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Enzyme-linked Immunosorbent Assay of Versicolorin A and Related Aflatoxin Biosynthetic Precursors

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ABSTRACT

An immunochemical approach is described for the detection of versicolorin (VA) and other aflatoxin precursors in *Aspergillus parasiticus* cultures. VA was purified from *A. parasiticus* ATCC 36537 cultures by semipreparative high performance liquid chromatography and confirmed by mass spectrometry and ultraviolet (UV) absorption. To be rendered immunogenic, VA was converted to a hemiacetal and conjugated to bovine serum albumin (BSA) by reductive alkylation. Rabbit polyclonal antiserum prepared against the VA hemiacetal-BSA conjugate was employed in a competitive ELISA using VA hemiacetal-horseradish peroxidase conjugate as the marker ligand. Based on the amount of VA analogue required to inhibit binding by 50% in competitive ELISA, cross-reactivity relative to VA for VA hemiacetal, averufanin, averantin, norsolorinic acid, averufin, sterigmatocystin, and aflatoxin B_1 were 106, 85, 7, 6, 2, <1, and <1%, respectively. The ELISA was used to monitor enhanced production of VA equivalents by *A. parasiticus* ATCC 36537 in a modified culture procedure. The VA antibody should be extremely useful in the biochemical and genetic investigation of aflatoxin biosynthesis.

Aflatoxin B_1 (AFB_1) is a potent, naturally occurring hepatotoxin and hepatocarcinogen produced by fungal species *A. flavus* and *A. parasiticus*. Based on analysis of blocked mutants and isotope labeling, a partial putative pathway for aflatoxin biosynthesis has been proposed to be: acetate ->norsolorinic acid -> averantin -> averufanin -> averufin -> versiconal hemiacetal acetate -> versicolorin A -> sterigmatocystin -> O-methylsterigmatocystin -> aflatoxin B_1 (*2,9*). It has been well established that the chemical site responsible for biological activity of aflatoxins and related compounds is the C_2-C_3 double bond in the dihydrofuran moiety of these molecules (*15*). Versicolorin A (VA) is perhaps the most critical of the aflatoxin precursors because it is the first in the pathway to contain this essential moiety. Hence, insight into the biosynthesis of VA and its control is of fundamental importance in understanding regulation of aflatoxin biosynthesis.

Current methods for quantitative and qualitative measurement of production of VA and other aflatoxigenic precursors generally include thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). These methods have the disadvantage of requiring extensive extraction and cleanup and are not readily amenable to genetic and biochemical studies of aflatoxin biosyntheses. Immunochemical assays, specifically enzyme-linked immunosorbent assays (ELISAs), are now commonly used for analytical detection of mycotoxins (*13*). These assays demonstrate high specificity and sensitivity based upon the use of antibodies produced against the compound of interest. Several approaches for preparation of aflatoxin conjugates have been used to produce aflatoxin B_1 antibodies (*7,14*). Recently, our laboratory utilized a sterigmatocystin antibody to assess production of that precursor in *Aspergillus* sp. under a variety of culture conditions (*5*). It is further desirable to develop antibodies as immunochemical probes for the early steps of the aflatoxin biosynthetic pathway. This report describes the production and characterization of antibody against VA and its application in ELISA for evaluation of VA production in *Aspergillus parasiticus*.

MATERIALS AND METHODS

Chemicals

All inorganic and organic solvents were reagent grade quality or better. Bovine serum albumin (BSA) (fatty acid free), horseradish peroxidase (HRP) type VI, Tween 20, 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS), AFB_1, sterigmatocystin, and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO). Complete and incomplete Freund's adjuvants were purchased from Difco Laboratories (Detroit, MI). Standards of versicolorin A, norsolorinic acid, averantin, averufin, and averufanin were kindly supplied by L. Lee and D. Bhatnagar (USDA Agriculture Research Service, New Orleans, LA).

