Production of a Sensitive Monoclonal Antibody to Sterigmatocystin and Its Application to ELISA of Wheat

Suzhen Li,[†] Pu Yan Chen,[‡] Ronald R. Marquardt,*[,†] Zhengkang Han,[§] and James R. Clarke[⊥]

Departments of Animal Science and Food Science, Faculty of Agriculture and Food Sciences, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2, and Laboratory of Veterinary Infectious Diseases and Laboratory of Physiology and Biochemistry, College of Veterinary Science, Nanjing Agricultural University, Nanjing 210095, China

A hybridoma cell line capable of secreting sensitive and specific monoclonal antibody for sterigmatocystin (STG) was produced by fusing SP2/0 myeloma cells with spleen cells of female Balb/C mice immunized with STG hemiacetal−bovine serum albumin conjugate. The concentration of STG required to inhibit 50% of the binding of the monoclonal antibody in a competitive ELISA (cELISA) was 2.5 ng/mL. The apparent affinity dissociation constant of the monoclonal antibody was $2.3 \times 10^{-9} \pm 1.4 \times 10^{-10}$ M^{-1}. The cross-reactions of STG, *O*-methyl-STG, STG hemiacetal, aflatoxin B_1 (AFB_1), AFB_2, AFG_1, AFG_2, and AFB_1 hemiacetal with the antibody in the cELISA were 100, 0.4, 12.5, 0.4, 0.1, <0.1, <0.1, 6.25%, respectively. Sterigmatocystin could be detected reproducibly using the cELISA and quantified in spiked wheat samples at concentrations greater than 31 ppb using a simple methanol and aqueous potassium chloride extraction procedure.

Keywords: *Sterigmatocystin; ELISA; monoclonal; antibody; wheat*

INTRODUCTION

Sterigmatocystin (STG) is a highly toxic secondary fungal metabolite as well as a potent carcinogen and mutagen (Berry, 1988; Curry et al., 1984; Enomoto et al., 1982; Ueda et al., 1984; Purchase and van der Watt, 1970). Sterigmatocystin is produced by certain species of *Aspergillus, Penicillium, Chaetomium*, and *Bipolaris* (Betina, 1989; CAST, 1989), with *Aspergillus versicolor* being the main producer of this toxin. The mold occurs frequently in grains such as wheat or barley (Abramson et al., 1983; Scott et al., 1972). This mycotoxin has been implicated in the deaths and/or symptoms of bloody diarrhea and reduced milk production in dairy cattle fed STG-contaminated feed (Vesonder and Horn, 1985). Numerous studies on the analysis of this mycotoxin using thin-layer chromatography (Shannon and Shotwell, 1976) and high-performance liquid chromatography (Steyn et al., 1991) have been published. These instrumental−chromatographic techniques are specific and sensitive but lack ease and speed as they require extensive sample cleanup and in certain cases expensive instrumentation for separation and detection. Quantitative cELISAs for STG have been successfully developed using rabbit antisera (Li and Chu, 1984; Morgan et al., 1986). The antisera were highly sensitive but required the toxin to be converted to the hemiacetal form (STG hemiacetal) for quantitation. The reason suggested for conversion was the dramatic improvement in the water solubility of this toxin and the antisera's singular specificity for this form. Murine monoclonal antibodies have been successfully generated against most of the mycotoxins (Chu, 1990). This type of approach offers many advantages which include high specificity, large-scale production potential, and high quality and uniformity. It would therefore be advantageous to produce a cELISA by which STG can be measured directly without the need for derivitization, which provides a high degree of sensitivity and specificity, and which would be practical for routine analysis. This study describes the development of a cELISA based on a high-affinity monoclonal antibody which requires no derivitization and provides a high level of sensitivity, specificity, and reproducibility.

