# Development of an Enzyme-Linked Immunosorbent Assay for Analysis of Sporidesmin A and Its Metabolites in Ovine Urine and Bile

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Monoclonal and polyclonal antibodies have been produced for the development of competitive enzyme-linked immunosorbent assays for sporidesmin A, the mycotoxin that causes facial eczema in grazing livestock. Hemisuccinyl derivatives of sporidesmin conjugated to a number of proteins were used as immunizing and coating antigens. Two immunoassays have been developed. Each detects the presence of a different region of the sporidesmin molecule, therefore allowing the two major sporidesmins found in grass or in culture to be distinguished as well as allowing the detection of metabolites in ovine urine and bile. An analytical procedure that did not include organic solvent extraction was developed so that water-soluble metabolites would also be detected. Matrix effects occurring in the immunoassay of bile have been overcome by dilution of samples in 1% Tween 20 followed by heat treatment at 70 °C for 30 min. Sporidesmin A in bile measured by immunoassay was compared with that measured by HPLC. The coefficient of determination $R^2$ was 0.97.

**Keywords:** *Sporidesmin A; facial eczema; ELISA; urine and bile*

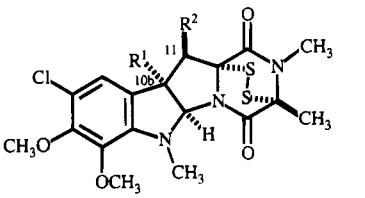
## INTRODUCTION

Ingestion of spores of the saprophytic fungus *Pithomyces chartarum* by grazing livestock causes the disease commonly known as facial eczema in sheep, cattle, deer, and goats. Outbreaks of facial eczema occur frequently in New Zealand, and they have been recorded periodically in Australia, in South Africa (White et al., 1977), and more recently in South America (Smith and Towers, 1984) and France (Bézille et al., 1984). This mycotoxicosis is responsible for significant reductions in growth, reproductive performance, and wool and milk production. Facial eczema is therefore of considerable economic significance to the agricultural industry, particularly in New Zealand.

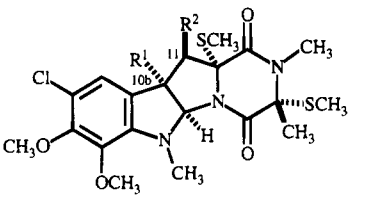
The spores contain the potent mycotoxin sporidesmin A (**1**) (Figure 1), which causes extensive liver damage, particularly to the biliary system, and the resulting impaired liver function often causes a secondary photosensitization of exposed areas of skin around the eyes, ears, and muzzle (Mortimer and Ronaldson, 1983).

The toxicity of sporidesmin A can be attributed to the presence of the disulfide group, which in a cyclic reduction/autoxidation with glutathione and other cellular thiols generates toxic "active oxygen" species (Munday, 1984). In the nontoxic analog sporidesmin D (**2**) the disulfide bridge is broken and the two sulfur groups are methylated. Sporidesmins A and D are the two major components of the sporidesmins that can be extracted from the spores of toxigenic strains of *P. chartarum* grown either in culture or on grass (A. D. Hawkes, AgResearch, Hamilton, New Zealand, 1993).

There is evidence that the metabolism of sporidesmin A in sheep involves destruction of the disulfide group (Fairclough and Smith, 1983), a process that would lead to detoxification. Furthermore, it is known that heritable differences exist among sheep with regard to their susceptibility to sporidesmin intoxication (Campbell et al., 1981), which may reflect differences in the rate of destruction of the disulfide bridge.



(1) $R^1 = R^2 = OH$
(3) $R^1 = OH$, $R^2 = OCO(CH_3)_2COOH$
(4) $R^1 = OCO(CH_3)_2COOH$, $R^2 = OH$



(2) $R^1 = R^2 = OH$

**Figure 1.** Structures of sporidesmin A (**1**), sporidesmin D (**2**), sporidesmin A 11-hemisuccinate (**3**), and sporidesmin A 10b-hemisuccinate (**4**).

To facilitate studies of the metabolism of sporidesmin in sporidesmin resistant and susceptible sheep, methods for the analysis of sporidesmins A and D and their metabolites in various body fluids were developed. Existing HPLC methods (Halder, 1980; Fairclough and Smith, 1983) involve extensive cleanup of samples before analysis, making the procedure time-consuming and inconvenient when the large number of samples generated from such a study are handled. Immunoassays, on the other hand, often require little or no sample cleanup, provide high sensitivity and rapid throughput, and, depending on the specificity of the antibodies, will detect compounds with similar structures such as

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metabolites. Immunoassays have been successfully developed for a number of mycotoxins [reviewed by Chu (1991)], suggesting that this would be a suitable alternative method for sporidesmin analysis.

We describe the production of polyclonal and monoclonal antibodies that bind to sporidesmins A and D and the development of two immunoassays, each detecting the presence of a different region of the sporidesmin molecule. Assay conditions were developed to overcome matrix effects and to allow analysis of samples without organic solvent extraction so that water-soluble metabolites may be detected and sample throughput increased. The specificities of the antibodies were selected to allow toxic sporidesmin A and the nontoxic analog sporidesmin D to be distinguished as well as to allow the detection of metabolites in urine and bile.

