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Production of Polyclonal Antibodies against Territrem B and Detection of Territrem B in the Conidia of *Aspergillus terreus* 23-1 by Immunoelectron Microscopy

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Territrem B, a fungal metabolite isolated from *Aspergillums terreus* 23-1, is a tremorgenic mycotoxin. Immunoelectron microscopy using anti-territrem B polyclonal antibody was used to detect territrem B in the fungal body of *A. terreus* 23-1 at different times of culture without shaking on potato dextrose (PD) agar medium. The anti-territrem B serum was produced by immunization of rabbits with 4β-hydroxymethyl-4β-demethylterritrem B-sccinate bound by a linker to keyhole limpet hemocyanin. This antiserum recognized territrems and immunoelectron microscopy using this antiserum, and colloidal gold-conjugated anti-rabbit IgG antibodies showed that territrem B was localized to the fungal body of *A. terreus* 23-1. Territrem B was first seen in the cytoplasm of the conidia after 4 days' culture on PD agar medium. Maximal territrem B production in the conidia was seen on the 14th day of culture; however, territrem B was not formed in the hyphae at any stage of culture. These results are consistent with the previous finding that the formation of territrems is related to fungal sporulation.

KEYWORDS: ***Aspergillus terreus* 23-1; anti-territrem B serum; immunoelectron microscopy; immunogold particles; indirect competitive ELISA; keyhole limpet hemocyanin; sporulation; territrem B**

INTRODUCTION

During a study of fungi contaminating stored unhulled rice in Taiwan, three tremorgenic mycotoxins, designated territrems A (**1**), B (**2**), and C (**3**), were isolated from a chloroform extract of a rice culture of *Aspergillus terreus* 23-1 (*1*, *2*). When injected intraperitoneally (ip) into rats and mice, territrems induce tremor, salivation, convulsion, liver or kidney congestion, and even death. Their main biochemical effect is inhibition of acetylcholinesterase (*3*−*6*). Experiments aimed at determing what culture conditions affected territrem formation showed that it was related to fungal sporulation. Sporulation and territrem formation occur simultaneously in unshaken cultures of *A. terreus* 23-1, whereas, in shaken cultures, spores are not formed and territrems are not produced. After the conidia and hyphae are separated from a sporulating culture by filtration on a stainless filter, territrems are detected by HPLC in the conidial fraction, but not in the mycelial fraction. Because of its sensitivity and specificity, ELISA is widely used for mycotoxin estimation, and an indirect competitive ELISA with polyclonal antibodies has been successfully used for mycotoxin detection (*7*−*10*). Therefore, to examine the location of the territrem formed in the fungal body, we have previously produced polyclonal antibodies of 3,4,5-trimethoxybenzoic acid (**4**) and used these for immunoenzyme staining and silver-enhanced immunogold staining (unpublished results). To produce this antiserum, because **2** has no group suitable for protein conjugation, **4**, which is a part of the **2** molecule, was coupled to bovine serum albumin (BSA) and the conjugate used to raise an antiserum of **4** in New Zealand White rabbits. This antiserum recognized both **4** and **2** in a competitive ELISA, and the antigen−antibody complex was detected in the cellulase-digested fungal body by conventional staining and light microscopy; however, this experiment was not performed on sections, and we could not assess whether the antibodies of **4** were able to penetrate the fungal cell wall. In this study the location of **2** in the fungal body was examined using immunogold electron microscopy.

MATERIALS AND METHODS

Reagents and Instruments. Ethylenediamine (EDA), 1-ethyl-3,3-dimethylaminopropylcarbiimide (EDPC), glutaraldehyde, horseradish peroxidase-conjugated goat anti-rabbit IgG, colloidal gold-conjugated goat anti-rabbit IgG, *p*-nitrophenyl diamine (OPD), *p*-nitrophenyl phosphate, osmium tetraoxide, paraformaldehyde, and 3,4,5-trimethoxy-

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benzoic acid were purchased from Sigma Chemical Co. (St. Louis, MO). 4-*N*,*N*-Dimethylaminopyridine, pyridine, succinic anhydride, and tetrahydrofuran (THF) were purchased from Merck (Darmstadt, Germany). Hydrogen peroxide and isobutylchloroformate were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Acetone, chloroform, ethanol, and triethylamine were purchased from RdH Laborchemikalien GmbH and Co. KG, Seelze, Germany. Keyhole limpet hemocyanin (KLH) was obtained from Pierce Biotechnology, Inc. (Rockford, IL). The Spurr resin kit was purchased from Polysciences Inc., Warrington, PA. **1**−**3** were isolated from rice cultures of *A. terreus* 23-1 (*3*). 4*β*-Hydroxymethyl-4*β*-demethylterritrem B (**2a**) was isolated from the incubation mixture of **2** and a liver microsomal fraction from male Wistar rats as described previously (*11*).