Purification of VA

The sucrose-based defined liquid medium formulated by Adye and Matales (*1*) and modified by Bennett et al. (*3*) for VA was used for culturing *A. parasiticus* ATCC 36537. Media were dispensed in 100-ml volumes in 250-ml Erlenmeyer flasks. Each flask was inoculated with a 1-ml spore suspension of ATCC 36537 containing approximately 1×10^7 conidia and incubated at 28°C for 6 d at 200 rpm.

VA was extracted in a manner similar to that described by Lee et al. (*10*). Briefly, mycelia were soaked overnight in acetone and then blended into a macerated slurry. The slurry was filtered and repeatedly blended in acetone until extracts were colorless. Extracts

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were dried, resuspended in 30% acetone, and reextracted with hexane.

An analytical HPLC procedure developed by McCormick et al. (*12*) was modified for purification of VA on a Partisil 10 ODS Magnum 9/25 prepacked 25 cm x 9.4 mm i.d. (Whatman Chemical Separations, Inc., Clifton, NJ) reverse phase semipreparative HPLC column. Briefly, 1-2 mg of crude VA dissolved in 2 ml of methanol:tetrahydrofuran:0.1 M acetic acid (4:2:1) were injected onto the column using a Model 396-89 mini-pump (Laboratory Data Control, Milton Roy Corp., Riviera Beach, FL) attached to a Valco C6U sample loop injector (Valco Instruments Co., Inc., Houston, TX) connected to a guard column (CSK I, Whatman) packed with Pellicular ODS (Whatman). Using a flow rate of 3 ml/min, VA began eluting at a retention time of 9-10 min. Collected fractions were stored overnight at 4°C where VA crystallized into fine, yellow needles. VA was verified by mass spectrometry using a direct insertion probe with an electron impact ionization mode and by UV absorption (*6*).

Preparation of versicolorin-protein conjugates

In order to produce a reactive group for attachment to a carrier protein, VA was first converted to the hemiacetal form using a method similar to that described by Li and Chu (*11*) for modifying sterigmatocystin. VA (20 mg) was dissolved in 2 ml acetone and refluxed with 20 µl 10% H_2S0_4 for 2-3 h at 56°C. The reaction was monitored on Whatman LHP-K high performance TLC plates in benzene:acetic acid (95:5) (*10*). Rfs for VA and VA hemiacetal were 0.4 and 0.1, respectively. The reaction mixture was dried, resuspended in water, and extracted with chloroform. Chloroform extracts were loaded onto a 900-mg silica gel cartridge (Alltech Maxi-Clean, Alltech Assoc., Deerfield, IL) connected to a 5-ml glass syringe. Unreacted VA eluted with chloroform. VA hemiacetal eluted with 50% acetone in chloroform.

Versicolorin hemiacetal was conjugated to BSA for use as immunogen by reductive alkylation. Briefly, VA hemiacetal, 0.5 mg, was dissolved in 0.75 ml methanol and added dropwise to 5 mg BSA in phosphate buffer while stirring slowly. The mixture was incubated at 37°C for 30 min. Fifty (50) µl sodium borohydride (6.5 mM) was added, the reaction mixed for 30 min, and the 25 µl of hydrochloric acid (50 mM) was added to destroy excess sodium borohydride (*4*). The conjugate solution was dialyzed for 48 h against 0.05 M phosphate buffer (pH 7.4). Conjugation ratio of hapten to carrier was estimated spectrophotometrically at 456 nm. Conjugation to HRP as a marker ligand for ELISA was conducted in a similar manner.

Immunization of rabbits

Albino male rabbits were initially injected intradermally with 500 ug VA-BSA (1 ml conjugate in a 1:2 ratio of saline and Freund's complete adjuvant) at approximately 30 sites on the back of each rabbit. Subsequent intramuscular injections consisted of 250 µg VA-BSA (0.5 ml conjugate in a 1:2 ratio of saline and Freund's complete adjuvant). Rabbits were bled via the marginal ear vein. Antiserum was purified from serum by ammonium sulfate precipitation (*8*). ELISA was used to determine titer and sensitivity of antibodies.