## MATERIALS AND METHODS

**Materials.** Sporidesmin A, B, and D were extracted and purified in our laboratories. Sporidesmin A derivatives were available from earlier work (Ronaldson, 1978). Additional compounds structurally related to sporidesmin A were obtained from AgResearch, Wallaceville, New Zealand. Thyroglobulin, bovine serum albumin (BSA) ELISA grade A-7030, ovalbumin, conalbumin, pristane, Freund's complete and incomplete adjuvants, penicillin, streptomycin, poly(ethylene glycol) (MW 3000–3700), goat anti-rabbit IgG peroxidase conjugate (anti-rabbit–HRP), 50× hypoxanthine and thymidine (HT), and 50× hypoxanthine, aminopterin, and thymidine (HAT) were obtained from Sigma Chemical Co. (St. Louis, MO). Iscoves modified Dubeccos medium (IMDM), minimum essential medium (MEM) nonessential amino acids (NEAA), and fetal calf serum (FCS) were from Gibco Laboratories (Grand Island, NY). Donkey anti-mouse immunoglobulin (gamma and light chains) horseradish peroxidase conjugate (anti-mouse–HRP) was purchased from Silenus Laboratories Pty. Ltd. (Victoria, Australia). The immunoglobulin subclass identification kit, Mouse Typer, was purchased from Bio-Rad Laboratories (Richmond, CA). 3,3′,5,5′-Tetramethylbenzidine (TMB) was from Boehringer Mannheim (Germany). Protein G Sepharose 4 Fast Flow was obtained from Pharmacia LKB (Uppsala, Sweden). Peroxidase conjugated rabbit anti-sheep IgG (anti-sheep–HRP) was provided by USB Immunochemicals (Cleveland, OH). The myeloma cell line P3/NS-1/1-AG4-1 (NS-1) (ATCC TIB 18) was obtained from the American Type Culture Collection (Rockville, MD). Mice (BALB/c) were obtained from AgResearch (Hamilton, New Zealand). Maxisorp immunoplates and tissue culture plasticware were purchased from Nunc (Roskilde, Denmark), and all inorganic chemicals and organic solvents were of reagent grade or better.

**Buffer Solutions:** plate coating buffer, carbonate–bicarbonate buffer, 50 mM, pH 9.6; phosphate-buffered saline (PBS), pH 7.4, NaCl (8.0 g), KCl (0.2 g), $Na_2HPO_4$ (1.15 g), $KH_2PO_4$ (0.2 g), and distilled water (1 L); washing buffer, 0.05% Tween 20 in PBS; sample buffer, 2% methanol (v/v) in washing buffer; standard buffer, 1% methanol (v/v) in washing buffer; antibody buffer; 1% BSA (w/v) in washing buffer; enzyme substrate, 42 mM TMB in dimethyl sulfoxide (1 vol), 100 mM acetate buffer, pH 5.5 (10 vol), and 1.3 mM $H_2O_2$ (1 vol).

**Sporidesmin–Protein Conjugates.** Sporidesmin A hemisuccinate isomers (**3**, **4**) available from earlier studies (Gallagher et al., 1992) were conjugated to BSA and thyroglobulin to produce the immunizing conjugates or to ovalbumin and conalbumin to produce coating conjugates, using the active ester method of Bauminger and Wilchek (1980).

**Immunization.** *1. Mice.* Three groups of five BALB/c mice (4–6 weeks old) were each immunized with 120 μg of sporidesmin A 11-hemisuccinyl BSA, sporidesmin A 10b-hemisuccinyl BSA, or sporidesmin A 11-hemisuccinyl thyroglobulin in 100 μL of PBS. The primary injection was given intrasplenically (Spitz et al., 1984). Booster injections were given intraperitoneally as an emulsion containing an additional 100 μL of Freund's adjuvant. Complete adjuvant was used in the first boost and incomplete adjuvant in subsequent injections. Five immunizations were given at 3 week intervals followed by injections at intervals that varied from 1 to 4 months over the next year. After seven immunizations, the first mouse was taken for hybridoma production and the remaining mice received further injections. One week after each immunization, 100 μL blood samples were removed from the lateral tail vein and antibody titers of the resulting antisera determined by an indirect enzyme immunoassay as described below. Three days prior to fusion, and 4 weeks after the last immunization, 250 μg of the immunogen was dissolved in 100 μL of sterile PBS and injected into the lateral tail vein.

*2. Rabbits.* Four New Zealand White female rabbits were each immunized with a subcutaneous injection of 100 μg of the sporidesmin A 11-hemisuccinyl BSA conjugate in 400 μL of PBS and adjuvant (1:2 v/v). The emulsion was injected into four different sites in the shoulder and hip region, i.e. 100 μL/leg. Complete Freund's adjuvant was used in the first injection and incomplete adjuvant in the following injections. A total of four immunizations at monthly intervals were followed by a rest period of 4 months and then three further monthly injections. Immunizations were followed 1 week later by the collection of 1 mL of blood from the marginal ear vein and the sera obtained monitored for antibody production and specificity. Two selected animals were bled by cardiac puncture and the antisera prepared and then stored at −20 °C.

*3. Sheep.* Twenty sheep were each injected with 250 μg of sporidesmin A 11-hemisuccinyl BSA in 1 mL of PBS and Freund's adjuvant (1:4 v/v). The emulsion with complete adjuvant was first injected subcutaneously into two sites in the neck region, i.e. 0.5 mL into each site. Subsequent monthly immunizations were given with incomplete adjuvant, injected intramuscularly into two sites on the hind legs. One week after each immunization, a 10 mL blood sample was taken from the jugular vein. The antisera were monitored for antibody production. After seven immunizations, during which there was a rest period of 4 months after the fourth immunization, 400 mL of blood was collected from selected animals into blood donor bags and antisera were prepared and then stored at −20 °C. The collection was repeated a month later.

**Indirect Enzyme-Linked Immunosorbent Assay (ELISA).** Antibody titers and binding of monoclonal antibodies to plate coating antigens were determined by ELISA. Microtiter plates were coated with 10b- or 11-derivatized sporidesmin conjugated to ovalbumin (2 μg/mL) in coating buffer (100 μL/well). The plates were sealed and incubated for 16 h. The coating solution was then removed, and the wells were washed three times with PBS. Nonreacted sites were blocked for 75 min with 200 μL/well of PBS containing 1% BSA. This was followed by two washes with washing buffer. To each well was added 50 μL of sample buffer followed by 50 μL of various dilutions of supernatant fluid or antiserum in antibody buffer. The plate contents were gently mixed and incubated for 1 h. After four washes, 100 μL of anti-mouse–HRP diluted 1:2500, anti-sheep–HRP diluted 1:10000, or anti-rabbit–HRP diluted 1:4000 in antibody buffer was added. The plates were incubated for 2 h. Wells were washed four times, and then 100 μL/well of freshly prepared TMB substrate solution was added. After 25 min, the reaction was stopped by adding 100 μL of 10% sulfuric acid. All procedures were carried out at 20 °C.

