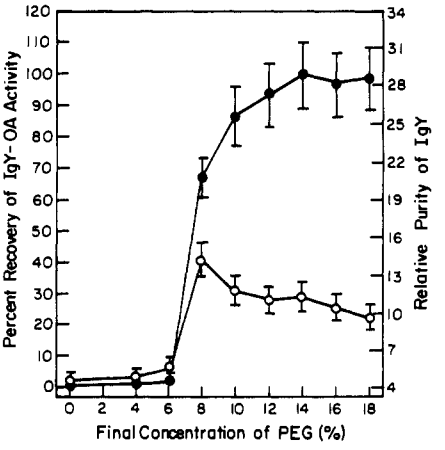

Figure 1. Percent recovery of IgY anti-OA (●) activity and relative fold purification of the protein precipitate (○) following addition of different concentrations of PEG-6000 to aqueous extract of yolk. Percent total protein recovery and IgY activity values are the means of eight replicates, and the vertical bars represent the standard error of the means.

Table III. Percent Recovery of Total Protein and Anti-OA IgY Activity and Corresponding Relative Fold Purification following Successive Precipitation with PEG-8000[a]

	% of total protein recovered[b] (x)	% of anti-OA IgY activity recovered[b] (y)	relative fold purification (x/y)
whole yolk	100.0 ± 7.0	100.0 ± 3.9	1.0 ± 0.1
aqueous supernatant protein	21.5 ± 1.5	85.7 ± 3.4	4.0 ± 0.4
precipitate 1	7.5 ± 0.6	82.5 ± 3.3	11.0 ± 1.2
precipitate 2	5.4 ± 0.5	75.0 ± 5.0	14.0 ± 1.8
precipitate 3	5.2 ± 0.6	44.2 ± 4.4	8.5 ± 1.6
precipitate 4	4.8 ± 0.6	31.2 ± 3.1	6.5 ± 1.3

[a] The procedure used was as outlined in Figure 2 and described under Materials and Methods. [b] Values shown are the mean and standard error of at least four replicates.

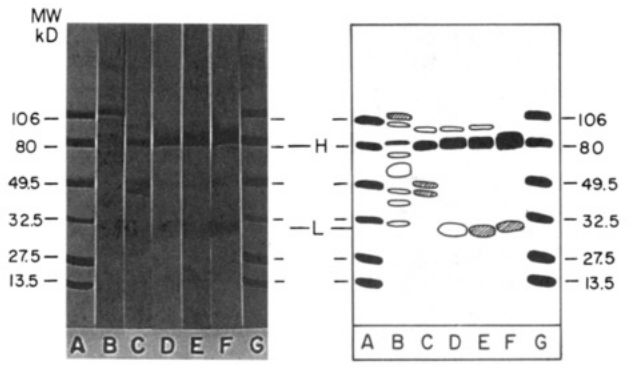

Figure 2. Reducing SDS–PAGE electrophoretic evaluation of extracted proteins following different steps of antibody purification. (Lanes A and G) Prestained, low molecular protein standards (Bio-Rad Laboratories Ltd.); (lane B) diluted whole egg yolk; (lane C) aqueous supernatant following lipid solvent extraction; (lanes D and E) aqueous supernatant following one and two 14% PEG-6000 precipitations; (lane F) commercially prepared IgY standard. H and L denote heavy and light chains of IgY, respectively.