Determination of antibody titers

For determination of antibody titers by direct ELISA, 100 µl of antisera diluted in 0.05 M carbonate buffer (pH 9.6) was added to microtiter wells and incubated at 37°C in a forced air incubator. Plates were washed twice with 0.1 M phosphate buffered (pH 7.5) saline containing 0.20% (v/v) Tween 20 (PBS-Tween). Unbound sites were blocked by addition of 300 µl of 1% (wt/vol) ovalbumin in PBS (OA-PBS) to each well followed by incubation for 30 min at 37°C. The plate was washed three more times with PBS-Tween. VA hemiacetal-HRP conjugate (0.5 mg/ml) was diluted 1:500 in OA-PBS and 100 µl added to each well. Plates were incubated for 60 min at 37°C. Unbound enzyme conjugate was removed by washing eight times with PBS-Tween. Bound peroxidase activity was determined by addition of 100 µl substrate solution consisting of 0.4 mM ABTS, 1.2 mM hydrogen peroxide in 50 mM citrate buffer, (pH 4.0) to each well (*14*). The reaction was stopped by adding 100 µl stopping solution consisting of 300 mM citric acid containing 15 mM sodium azide to each well. Absorbance was read at 405 nm. Titers were defined as the highest dilution of antiserum resulting in absorbance greater than the control.

Competitive ELISA and specificity determination

Competitive ELISA for VA detection was performed as described above using a 1:500 - 1:1000 antibody dilution, with the following modifications. After blocking plates with OA-PBS, 100 µl of VA standards or samples diluted in 20% methanol in PBS (MEOH-PBS) was added to each well immediately prior to addition of VA hemiacetal-HRP enzyme conjugate (1:250). Direct competitive ELISA was also used to analyze for cross-reactivity with averufin, averantin, averufanin, norsolorinic acid, versicolorin hemiacetal, sterigmatocystin, and aflatoxin B_1.

Miniscale production of VA

Direct competitive ELISA was used to determine the time course of VA accumulation by *A. parasiticus* ATCC 36537 when grown in miniscale cultures. Ten ml of culture media was added to 50-ml flasks previously treated with an organosilane surface treating agent. Five 6-mm (diameter) glass beads were added to half of the flasks to reduce potential clumping of mycelia that tended to occur during incubation. Flasks were inoculated with 106 conidia, plugged with foam stoppers, covered with foil, and incubated in the dark at 28°C with continuous shaking (200 rpm.) for 1, 2, 3, 4, 5, and 6 d. VA was extracted from mycelia with two 10 ml volumes of methanol. Direct competitive ELISA was used to determine VA content. Mycelial dry weight was determined after drying overnight at 50°C.

RESULTS AND DISCUSSION

Final recovery for VA hemiacetal from pure VA was 83%. Results of conjugation indicated a 16:1 molar ratio of VA hemiacetal:BSA. These results are comparable to the 74% recovery and 14:1 molar ratio reported by Li and Chu (*11*) for sterigmatocystin hemiacetal and sterigmatocystin-BSA conjugation, respectively.

Final titers for five rabbits after 23 weeks ranged from 2000 to 4000. A competitive ELISA based on inhibition of VA hemiacetal-HRP binding to the antibody solid phase by free VA was developed to measure VA antisera. Two of 5 rabbits immunized produced antibodies with high relative affinity for VA based on this ELISA. Fifty percent inhibition of end-product absorbance for these antibodies occurred between 100 and 1000 ng/ml VA. The minimal limit of detection for VA was 10 ng/ml.