The absorbance at 450 nm was determined in an ELISA reader (Bio-Rad Model 3550 microplate reader). Curve fits of log concentration versus linear absorbance were determined with a logit transformation using the Bio-Rad Microplate Manager data analysis software.

**Competitive Indirect Enzyme-Linked Immunosorbent Assay (cELISA).** cELISA was used to determine sensitivity and specificity of antibody binding to sporidesmins A and D. Plates were coated and blocked as described for monitoring antibody titers. Sporidesmin standards were prepared in methanol and diluted in standard buffer to give a methanol concentration of 2%. Standard solutions (50 μL) were then added to the wells followed by 50 μL of antibody at an

**Table 1. Immunoassay Conditions for Bile and Urine Analysis**

| analyte | antibody group, dilution | body fluid | coating antigen,[a] concn | second antibody dilution |
|---|---|---|---|---|
| sporidesmin A | A, 1:375000 | bile | hapten 11-ovalbumin, 2 μg/mL | 1:2000 |
| sporidesmin A and metabolites | B, 1:60000 | bile | hapten 11/10b-ovalbumin, 4 μg/mL | 1:2000 |
| sporidesmin metabolites | B, 1:100000 | urine | hapten 11/10b-ovalbumin, 4 μg/mL | 1:2500 |

[a] Hapten 11 represents sporidesmin A 11-hemisuccinyl, and hapten 10b is sporidesmin A 10b-hemisuccinyl.

appropriate dilution in antibody buffer. All samples were analyzed in duplicate. The selected antibody dilution was that which gave approximately 50% of the maximum absorbance recorded with excess antibody. cELISA was completed as described for ELISA.

**Hybridoma Production.** NS-1 myeloma cells were grown in IMDM with 10% FCS, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 0.1 mM NEAA, 30% NS-1 conditioned media, and HT (100 μM hypoxanthine, 16 μM thymidine). Spleen cells ($5 \times 10^7$) from selected immunized mice were fused with myeloma cells ($1 \times 10^8$), using poly(ethylene glycol) (Galfrè and Milstein, 1981). Fused cells were resuspended in IMDM with 20% FCS and selected for growth in HAT (HT with 0.4 μM aminopterin). They were distributed into 96 well plates together with mouse macrophage feeders at a cell density of $10^4$ cells/well (Sugasawara et al., 1985). Five days after the fusion, 100 μL of the culture fluid was removed and replaced by freshly prepared HAT medium. When the colonies covered approximately half of the well area, 100 μL of the culture supernatants was removed and screened by ELISA for the presence of antibodies binding to the sporidesmin coating antigens. Positive cultures were tested by cELISA for sporidesmin A and sporidesmin D binding antibodies. After 2 weeks, HAT-containing medium was gradually eliminated by replacement with HT medium containing 10–15% FCS.

Hybridoma lines producing selected antibodies were cloned by dilution to calculated densities of 2.5, 1.0, and 0.5 cells/well (Zola, 1987), and growth of the diluted cells was supported with macrophage feeder cells. Wells containing single colonies were identified and screened for antibody production by ELISA and cELISA. Hybridoma cells from wells showing a positive response were expanded and subcloned again twice. All subclones produced antibodies and were presumed to be monoclonal in origin.

**Monoclonal Antibody Production and Purification.** The isotype classes of the monoclonal antibodies were determined using an isotyping kit, based on an enzyme immunoassay method.

The two cell lines selected for antibody production were grown in 100 mL batch cultures to stationary phase, and the culture fluids collected were stored at −20 °C. BALB/c mice, 8–10 weeks old, were injected intraperitoneally with 0.5 mL of pristane 10 days before an intraperitoneal inoculation with $1-5 \times 10^6$ hybridoma cells in 500 μL of serum-free IMDM. The ascitic fluid that accumulated in the peritoneal cavity was drained from the animal and immediately after collection was centifuged for 15 min at 2000*g* and stored at −20 °C.

Monoclonal antibodies were purified from culture supernatant and ascitic fluids using affinity chromatography with Protein G Sepharose according to the manufacturer's instructions. Bound antibodies were eluted with 0.1 M glycine buffer at pH 2.7. Fractions were collected into tubes containing sufficient 1 M Tris buffer, pH 8.0, to adjust the eluted antibodies to approximately pH 7.0. Total protein concentration was determined using the Bio-Rad microplate procedure (Bio-Rad Laboratories, 1984) based on the Bradford method. Antibodies were stored in aliquots at −20 °C and at a protein concentration of 1 mg/mL in PBS.

**Determination of Cross-Reactivities.** To obtain an indication of the location of the antigen binding sites for these antibodies, cross-reactivity with compounds that were structurally related to sporidesmin A were determined from standard curves of the tested compounds in cELISA. Cross-reactivities were calculated according to the formula % cross-reactivity (% CR) = ($I_{50}$ sporidesmin A/$I_{50}$ analog) × 100, where $I_{50}$ is the molar concentration of compound giving 50% inhibition of antibody binding to the coating antigen.

**Immunoassay of Urine and Bile.** *1. Bile.* Hepatic bile was collected from cannulated sheep that were dosed orally with a single dose of sporidesmin A (0.4 mg/kg of body weight). Samples were taken at intervals over 48 h and stored at −20 °C before analysis.

Immunoassay reagents were diluted as listed in Table 1, and the analytical protocol was as described for cELISA with the following modifications to remove matrix effects. Samples were diluted 1:50 and 1:100 in sample buffer in which the Tween 20 concentration had been increased from 0.05 to 1.0% (w/v). This was followed by mixing on a vortex mixer and incubation in a water bath for 30 min at 70 °C (Dhar and Ali, 1992). Standards were prepared in buffer containing 1% Tween 20 and were treated the same as the samples, although preparation of standards in this manner did not significantly change the standard curve. After cooling to 20 °C, 50 μL of the samples or standards was added to the plate and assayed as previously described.

*2. Urine.* Urine was collected by catheter from the sheep dosed with sporidesmin A. The urine was collected over intervals for 72 h and stored at −20 °C before analysis. Samples were diluted 1:50 and 1:100 in sample buffer modified by the addition of 1% BSA. Standard buffers also contained 1% BSA.