MATERIALS AND METHODS

Materials. Sterigmatocystin, *O*-methyl-STG, AFB_1, AFB_2, AFG_1, AFG_2, bovine serum albumin (BSA), human serum albumin (HSA), complete and incomplete Freund's adjuvant (CFA, IFA), pristane, *p*-nitrophenyl phosphate, diethanolamine, Tween 20, and rabbit anti-mouse IgG coupled to alkaline phosphatase were purchased from Sigma Chemical Co., St. Louis, MO. Poly(ethylene glycol) (PEG 1000) was from E. Merck, Darmstadt, Germany. RPMI-1640 and HAT media were from Gibco BRL Products, Gaithersburg, MD, and Carnation instant skim milk powder was from Nestlé, Don Mills, ON, Canada. The STG that was used as the immunogen was a gift from J. Lou, Laboratory of Veterinary Internal Medicine, while fetal bovine serum was supplied by the Laboratory of Veterinary Infectious Diseases, Nanjing Agricultural University. Sodium borohydride was purchased from Aldrich Chemical Co., Milwaukee, WI. Sterigmatocystin hemiacetal (STG-HA), STG hemiacetal−bovine serum albumin (STG-HA−BSA), and STG-HA−human serum albumin (STG-HA−HSA) were prepared as previously described (Li and Chu, 1984). Spectrophotometric analysis of the conjugates revealed that greater than 11 mol of STG-HA was bound to each mole of protein. AFB_1 hemiacetal (AFB_1-HA) was prepared in a manner similar to that previously described (Pohland et al., 1968). The mouse monoclonal antibody isotyping kit was purchased from Bio-Rad Laboratories Ltd., Mississauga, ON, Canada. Microtiter plates (Falcon 3911, MicroTest III) were obtained from Becton Dickson Labware, Oxnard, CA. All other solvents and reagents were of analytical quality or better.

Immunization. Five female Balb/C mice, approximately 8 weeks of age, were immunized and boosted with the

* Author to whom correspondence should be addressed.

[†] Department of Animal Science.

[‡] Laboratory of Veterinary Infectious Diseases.

[§] Laboratory of Physiology and Biochemistry.

[⊥] Department of Food Science. Present address: Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824-1224.

0021-8561/96/1444-0372$12.00/0

immunogen (STG-HA−BSA), and the spleens were removed for monoclonal antibody production. The initial immunogen consisted of 50 μg of STG-HA−BSA that was prepared in 0.25 mL of distilled water followed by emulsification with an equal volume of CFA. The mixture was administered subcutaneously in each mouse at two sites. Four boosters were given at 4 week intervals and consisted of 50 μg of STG-HA−BSA in 0.25 mL of distilled water emulsified with an equal volume of IFA. The boosters were also given subcutaneously at multiple sites into the abdomen. An indirect competitive ELISA similar to that previously described (Morgan et al., 1986) was used to screen serum samples with the exception that the plate coating antigen was replaced with STG-HA−HSA. Mice showing specific inhibition by STG were selected for fusion. The mice were injected with 50 μg of STG-HA−BSA in 0.5 mL of distilled water, intraperitoneally, 3 days prior to removal of spleen and subsequent fusion.

Cell Fusion and Cloning Protocol. The nonsecreting myeloma cell line, SP2/0-Ag14, was used to fuse immunized mouse spleen cells in a ratio of 1:4 with a poly(ethylene glycol) solution using a procedure similar to that previously described (Candlish et al., 1988). Cell suspensions (100 μL) were distributed into 96-well culture plates, and the resulting hybridoma cells were selected using bicarbonate-buffered RPMI 1640 medium supplemented with 20% (v/v) fetal calf serum, 2 mmol/L glutamine, and HAT solution (1×10^{-4} mol/L hypoxanthine, 4×10^{-7} mol/L aminoterin, and 1.6×10^{-5} mol/L thymidine). Hybridomas were cloned according to the limited dilution method, and positive clones were screened for specificity and sensitivity in the indirect competitive ELISA. The cells at a concentration of approximately 5×10^{-6} cells/mL were subjected to six 10-fold dilutions to yield final cell concentrations of 5−10 cells/mL. Specific antibody-secreting hybridomas were selected by indirect ELISA for STG. Positive clones were subsequently grown in flasks and as ascites in Balb/C mice primed with 0.5 mL of pristane, 10 days prior to injection. Ascites fluid was collected after 14 days, and the suspension was centrifuged at 3000*g* for 10 min and precipitated with 50% ammonium sulfate. Samples were reconstituted with a solution of saline and glycerol (1:1 v/v) and stored at −70 °C. The class of antibody was determined following the procedures outlined in the manufacturer's manual, which were similar to those outlined by Harlow and Lane (1988).