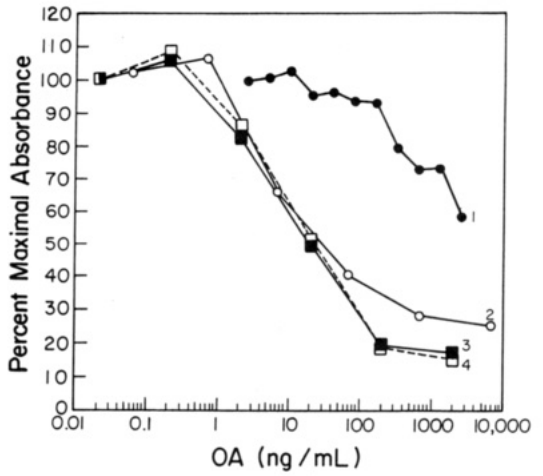

Figure 3. Reduction in background binding and concurrent increase in sensitivity due to the substitution of coating antigens (CA) and addition of BSA–MA mock protein conjugate: (1) MA prepared CA, 0 mg mL^{-1} BSA–MA (●); (2) NHS prepared CA, 0 mg mL^{-1} BSA–MA (○); (3) MA prepared CA, 3 mg mL^{-1} BSA–MA (■); (4) NHS prepared CA, 3 mg mL^{-1} BSA–MA (□). Standard errors (n = 6) in all cases were less than 10%.

Table IV. Cross-Reactivity of Chicken Anti-OA IgY with Various Ochratoxin A-like Molecules in an Indirect Competitive ELISA

structurally related molecules	cross-reactivity[a] (%)
ochratoxin A	100.0
ochratoxin B	100.0
ochratoxin α	33.3
ochratoxin C	400.0
L-phenylalanine	0
citrinin	1.9

[a] Cross-reactivity was determined by comparing the concentrations of toxin required to give 50% maximal absorbency in the indirect competitive ELISA. All analyses were performed at least in triplicate.

the final IgY preparation was reasonably pure as it contained between 8 and 14% of non-IgY protein (Figure 2). The final IgY recovery and purity for the optimized procedure are shown in comparison to the results of the earlier suggested purification procedures (Table I). The optimized procedure is consequently an excellent means of preparing nearly pure and immunologically active IgY in relatively short periods of time and avoids the use of cumbersome column chromatographies.

Cross-Reactivities of the Laying Hen Ochratoxin A Specific Antibody Preparation. The anti-OA IgY preparation used for the development of the OA indirect competitive ELISA appeared to have antibodies with noticeable specificity differences, i.e., the presence of nontoxin specific antibodies. In a normal indirect competitive ELISA using a mixed anhydride prepared coating antigen (OA–OV) and OA as the competing antigen, full inhibition of antibody response by the free ochratoxin A was not possible (Figure 3). This observation is consistent with the findings of Gendloff et al. (1986), who also reported the presence of high backgrounds in their mycotoxin-specific ELISAs. Their experiments confirmed the presence of new antigenic determinants and attributed their presence to the mixed anhydride coupling reaction which was used to prepare the toxin–protein immunogen. One solution proposed by this group was to replace the plate coating antigen coupling chemistry. They selected NHS activated ester procedures instead of MA coupling chemistries to prepare the plate coating antigen. Although they found the technique to be generally useful, the approach was shown to not work in all cases; therefore, alternative techniques needed to be developed. In the current study, the MA coupling chemistry used to prepared the OA–OV plate coating antigen was replaced with the NHS activated ester coupling reaction. This technique as demonstrated in Figure 3 reduced backgrounds significantly. Alternatively, the high background binding observed could be reduced by the preincubation of anti-OA IgY preparation with BSA which had been treated with the mixed anhydride coupling reagents (BSA–MA). This course of action should prove to be useful when alternation of plate coating antigen chemistries fail. The combination of both techniques yielded backgrounds sufficiently low for routine analysis and was therefore included in the final ELISA used to quantitate OA in swine finisher diets.