To evaluate any potential matrix effects, sporidesmin A standard curves were prepared in diluted urine or bile samples and compared to standard curves prepared in the standard buffers alone. The optimized methods were validated by spiking urine and bile samples with sporidesmin A in methanol by adding 10 μL of standard to 990 μL of the undiluted sample. Samples were spiked with at least three different concentrations of analyte. Three samples were spiked at each concentration, and each sample was assayed in eight replicates duplicated each day on two plates. Recoveries were determined by comparing the cELISA results with the calculated sporidesmin concentrations. The intra-assay (intra-plate) variation was determined from the variation of the mean sporidesmin concentration of each of the three spiked samples for the three different concentrations on one plate. The inter-assay (inter-plate) variation was determined from the variation of the mean sporidesmin concentrations determined on each plate over 2 days.

Bile and urine samples were also analyzed for sporidesmin A using the HPLC conditions reported by Miles et al. (1992). A 2 mL sample of bile was extracted twice with 2 mL of diethyl ether, and 10 mL of urine was extracted twice with 10 mL of dichloromethane. The extracts were taken to dryness by evaporation under nitrogen and dissolved in 500 μL of methanol, and a sample volume of 10 μL was injected into the HPLC.

## RESULTS

**Screening of Sera.** When antisera were monitored for the potential for polyclonal immunoassay and monoclonal antibody production, the highest titers and cELISA sensitivities were found in the sheep antisera (Table 2).

The sheep and rabbit antisera preferentially bound sporidesmin A, while the majority of the mouse antisera had greater affinity for sporidesmin D than for sporidesmin A (Table 3).

**Table 2. Immune Response of Animals Immunized with Sporidesmin A 11-Hemisuccinyl BSA**

| species | no. of animals | no. of immuni-zations | mean titer[a] | dilution range | $I_{50}$[b] sporidesmin A (ng/mL) |
|---|---|---|---|---|---|
| sheep | 17 | 4 | 1:5000 | 1:1000–1:20000 | 15 |
| rabbits | 4 | 4 | 1:2000 | 1:1000–1:4000 | 1000 |
| mice | 5 | 5 | 1:1000 | 1:500–1:1000 | 800 |

[a] Antisera dilution required to reduce ELISA absorbances to 50% of the maximum absorbance. [b] Concentration of analyte giving 50% inhibition of antibody binding to the coating antigen as determined by cELISA. The lowest $I_{50}$ obtained for the antisera screened in each species is recorded.

Larger volumes of mouse antisera were collected at the time the spleens were removed for fusion, and this enabled more extensive screening to be carried out (Table 4). The results suggested that sporidesmin A 11-hemisuccinyl ovalbumin was the most suitable coating antigen for screening hybridomas. In some instances (i.e., mice 1 and 5), inhibition of polyclonal antisera binding to the sporidesmin A 10b-hemisuccinyl coating antigens by sporidesmins A and D did not occur.

**Monoclonal Antibody Production.** *Fusion I.* A mouse was selected for hybridoma production because together with a high antibody titer it produced antiserum that had the highest sensitivity for sporidesmin A in cELISA. Spleen cells from this animal, which had been immunized seven times with sporidesmin A 11-hemisuccinyl BSA over 5 months, were fused with myeloma cells. The resulting hybridomas were screened by ELISA, and 17 cell lines were selected for production of antibodies which bound to the sporidesmin A coating antigens. These antibodies, however, did not bind to free sporidesmin A when screened in cELISA. Changes in assay conditions such as variation in pH, blocker, detergent, and substitution of Tris for phosphate buffer did not increase the sensitivity of the cELISA when mouse polyclonal antisera were used, and competition between sporidesmin A and the sporidesmin A coating antigen for binding to the monoclonal antibodies was not observed. To eliminate the possibility that the inability of these antibodies to bind free sporidesmin A arose from the selection of hybridomas producing antibodies which bound to the hemisuccinyl group rather than to the sporidesmin moiety, the plate coating was changed to sporidesmin A 11-hemiglutaryl ovalbumin. The antibodies still bound to the plates, but again there was no evidence of ability to bind to free sporidesmin A in cELISA.

*Fusion II.* The remaining mice were immunized over a longer period of time in the hope that the maturation of the immune response would result in antibodies with greater affinity for free sporidesmin A than for the immobilized plate coating antigens.

After nine immunizations with sporidesmin A 11-hemisuccinyl BSA over 10 months, a second mouse was chosen for its high antibody titer and sensitivity for sporidesmin A in cELISA. Hybridoma cells were produced and screened as above, and 16 cell lines were found to secrete antibodies binding to free sporidesmins A and D in cELISA. Six lines were selected for subcloning. The antibodies produced had greater affinity for sporidesmin D than for sporidesmin A.

*Fusion III.* In an effort to produce monoclonal antibodies specific for sporidesmin A, spleen cells from the only mouse producing antisera that showed a predominant specificity for sporidesmin A were fused with myeloma cells. This mouse had 10 immunizations with sporidesmin A 11-hemisuccinyl BSA over 14 months. After screening by ELISA and cELISA, 30 cell lines producing sporidesmin-binding antibodies were selected. Twenty hybridomas produced antibodies that preferentially bound sporidesmin A, some of which did not cross-react with sporidesmin D even at assay concentrations of 1 μg/mL. Two of these cell lines were subcloned by limiting dilution.

**Antibody Selection.** Thirteen antibodies specific for sporidesmin A (group A antibodies) and nine antibodies specific for sporidesmins A and D (group B antibodies) were screened for binding to the following coating

**Table 3. Antisera Specificity to Sporidesmins A and D**

| species | no. of animals | immunogen | immunization route | specificity of sporidesmin A[a] | specificity to sporidesmin D[a] |
|---|---|---|---|---|---|
| sheep | 16 | hapten 11–BSA | subcutaneous and intramuscular | ++ | + |
| | 1 | hapten 11–BSA | | ++ | – |
| rabbits | 4 | hapten 11–BSA | subcutaneous | ++ | + |
| mice | 2 | hapten 11–BSA | intrasplenic and intraperitoneal | + | ++ |
| | 3 | hapten 10b–BSA | | + | ++ |
| | 3 | hapten 11–thyroglobulin | | + | ++ |
| | 1 | hapten 11–BSA | | ++ | + |
| | 1 | hapten 10b–BSA | | ++ | ++ |

[a] ++ indicates antibody binding was inhibited by sporidesmins A and D at assay concentrations of <1 μg/mL; + indicates inhibition of binding at 1 μg/mL, and – indicates no inhibition of binding at 1 μg/mL.