Preparation of Samples from Wheat Spiked with STG. Spiked amber durum wheat samples were prepared by adding STG in methanol to ground wheat (grinder was fitted with a 1 mm sieve) at concentrations ranging from 8 to 500 ppb (ng/g) and allowing the solvent to evaporate before proceeding with the extraction.

The extraction conditions used in this study were similar to those previously described for cereals and soybeans but avoided sample cleanup (Shannon and Shotwell, 1976). In brief, 5 g of the ground wheat sample was mixed with 10 mL of methanol and a 4% KCl aqueous solution (9:1) in a capped Nalgene centrifuge tube and subsequently shaken for 30 min. The mixture was then centrifuged for 30 min at 10000*g*, and the cleared supernatant was recovered by decanting. Samples from the supernatant were directly analyzed in the indirect competitive ELISA.

Indirect Competitive ELISA. Microtiter plate wells were coated with 0.5 μg of STG-HA−HSA in 200 μL of PBS overnight at 4 °C. The plates were washed three times with PBS and subsequently blocked with 0.1% skim milk in PBS (pH 7.2, 0.1 M sodium phosphate buffer) for 1 h at 37 °C. Plates following blocking were washed three times with PBS-T (0.05% Tween 20 in PBS). The standards or sample (10 μL) was added to 65 μL of PBS-T in each well followed by the addition of the mouse anti-STG monoclonal antibody solution (75 μL). The anti-STG solution was initially diluted 1:1000 with PBS-T containing 0.4% ovalbumin. The reaction was allowed to take place for 2 h at 37 °C, followed by washing with PBS-T as described above. Following washing, 150 μL of rabbit anti-mouse IgG−alkaline phosphatase conjugate diluted in PBS-T (1:1000) was added to all wells and allowed to incubate at 37 °C for 1 h. Following incubation and extensive washing with PBS-T (six washing cycles), 150 μL of the substrate solution (*p*-nitrophenyl phosphate, 1 mg/mL, in 0.1 M diethanolamine buffer, pH 9.8) was added. The plates were allowed to develop for 45 min at 37 °C, and absorbances were determined on an ELISA plate reader (Bio-Rad Laboratories, Model 450) fitted with a 405 nm filter. The data was plotted graphically using Sigma Plot (Kao and Norby, 1992).

The competitive indirect ELISA for STG was used to determine the apparent affinity dissociation constant (K_d) of the monoclonal antibody. The K_d value was determined by Klotz plot analysis similar to that previously described (Candlish et al., 1988). The times of incubation were reduced from overnight at 25 °C to 2 h at 37 °C.

Table 1. Specificity of Monoclonal Antibody As Determined by Indirect Competitive ELISA

compound	concn of toxin to obtain 50% inhibition (ng/mL)	cross-reactivity[a] (%)
sterigmatocystin	2.5	100
O-methylsterigmatocystin	600	0.4
sterigmatocystin hemiacetal	20	12.5
aflatoxin B_1	625	0.4
aflatoxin B_2	3000	0.1
aflatoxin G_1	>3000	<0.1
aflatoxin G_2	>3000	<0.1
aflatoxin B_1 hemiacetal	47	6.25

[a] Cross-reactivity was determined by comparing the concentration of toxin required to give 50% maximal absorbency in the cELISA. All samples were analyzed in triplicate.