Relative cross-reactivity of the VA antiserum for VA analogues was determined in the competitive ELISA (Fig. 1). Concentrations required for 50% inhibition for VA and other compounds analyzed were compared to versicolorin hemiacetal and percentage cross-reactivity estimated based on the amount of VA analogue required to inhibit binding by 50% (Fig. 2). The results demonstrated that VA hemiacetal, which was used in immunogen preparation, required a lower concentration for 50% inhibition than VA. Similar results were reported by Li and Chu (*11*) when producing

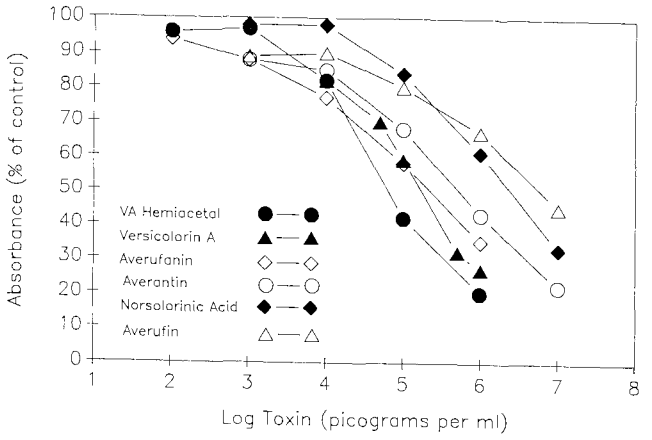

Figure 1. *Direct competitive ELISA of versicolorin A and related analogues.*

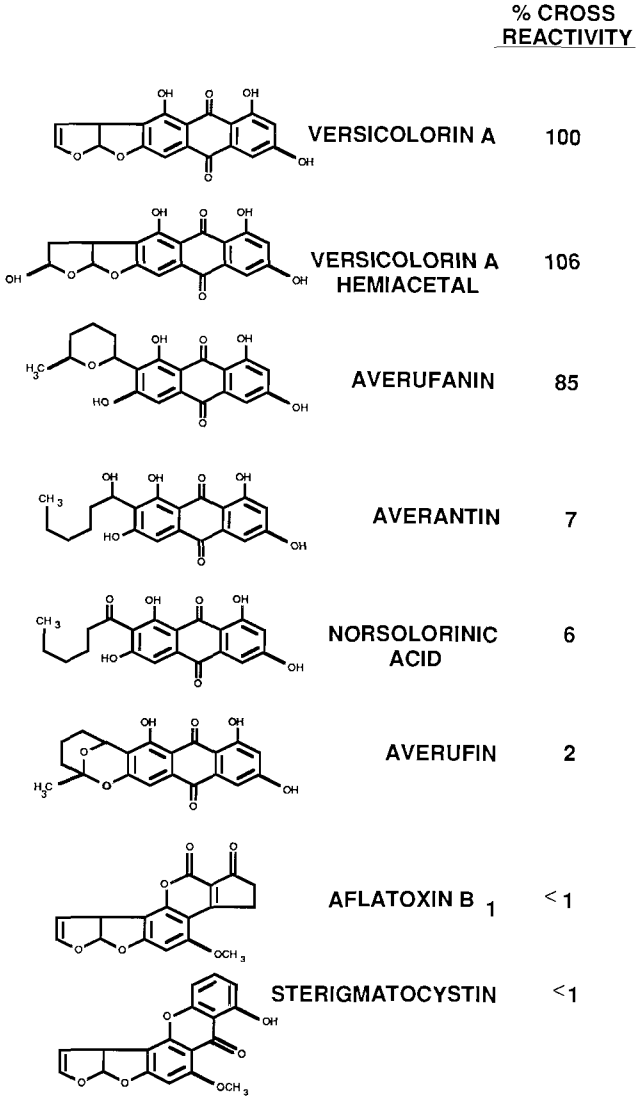

Figure 2. *Relative cross-reactivity of versicolorin A antibody. Percentage cross-reactivity was calculated by ELISA as follows:*

$$\text{Percentage cross-reactivity} = \frac{\text{ng VA analogue required for 50\% inhibition}}{\text{ng VA required for 50\% inhibition}} \times 100.$$