**Table 4. Effect of Coating Antigen on the Sensitivity ($I_{50}$)[a] of cELISA Using Mouse Antisera Collected at Fusion**

| | | coating antigen | | | |
|---|---|---|---|---|---|
| antisera | competing analog[b] | hapten 11–thyroglobulin | hapten 11–ovalbumin | hapten 10b–conalbumin | hapten 10b–ovalbumin |
| mouse 1 | Spdm A | >1.0 | 0.078 | >1.0 | >1.0 |
| | Spdm D | 0.025 | 0.003 | >1.0 | >1.0 |
| mouse 5 | Spdm A | >1.0 | 0.087 | >1.0 | >1.0 |
| | Spdm D | >1.0 | >1.0 | >1.0 | >1.0 |
| mouse 11 | Spdm A | 0.155 | 0.378 | 0.105 | 0.159 |
| | Spdm D | 0.037 | 0.015 | 0.011 | 0.012 |

[a] $I_{50}$ values are expressed in micrograms per milliliter. [b] Spdm A represents sporidesmin A and Spdm D, sporidesmin D. The immunogen used to raise the antisera was sporidesmin A 11-hemisuccinyl BSA.

antigens: sporidesmin A 11-hemisuccinate conjugated to thyroglobulin and ovalbumin, and sporidesmin A 10b-hemisuccinate conjugated to conalbumin and ovalbumin. Group A antibodies did not bind to the sporidesmin A 10b-hemisuccinyl coating antigens, whereas group B antibodies bound to all four coatings.

Of all the monoclonal and polyclonal antibodies produced, two monoclonal antibodies showed the highest sensitivities for binding with sporidesmins A and D and were, therefore, selected for use in cELISA. One was from group A antibodies and the other from group B. Antibody isotypes were determined by means of an isotyping kit. The group A antibody was found to be $IgG_{2b}$ and the group B antibody, $IgG_1$.

**Cross-Reactivities.** The compounds that cross-reacted with these antibodies fell into two groups, those bound by the group B antibody only and those that were bound by both antibodies (Table 5).

**Immunoassay Development.** Matrix problems were encountered when the purified monoclonal antibodies were used under standard ELISA conditions for the assay of sporidesmin A and its metabolites in ovine bile and urine. In the case of bile, these could be eliminated by diluting the sample (1:50) in 1% Tween 20 followed by heat treatment at 70 °C for 30 min (Figures 2 and 3). Urine matrix effects were removed by 1:50 or greater sample dilution in buffer containing 0.05% Tween 20 and 1% BSA (Figure 4). Heat treatment of the sample was not required in this instance. At dilutions of less than 1:50 matrix effects resulted in a significant overestimation of sporidesmin A recovery, particularly at lower levels of the assay working range.

**Assay Validation.** The cELISA for bile using the group B antibody gave $I_{50}$ values for sporidesmins A and D of 30 and 0.7 ng/mL, respectively. The effective working ranges (defined as the analyte concentration in the original sample giving 80 and 20% of the maximum absorbance) were 500–10000 ng/mL of bile using a sporidesmin A standard, and 15–300 ng/mL of sample using a sporidesmin D standard. Recoveries of sporidesmin A from spiked bile containing 10, 5, 4, 3, 2.5, and 1.5 μg/mL were 95.6, 85.7, 105.9, 106.9, 79.8, and 122.0%, respectively, and the mean recovery was 99.3%. Mean intra-assay (intra-plate) coefficient of variation was 7.6%, while inter-assay (inter-plate) variation was 5.2%. A standard bile sample was assayed on every ELISA plate. The mean daily inter-assay coefficient of variation for this sample after five assays was 9.1%. In practice, the cELISA using the group B antibody would detect both sporidesmins A and D and metabolite(s).

The cELISA for bile using group A antibody gave an $I_{50}$ for sporidesmin A of 40 ng/mL. The effective working range was between 0.5 and 20 μg/mL of bile. Recoveries of sporidesmin A from bile spiked with 10.0, 5.0, and 1.5 μg/mL were 85.6, 89.1, and 114.6%, respectively, and the mean recovery was 94.6%. The mean intra-assay coefficient of variation was 11.7%, while the mean inter-assay variation was 7.8%. In practice, the cELISA using the group A antibody would detect only sporidesmin A.

Preliminary investigations with cELISA using the group A antibody indicated that unmetabolized sporidesmin was not present in urine collected from sporidesmin A-dosed sheep (0.4 and 0.2 mg/kg of body weight). Sporidesmin A was also not detected by HPLC (limit of detection, 45 ng/mL of urine). Sporidesmin metabolite, however, could be detected with the group B antibody. Therefore, only the cELISA using group B antibody was validated for use with the urine samples. The $I_{50}$ values for sporidesmins A and D with this antibody were 7.0 and 0.3 ng/mL, respectively. The effective working range was 200–3500 ng of sporidesmin A/mL of urine. With a sporidesmin D standard the range was 3–60 ng/mL. Recoveries of sporidesmin A from spiked urine containing 3, 1, and 0.4 μg/mL of urine were 92.1, 89.1, and 93.0%, respectively, with the mean recovery 91.4%. The mean intra-assay coefficient of variation was 8.1%, while the mean inter-assay coefficient of variation was 7.9%. A standard urine sample was assayed on every ELISA plate. The mean daily inter-assay coefficient of variation for the sample after 12 assays was 11.7%.