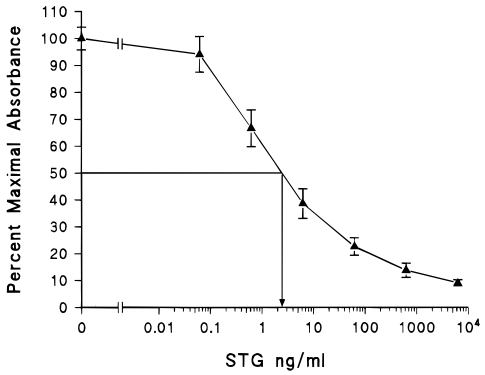

Figure 1. Typical competitive ELISA standard curve for STG. Values represent means ± SD implicate analysis. See Materials and Methods for further detail.

RESULTS AND DISCUSSION

The isolation of a STG-specific antibody secreting cell line has facilitated the development of a quantitative cELISA for STG in wheat. The approach that was used allowed the selection of an antibody with a high specificity and sensitivity for STG (Table 1) and yielded a uniform quality antibody preparation. The subclass of the antibody isolated was determined to be IgG2a with a kappa light chain. This class of antibody can be used for the preparation of antibody−enzyme conjugates and affinity columns (Harlow and Lane, 1988). The affinity dissociation constant of this antibody was $2.3 \times 10^{-9} \pm 1.4 \times 10^{-10}$ M^{-1} as determined by Klotz plot analysis (data not shown). This affinity corresponds closely to the affinity observed for an ochratoxin A monoclonal antibody (Candlish et al., 1988). The concentration of STG required to inhibit 50% of the binding in the cELISA was 2.5 ng/mL, with the lowest detectable concentration being <0.5 ng/mL (Figure 1).

The sensitivity of the assay for STG was somewhat lower than that reported by Morgan et al. (1986) and Li and Chu (1984) for STG hemiacetal (2.5 vs ~0.6 ng/

mL) but was much higher than that reported for STG or dehydro-STG. In this regard, Morgan et al. (1986) reported that STG essentially did not react with their polyclonal antibody (i.e., the cross-reactivity was less than 0.01% compared to STG hemiacetal), while Li and Chu (1984) did not report a value for the reactivity of STG with their polyclonal antibody preparation. They reported, however, that dehydro-STG was approximately 16 times less reactive to the antibody than STG hemiacetal.

The monoclonal antibody that was used in the current study, in addition to showing a strong reaction with STG, was also highly specific (Table 1) as the antibody essentially did not react with *O*-methyl-STG or any of the nonconjugated aflatoxins. The antibody, however, had low (less than 12%) cross-reactivity with the hemiacetal form of both STG and aflatoxin B_1. The aflatoxins were included in the study as they, like STG, possess a bis(dihydrofuran) ring structure and therefore are chemically and structurally similar to STG (Betina, 1989). Sterigmatocystin being a possible precursor in the biosynthetic pathway to AFB_1 could also co-occur with this mycotoxin. The reduced cross-reaction with STG hemiacetal was unexpected, as this form was used in the formation of the immunogens. The two earlier papers, in contrast, demonstrated that the polyclonal antibodies have a high and exclusive specificity for this form. The unique specificity of the monoclonal antibody can be attributed to the selection process that was used, that is, the selection of a clone that produced antibodies that would specifically react with STG instead of STG hemiacetal. The low but significant cross-reaction with AFB_1 hemiacetal suggests that the hemiacetal derivitization approach, as used by the previous researchers, is probably inappropriate for routine analysis of natural commodities for STG due to the high coincidence of aflatoxin in these commodities (CAST, 1989) and the probability that many other reactive hemiacetals would form when a crude extract is subjected to this reaction prior to the ELISA. Under such conditions it would probably be impossible to detect STG using polyclonal antibodies as background values would be high. It is not known, however, if this problem would arise as the previous authors did not verify their procedure using spiked grain samples. Such reactions have been reported before with polyclonal antibodies and are attributed to the nonspecific binding of antibody to the bridge between the hapten and the protein component of the immunogen (Nordblom et al., 1981; Wang et al., 1996).