The antibody was found to be ochratoxin group specific as it exhibited cross-reactivity with several other ochratoxin A-like molecules in an indirect competitive ELISA (Table IV). The cross-reactivities are of interest as ochratoxins B and C are toxic, while ochratoxin α and L-phenylalanine are not (Marquardt et al., 1990). The general specificity of this antibody preparation is not expected to interfere with quantitation of OA by ELISA in grain-based diets, as OA is the most predominant member of the group to occur naturally, but may depend on substrate and particular fungi infecting the grain. In grains such as corn and barley, OB is reduced in comparison to OA, but in peanuts, soybean, and rapeseed, OB production can be enhanced (Roth et al., 1989; Madhyastha et al., 1990). Ochratoxin C, which competes extensively in this ELISA, is a rare molecule and is unlikely to interfere with OA quantitation under normal conditions (Candlish

Table V. Effect of Sample Preparation on Recovery (Percent) of Ochratoxin A from Swine Finisher Diet by HPLC[a]

toxin added to diet (ng/g; spiked level)	simplified sample preparation procedure	comparison sample preparation procedure
50	100.0 ± 4.0	100 ± 4.0
100	100.0 ± 10.0	100.0 ± 10.0
200	110.0 ± 4.5	na[b]
1000	85.0 ± 23.0	96.0 ± 2.1
2000	93.0 ± 5.9	100 ± 3.5
5000	97.2 ± 5.9	na[b]

[a] Mean and standard deviation. Each sample analyzed by HPLC was done at least in triplicate. The correlation between OA recoveries for the two extraction procedures was $r = 0.98$ ($P < 0.05$) as determined by HPLC. [b] na, not analyzed.

Table VI. Recovery of Ochratoxin A from Swine Finisher Diet Samples As Determined by the Quantitative Indirect Competitive ELISA Using the Simplified Sample Preparation Procedure[a]

toxin added to diet (ng/g; spiked level)	toxin detected by ELISA (ng/g)	recovery (%)
50	55 ± 5	110.0 ■ 9.1
100	115 ± 20	115.0 ± 17.4
200	215 ± 30	107.5 ± 14.0
1000	815 ± 80	81.5 ± 9.8
2000	1945 ± 150	97.2 ± 7.7
5000	4565 ± 400	91.3 ± 8.7

[a] Mean and standard deviation. Each sample analyzed by the ELISA was done at least in triplicate. Recoveries by ELISA and HPLC (Table V) using the simplified extraction gave a correlation of $r = 0.99$ ($P < 0.05$).

et al., 1986; Fuchs et al., 1984). Ochratoxin α has not been reported in grains but is an excellent indicator of past OA contamination (Screemannarayana et al., 1988; Xiao et al., 1991). Identification of OA and citrinin toxicosis is possible as the antibody showed no or little cross-reactivity. Citrinin often co-occurs with OA and has similar modes of toxicity (Damoglou et al., 1984; Krogh, 1973b). Although there exists the possibility of erroneous OA analysis, the antibody developed can be considered relatively specific for grain analysis.

Development of a Quantitative Indirect Competitive ELISA for Determination of OA in Swine Finisher Diets. The development of a quantitative ELISA for OA in swine finisher diets is a practical and potentially useful model application of this antibody since swine finisher diets are composed of a myriad of complex and potentially interfering matrixes which could conceivably be encountered in routine grain testing.

The indirect competitive ELISA that was developed using a simple sample preparation procedure which avoided extensive sample cleanup. The proposed sample extraction procedure was validated by conventional HPLC recovery analysis using a published comparison procedure involving the use of a more elaborate OA extraction protocol (Table V). An important feature of the simplified procedure for sample preparation is its use of methanol. This solvent has many advantages over chloroform, a ubiquitous OA extraction solvent, in that it is less toxic, is easily disposed, has comparable OA extraction recoveries, and is readily diluted with aqueous buffers necessary for the ELISA. It is also known that acetonitrile can be used effectively for the extraction of OA (Lacey et al., 1991); however, it also has considerable toxicity and is in general more expensive for routine analysis. The ability to quantitate OA by the ELISA was confirmed by correlation of observed OA contamination in OA-spiked diets (Table VI). Ochratoxin A in swine finisher diets was reproducibly detectable at 5 ppm with the lower limits of detection being 50 ppb. The high sensitivity of the ELISA for OA coupled with the simplified sample preparation procedure and the ease of antibody purification makes this assay attractive for routine OA analysis.

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