**Immunoassay of Bile and Urine Samples.** After sheep were dosed with sporidesmin A, it was possible to monitor sporidesmin A and metabolite output in the urine and bile samples using the appropriate optimized immunoassay methods. In bile, maximal levels were observed at 6 h after dosing and immunoreactive material could not be detected after 48 h (Figure 5). Quantitation of sporidesmin A by cELISA was highly correlated with HPLC results (Figure 6), giving a slope of 0.81. Sporidesmin metabolites were detected in the urine 1 h after dosing, reaching a maximum at 5 h. They began to decrease after 8 h and could no longer be detected at 48 h (Figure 7).

## DISCUSSION

Monoclonal and polyclonal antibodies that bind sporidesmin D and/or sporidesmin A in cELISA have been produced. These two compounds are the major sporidesmins extracted from spores produced by toxigenic strains of *P. chartarum*. Sheep and rabbit antisera were more specific for sporidesmin A, whereas the majority of mice antisera were more specific for sporidesmin D (Table 3). This may be attributed either to the difference in the immunization routes used or to species differences in toxin metabolism.

In this study it appears that the maturation of the immune response was important in the production of monoclonal antibodies suitable for use in cELISA measuring sporidesmin. Only after a lengthy immunization protocol including rest periods were high-affinity antibodies that would bind to the free mycotoxins obtained. The results also emphasize the need for careful selection of the coating conjugates. Not only did variation in the hapten and protein carrier used in the plate coating affect the sensitivity and specificity of the resulting assays (Table 4), but it was also particularly important to the results obtained when hybridoma supernatant fluids were screened. For example, the group A antibodies did not bind to the sporidesmin A 10b-hemisuccinyl conjugates and would not have been detected had this coating antigen been used for ELISA screening. This lack of binding may be explained by structural hindrance of antibody binding by the protein conjugated to sporidesmin A on the same face of the molecule as the disulfide group. Alternatively, the unmodified 10b-hydroxyl group could be required for antibody binding to coating antigens.

Cross-reactivity studies indicated that the group A antibody appears to have a binding site which requires the disulfide bridge to be intact. Cross-reactivities were greater than 100% when the analogs contained an 11-ester grouping, suggesting that the epitope for this antibody may include the immunogen hemisuccinate bridge. Group B antibody appears to bind to a region

**Table 5. Antibody Cross-Reactivities with Compounds Structurally Related to Sporidesmin A**

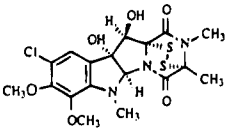
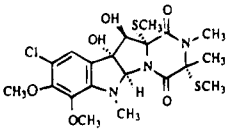
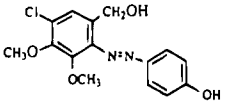
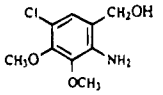
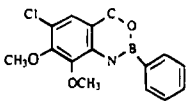
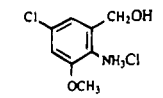
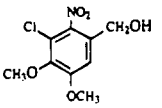
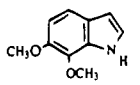
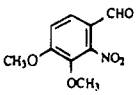
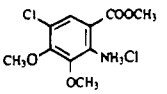
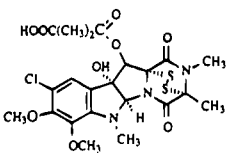
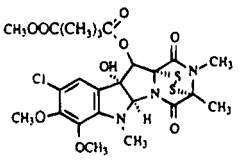
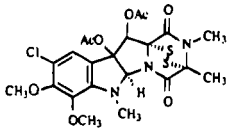
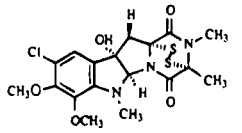
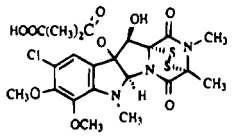
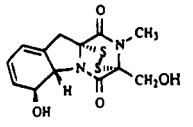
| compound | structure | %CR group A antibody | %CR group B antibody |
|---|---|---|---|
| sporidesmin A |  | 100.0 | 100.0 |
| sporidesmin D |  | <0.1 | 1550.0 |
| *a* |  | <0.1 | 3.6 |
| *b* |  | <0.1 | 2.2 |
| *c* |  | <0.1 | 1.9 |
| *d* |  | <0.1 | 1.7 |
| *e* |  | <0.1 | 0.9 |
| *f* |  | <0.1 | 0.4 |
| *g* |  | <0.1 | 0.1 |
| *h* |  | <0.1 | 0.1 |
| sporidesmin A 11-hemisuccinate |  | 50000.0 | 34.0 |

**Table 5 (Continued)**

| compound | structure | %CR group A antibody | %CR group B antibody |
|---|---|---|---|
| sporidesmin A 11-methylglutarate |  | 32240.0 | 115.0 |
| sporidesmin A diacetate |  | 1775.0 | 72.0 |
| sporidesmin B |  | 48.0 | 207.0 |
| sporidesmin A 10b-hemisuccinate |  | 32.0 | 9.0 |
| gliotoxin |  | <0.1 | <0.1 |

Microtitre plates were coated with sporidesmin A 11-hemisuccinyl ovalbumin for assay using group A and group B antibodies (2 and 4 μg/mL of coating antigen, respectively). cELISAs were carried out as previously described with group A antibody diluted 1:300000, class B antibody diluted 1:100000, and anti-mouse–HRP conjugate diluted 1:2500. Mean $I_{50}$ values of sporidesmin A with group A antibody and group B antibody were 33.7 and 16.7 ng/mL, respectively, and were determined from five different assays. [a] 5-Chloro-3,4-dimethoxy-2-azo-4′-phenylbenzyl alcohol. [b] 2-Amino-5-chloro-3,4-dimethoxybenzyl alcohol. [c] 2-Amino-5-chloro-3,4-dimethoxybenzyl benzeneboronate. [d] 2-Amino-5-chloro-3-methoxybenzyl alcohol hydrochloride. [e] 3-Chloro-4,5-dimethoxy-2-nitrobenzyl alcohol. [f] 6,7-Dimethoxyindole. [g] 3,4-Dimethoxy-2-nitrobenzaldehyde. [h] Methyl 2-amino-5-chloro-3,4-dimethoxybenzoate hydrochloride.