The extraction procedure selected for the wheat sample proved to be adequate for routine quantitation. The solvents were found to be compatible with the ELISA, although originally designed for chromatography-based assays. The parent form, STG, was readily extracted from the spiked wheat samples as demonstrated by the low error in the indirect competitive ELISA and the reasonably good recovery of the toxin in most samples (Table 2). The lowest reproducible amount of STG that could be quantitated in wheat was 31 ppb, with a maximum working range up to 250 ppb. The recoveries and associated errors, however, were unacceptable at values less than 31 ppb or greater than 250 ppb. The solvent system, presumably, is also sufficiently hydrophobic for the solubilization of STG (Neely and Emerson, 1990; Septien et al., 1993), a feature overlooked in earlier ELISA studies. The observed matrix effects due to the use of this solvent system were less than 5% of the maximal absorbance (data not shown). The inclusion of a sample cleanup step could further increase the reliability of the assay, while the dilution of samples that are higher than 250 ppb in concentration would allow more accurate quantitation of STG over a broad range. This is the first study that has assessed the recovery of STG from a grain matrix using the ELISA. Previous studies only established the matrix effect by diluting the standards in a grain extract. Further studies should be carried out to test the reliability of the assay over a broader range of the toxin and means of increasing the recovery of the toxin from the matrix. Also, studies should be carried out on naturally contaminated samples.

Table 2. Recovery of Sterigmatocystin from Spiked Wheat As Determined by the Quantitative Indirect Competitive ELISA[a]

toxin added to wheat (ng/g, spiked level)	toxin detected by ELISA (ng/g)	recovery (%)
8	4.1 ± 1.6	53.1
16	9.4 ± 2.2	60.2
31	25.3 ± 0.6	81.1
62	58.6 ± 1.6	92.2
125	104.0 ± 2.4	83.2
250	198.5 ± 19.6	79.4
500	304.1 ± 48.9	60.8

[a] Mean and standard deviation of triplicate analysis.

In summary, the monoclonal antibody developed in this assay was sufficiently sensitive for the routine detection of STG in wheat at low parts per billion levels. The antibody's unique specificity for the parent form of STG eliminated the need for sample derivitization prior to ELISA analysis. The solvents selected for the extraction were compatible with the ELISA and resulted in acceptable recoveries of toxin from wheat.

ACKNOWLEDGMENT

We thank Dr. Jianlong Lou for the antigen (STG) supply and Drs. Shiyou Chen and Andrew Frohlich for invaluable technical assistance.

LITERATURE CITED

Abramson, D.; Sinha, R. N.; Mills, J. T. Mycotoxin and odour formation in barley stored at 16 and 20% moisture in Manitoba Canada. *Cereal Chem.* **1983**, *60*, 350−355.

Berry, C. The pathology of mycotoxins. *J. Pathol.* **1988**, *154*, 301−311.

Betina, V. *Mycotoxins, Chemical, Biological and Environmental Aspects;* Elsevier: Amsterdam, The Netherlands, 1989.

Candlish, A. G.; Stimson, W. H.; Smith, J. E. Determination of ochratoxin A by monoclonal antibody-based enzyme immunoassay. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 961−964.

CAST. *Mycotoxins, Economic and Health Risks*; Task Force Report 116; Council for Agricultural Science and Technology, Ames, IA, 1989.

Chu, F. S. Immunoassay for mycotoxins: current state of the art, commercial and epidemiological applications. *Vet. Hum. Toxicol.* **1990**, *32*, 42−50.

Curry, P. T.; Reed, R. N.; Martino, R. M.; Kitchin, R. M. Induction of sister-chromatid exchanges in-vivo in mice by the mycotoxins sterigmatocystin and griseofulvin [contaminant of stored grains and nuts]. *Mut. Res. Genet. Toxicol.* **1984**, *137*, 111−115.