antibody to sterigmatocystin through hemiacetal derivatization. Cross-reactivity of VA hemiacetal was 106% relative to VA. Averufanin, a precursor which also contains the anthraquinone moiety, showed a high degree of cross-reactivity (85%). This higher cross-reactivity compared to other precursors may be due to structural similarity between the open ring of averufanin and the open ring of the stable VA hemiacetal-BSA conjugate used for immunization. Averantin (7%), norsolorinic acid (6%), and averufin (2%) demonstrated much lower cross-reactivity possibly due to their anthraquinone structures suggesting that the additional oxygenated ring of averufanin enhanced binding of the antibody. Sterigmatocystin and aflatoxin B_1 structurally share the difuran moiety with VA but showed less than 1% cross-reactivity. These results verify the desirability of using the difuran of VA for conjugation to carrier protein in order to elicit antibodies with specificity for the anthraquinone portion of the molecule.

The direct competitive ELISA was applied for detection and quantitation of VA equivalents in a modified miniscale culture procedure for enhanced VA production (Fig. 3). Cultures containing glass beads produced more VA than control cultures for the 2, 3, 4, 5, and 6 d incubation periods analyzed suggesting that the addition of glass beads to culture media increased VA production. Similarly, mycelial dry weights were higher for the bead-containing cultures compared to the control until day 6. Bead-containing cultures exhibited peak accumulation after 4 d of incubation. Notably mycelia produced by cultures grown with glass beads appeared as short threads of hyphae compared to control cultures which demonstrated hyphae that grew in small compact spheres.

In summary, hemiacetal derivatization of VA and con-

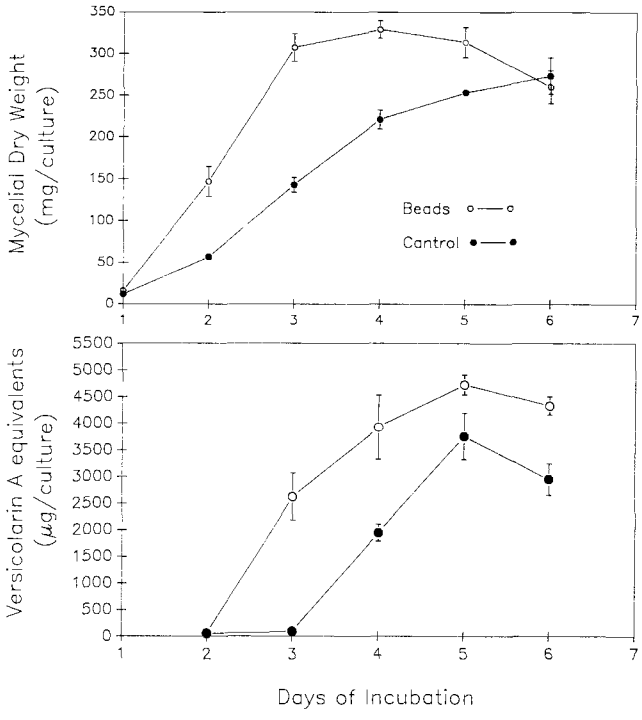

Figure 3. *Time course of mycelial dry weight and VA equivalent accumulation by* A. parasiticus *ATCC 36537. Cultures (10 ml in 50-ml flask) were shaken at 28°C with and without five 6-mm (diameter) glass beads.*

jugation to a carrier protein by reductive alkylation yielded an effective immunogen for production of antibody with specificity for the anthraquinone portion of the molecule. Therefore, the antibody produced in the present study could be used for detection of VA, averufanin and to a lesser extent, averantin, norsolorinic acid, and averufin. As illustrated here, ELISA offers a simple approach for monitoring the effect of culture conditions on production of VA and analogues. The antibody can similarly be applied to the rapid selection and identification of mutants blocked at or prior to the VA step in the aflatoxigenic pathway, detection of blocked mutants transformed back to VA biosynthesis with selected DNA segments, and rapid assay of VA/averufanin biosynthetic enzymes for facile purification. In addition, since VA is potentially carcinogenic, the ELISA could be used to test for *Aspergillus versicolor* contaminated feedstuffs.

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