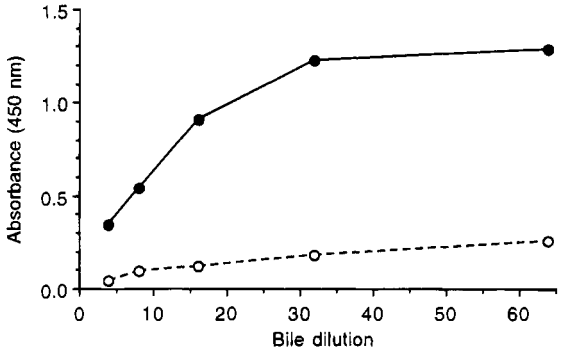



**Figure 2.** Effect of bile dilution on maximum ELISA absorbances with and without heat pretreatment of the sample: (●) bile with heat; (○) bile without heat. Assays used group B antibody.

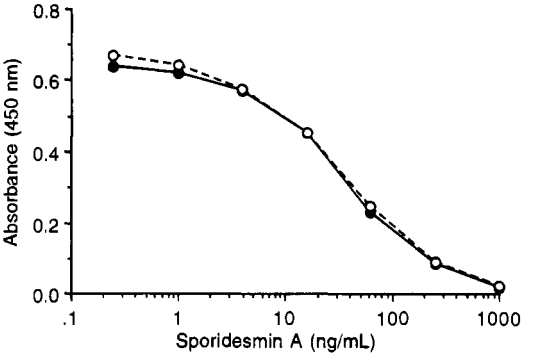



**Figure 3.** Coincidence of the modified sporidesmin cELISA standard curves including bile (●) diluted 1:40 with those without bile (○). Assays used group B antibody.

that includes or interacts with regions of the sporidesmin A molecule distal to the disulfide bridge, for example the chloride, methoxy, adjacent nitrogen, 10b hydroxyl groups, and associated ring structures. Unlike the group A antibody, cross-reactivities were not increased by the presence of an 11-ester group, suggesting that the immunogen hemisuccinate bridge is not included in the epitope. Therefore, the group A antibody would be expected to bind relatively unmodified sporidesmin A-like compounds containing the disulfide bridge and associated structures, whereas the group B antibody may be expected to also bind sporidesmins and sporidesmin metabolites in which the group A epitope is modified, so long as the distal region of the molecule is left sufficiently unchanged for binding to occur.

Group B antibody detected as yet unidentified urinary and biliary metabolite(s). As the cross-reactivities of these compounds with the antibody are not known, the

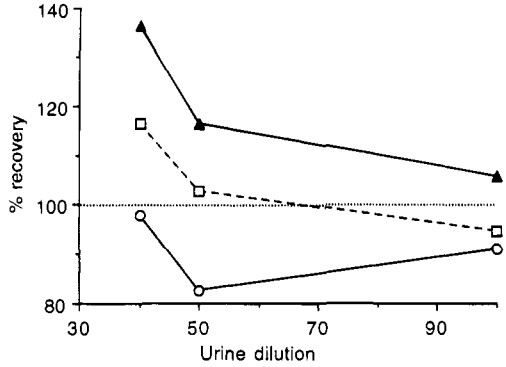



**Figure 4.** Effect of matrix dilution on the apparent recovery of sporidesmin A from urine determined by cELISA using group B antibody: recovery of (▲) 374, (□) 949, and (○) 2408 ng/mL of sporidesmin A.

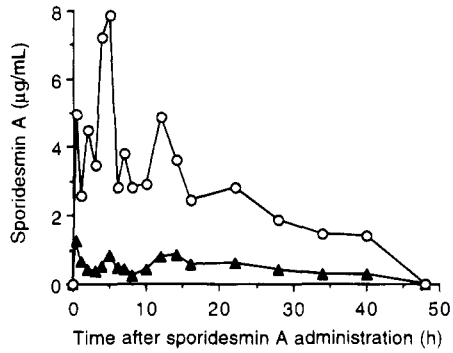



**Figure 5.** Biliary sporidesmin A and metabolite levels after oral dosing with sporidesmin A at 0.4 mg/kg of body weight analyzed by cELISA using group A antibody (▲) and group B antibody (○).

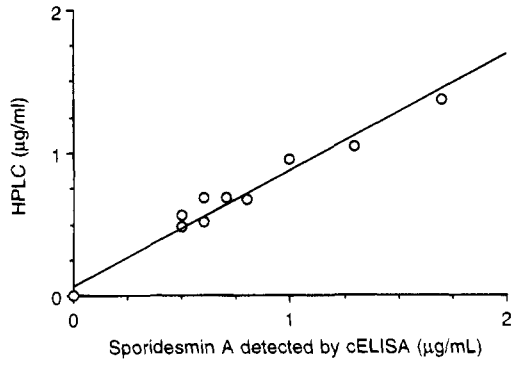



**Figure 6.** Correlation between cELISA (using group A antibody) and HPLC measurement of sporidesmin A in ovine bile. Regression data: $n = 10$; $R^2 = 0.97$; slope = 0.81.

cELISAs can only be used to quantitate these compounds in sporidesmin A or D immunoreactive equivalents. The possibility that there are additional metabolites present, to which neither antibody binds, cannot be excluded.

ELISA methods for the detection of sporidesmin A and its metabolites in ovine bile and urine have been developed. Standard cELISA conditions in the presence of these body fluids, particularly bile, gave reduced color development leading to overestimation of sporidesmin A. Diluting the sample did not overcome this problem. The influence of a number of assay parameters including pH, ionic strength, presence of organic solvents, plates with different binding properties, and different blockers, buffers, and detergents was investigated to determine suitable assay conditions. The method developed to overcome matrix effects in bile by sample dilution in 1% Tween 20 followed by heat treatment has also been successfully applied to cELISA for measuring sporidesmin A and metabolites in ovine whole blood and plasma (data not presented).