Enomoto, M.; Hatanaka, J.; Igarashi, S.; Uwanuma, Y.; Ito, H.; Asaoka, S.; Iyatomi, A.; Kuyama, S.; Harada, T.; Hamasaki, T. High incidence of angiosarcomas in brown fat tissue and livers of mice fed sterigmatocystin mycotoxins. *Food Chem. Toxicol.* **1982**, *20*, 547−550.

Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1988.

Kao, J.; Norby, J. *Sigma Plot, Scientific Graphing Software, User's Manual*; Jandel Scientific: San Rafael, CA, 1992.

Li, Y.; Chu, F. S. Production and characterisation of antibody against sterigmatocystin. *J. Food Saf.* **1984**, *6*, 119−127.

Morgan, M. R. A.; Kang, A. S.; Chan, H. W.-S. Production of antisera against sterigmatocystin-hemiacetal and its potential for use in an enzyme-linked immunosorbent assay for sterigmatocystin in barley. *J. Sci. Food Agric.* **1986**, *37*, 873−880.

Neely, F. L.; Emerson, C. S. Determination of sterigmatocystin in fermentation broths by reversed-phase high-performance liquid chromatography using post-column fluorescence enhancement. *J. Chromatogr.* **1990**, *523*, 305−311.

Nordblom, C. D.; Webb, R.; Counsell, R. E.; England, B. G. A chemical approach to solving bridging phenomena in steroid radioimmunoassays. *Steroids* **1981**, *38*, 161−173.

Pohland, A. E.; Cushmac, M. E.; Andrellos, P. J. Aflatoxin B1 hemiacetal. *J. Assoc. Anal. Chem.* **1968**, *51*, 907−910.

Purchase, I. F. H.; van der Watt, J. J. Carcinogenicity of sterigmatocystin. *Food Cosmet. Toxicol.* **1970**, *8*, 289−296.

Scott, P. M.; van Walbeek, W.; Kennedy, B.; Anyeti, D. Mycotoxins (ochratoxin A, citrinin and sterigmatocystin) and toxigenic fungi in grains and other agricultural products. *J. Agric. Food Chem.* **1972**, *20*, 1103−1109.

Septien, I.; Cutuli, M. T.; Garcia, M. E.; Suarez, G.; Blanco, J. L. Solubility and stability of steregmatocystin in different organic solvents. *Toxicon* **1993**, *31*, 1337−1340.

Shannon, G. M.; Shotwell, O. L. Thin-layer chromatographic determination of sterigmatocystin (carcinogens) in cereal grains and soybeans. *J. Assoc. Off. Anal. Chem.* **1976**, *59*, 963−965.

Steyn, P. S.; Thiel, P. G.; Trinder, D. W. In *CRC Mycotoxins and Animal Foods;* Smith, J. E., Henderson, R. S., Eds.; CRC Press: Boco Raton, FL, 1991.

Ueda, N.; Fugie, K.; Gotoh-Mimura, K.; Chattopadhyay, S. C.; Sugiyama, T. Acute cytogenetic effect of sterigmatocystin on rat bone-marrow cells in-vivo [toxic metabolite of *Aspergillus nidulans*]. *Mutat. Res.* **1984**, *139*, 203−206.

Vesonder, R. F.; Horn, B. W. Sterigmatocystin in dairy cattle feed contaminated with *Aspergillus versicolor*. *Appl. Environ. Microbiol.* **1985**, *49*, 234−235.

Wang, N.; Kierek-Jaszczuk, D.; Marquardt, R. R.; Frohlich, A. A.; Abramson, D. Use of a heterologous bridge coating antigen for the immunoassay of 3-acetyldioxynivalenol in barley. *J. Food Prot.* **1996**, in press.

Received for review April 11, 1995. Accepted October 17, 1995.[⊗] This work was supported in part by the Laboratory of Veterinary Infectious Diseases, Nanjing Agricultural University, China, by the Natural Sciences and Engineering Research Council of Canada, by the University of Manitoba, and by the Manitoba Department of Agriculture.

JF950258M

[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1995.