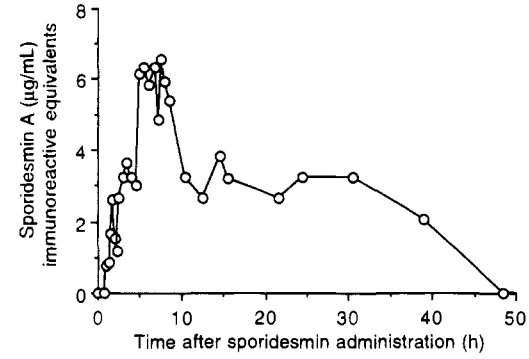



**Figure 7.** Urinary sporidesmin metabolite levels after oral dosing with sporidesmin A at 0.2 mg/kg of body weight analyzed by cELISA with group B antibody.

Plate "edge" effects were observed particularly in the assays with high detergent concentrations. These effects were removed when the coating antigen and second antibody concentrations were such that, in the absence of analyte, maximum absorbances were obtained.

The assays have been shown to be precise and reproducible, to require little sample preparation, and to provide a rapid analytical tool for the study of sporidesmin metabolism in sheep.

## ABBREVIATIONS USED

Hapten 11, sporidesmin A 11-hemisuccinyl; hapten 10b, sporidesmin A 10b-hemisuccinyl; Spdm A, sporidesmin A; Spdm D, sporidesmin D; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; ELISA, indirect enzyme-linked immunosorbent assay; cELISA, competitive indirect enzyme-linked immunosorbent assay.

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## LITERATURE CITED

Bauminger, S.; Wilchek, M. The Use of Carbodiimides in the Preparation of Immunizing Conjugates. *Methods Enzymol.* **1980**, *70*, 151–159.

Bézille; P.; Braun, J.-P.; Le Bars, J. First Identification of Facial Eczema in Sheep in Europe. *Rec. Med. Vet.* **1984**, *160*, 339–347.

Bio-Rad Laboratories. "Automated Protein Assay". Bio-Rad Technical Bulletin 1177 EG, Richmond, CA, 1984.

Campbell, A. G.; Meyer, H. H.; Henderson, H. V.; Wesselink, K. Breeding for Facial Eczema Resistance—a Progress Report. *Proc. N. Z. Soc. Anim. Prod.* **1981**, *41*, 273–278.

Chu, F. S. Development and Use of Immunoassays in the Detection of Ecologically Important Mycotoxins. In *Handbook of Applied Mycology. Mycotoxins in Ecological Systems*; Bhatnagar, D., Lillehoj, E. B., Arora, D. K., Eds.; Dekker: New York, 1991; Vol. 5, Chapter 10.

Dhar, T. K.; Ali, E. Direct Microtitre Plate Enzyme Immunoassay of Testosterone in Unextracted Serum. *J. Immunol. Methods* **1992**, *147*, 167–172.

Fairclough, F. J.; Smith, B. L. Sporidesmin Concentrations in the Bile of Sheep Resistant or Susceptible to Sporidesmin Dosing. *Proc. N. Z. Soc. Anim. Prod.* **1983**, *43*, 213–215.

Galfrè, G.; Milstein, C. Preparation of Monoclonal Antibodies: Strategies and Procedures. *Methods Enzymol.* **1981**, *73*, 3–46.

Gallagher, R. T.; Eichler, S. J.; Holland, P. T.; Wilkins, A. L.; Nicholson, B. K. Peptide Coupling Reactions of the Mycotoxin Sporidesmin: Synthesis of Sporidesmin Hemisuccinates, X-ray Crystal Sructure of Sporidesmin A 11-Hemisuccinate, and its Coupling with $N^{\alpha}$-*t*-Boc-L-Lysine Methyl Ester. *J. Agric. Food Chem.* **1992**, *40*, 701–706.

Halder, C. A. A Study of Sporidesmin and the Relationship of *Pithomyces spp.* to Swellhead Disease of Sheep in West Texas. Ph.D. Dissertation, Graduate College of Texas A&M University, Texas, 1980.

Miles, C. O.; Munday, S. C.; Wilkins, A. L. Improved Method for Purification of Epidithiodioxopiperazines from Fungal Extracts: Purification of Sporidesmin A. *J. Agric. Food Chem.* **1992**, *40*, 2458–2460.

Mortimer, P. H.; Ronaldson, J. W. Fungal-toxin-induced Photosensitization. In *Handbook of Natural Toxins. Plant and Fungal Toxins;* Keeler, R. F., Tu, A. T., Eds.; Dekker: New York, 1983; Vol. 1, Chapter 11.

Munday, R. Studies on the Mechanism of Toxicity of the Mycotoxin Sporidesmin. Evidence for Intracellular Generation of Superoxide Radical from Sporidesmin. *J. Appl. Toxicol.* **1984**, *4*, 176–180.

Ronaldson, J. W. Fungal Metabolite Chemistry: Sporidesmin, Crepidotine. D. Phil. Dissertation, University of Waikato, Hamilton, New Zealand, 1978.

Smith, B. L.; Towers, N. R. Pithomycotoxicosis (Facial Eczema) in New Zealand and the Use of Zinc Salts for its Prevention. In *Plant Toxicology. Proceedings of The Australia-U. S. A. Poisonous Plants Symposium*; Seawright, A. A., Hegarty, M. P., James, L. F., Keeler, R. F., Eds.; Queensland Poisonous Plants Committee: Yeerongpilly, 1985; Chapter 2, pp 70–79.

Spitz, M.; Spitz, L.; Thorpe, R.; Eugui, E. Intrasplenic Primary Immunization for the Production of Monoclonal Antibodies. *J. Immunol. Methods* **1984**, *70*, 39–43.

Sugasawara, R. J.; Cahoon, B. E.; Karu, A. E. The Influence of Murine Macrophage-conditioned Medium on Cloning Efficiency, Antibody Synthesis, and Growth Rate of Hybridomas. *J. Immunol. Methods* **1985**, *79*, 263–275.

White, E. P.; Mortimer, P. H.; di Menna, M. E. Chemistry of the Sporidesmins. In *Mycotoxic Fungi, Mycotoxins, Mycotoxicoses;* Wyllie, T. D., Morehouse, L. G., Eds.; Dekker: New York, 1977; Vol. 1, Chapter 2, pp 427–447.

Zola, H. Making Hybridomas. In *Monoclonal Antibodies: A Manual of Techniques;* CRC Press: Boca Raton, FL, 1987; pp 54–56.

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