Thin-layer chromatography (TLC) was performed on 0.25 mm SIL G-50 UV 254 silica gel-precoated glass sheets (Merck). For nuclear magnetic resonance (NMR) measurements, one-dimensional NMR data for 4*β*-hydroxymethyl-4*β*-demethylterritrem B-succinate (**2b**) were acquired using a Bruker AMX-400 spectrometer. ^{1}H and ^{13}C NMR spectra were obtained at 303 K using an AMX-400 Fourier transform NMR spectrometer (9.4 T, ^{1}H, 400.1 MHz; ^{13}C, 100.6 MHz) (Bruker Karlsruhe, Germany) and an Aspect 32 data-processing system (Spectrospin, Faellanded, Switzerland). Chemical shifts were referenced to the solvent peak (δH, 7.24 ppm; δC, 77.0 ppm). Mass spectra were obtained by fast-atom bombardment on a JMS HX-110 mass spectrophotometer (JEOL, Akishima, Tokyo) and the data reported as the relative intensity (*m*/*z*). UV−vis spectra were recorded on a Beckman DU-650 spectrophotometer. Sephadex G-25 was from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Antibody titers were measured on an MR-7000 ELISA plate reader (Dynex Technology, Chantilly, VA). Ultrathin sections of *A. terreus* 23-1 specimens were cut with a glass knife on an MT-2B Porter-Blum ultramicrotome (Ivan Sorvall Inc., Norwalk, CT). Morphology and gold particles in spores and hyphae of *A. terreus* 23-1 were observed with a JEOL 1200 EX transmission electron microscope operating at 80 kV. For gel electrophoresis and immunoblotting, protein was measured using the Lowry method (*12*).

Hapten Synthesis. Because **2a** has no reactive group for protein coupling, it was first converted to **2b** (**Figure 1**). Briefly, a 35 mg sample of **2a** was dissolved in 40 mL of dry THF at room temperature, then succinic anhydride (2.4 g) and 4-*N*,*N*-dimethylaminopyridine (473 mg) were added, and the reaction mixture was refluxed for 1.5 h at 90 °C in an oil bath. Following removal of the THF by evaporation, distilled water (10 mL) was added and the sample extracted with chloroform (3 × 10 mL). The pooled chloroform layers were evaporated to give 35 mg of amorphous residue, from which **2b** (28 mg, 80% yield) was separated and purified by preparative TLC using toluene/ethyl acetate/formic acid (5:4:1 v/v/v) as the mobile phase. The purity of **2b** was verified on a Gasukuro Kogyo model 576 liquid chromatograph equipped with a 10 μm Intersil 10 ODS C18 reverse phase column (4.6 × 250 mm i.d.) and a Shimadzu C-R18 Chromatopac integrator. The column was eluted for 15 min at a flow rate of 1 mL/min with acetonitrile/water (4.1:5.9 v/v). All chromatographic separations were performed at 25 °C, and column effluents were monitored at 335 nm with a Gasukuro Kogyo 520 U detector. ^{1}H NMR ($CDCl_3$) δ 5.89 (d, 1H, H-2, J = 10.32 Hz), 6.37 (d, 1H, H-3, J = 10.32 Hz), 1.71−1.95 (m, 2H, H-5), 1.78 (m, 1H, H-6α), 2.46 (m, 1H, H-6β), 6.36 (S, 1H, H-8), 3.37 (d, 1H, H-12α, J = 17.8 Hz), 2.83 (d, 1H, H-12β, J = 17.8 Hz), 1.26 (s, 3H, 4α-CH_3), 4.10−4.55 (d, 2H, 4β-CH_2, J = 10.9 Hz), 1.54 (s, 3H, 6α-CH_3), 1.43 (s, 3H, 12b-CH_3), 6.98 (s, 2H, H-2′), 6.98 (s, 2H, H-6′), 3.89 (s, 3H, 3′-OCH_3), 3.88 (s, 3H, 4′-OCH_3), 3.89 (s, 3H, 5′-OCH_3), 2.71 (m, 4H, 4β-succinate); ^{13}C NMR ($CDCl_3$) δ 203.8 (s, C-1), 125.1 (d, C-2), 147.9 (d, C-3), 45.9 (s, C-4), 78.6 (C-4a), 25.8 (t, C-5), 28.2 (t, C-6), 79.7 (s, C-6a), 161.9 (s, C-7a), 97.5 (d, C-8), 158.7 (s, C-9), 164.4 (s, C-11), 97.1 (s, C-11a), 27.9 (t, C-12), 76.2 (s, C-12a), 56.1 (s, C-12b), 19.8 (q, 4α-CH_3), 67.3 (t, 4β-CH_2), 21.4 (q, 6a-CH_3), 23.8 (q, 12b-CH_3), 126.7 (s, C-1′), 102.8 (d, C-2′), 153.5 (s, C-3′,5′), 140.4 (s, C-4′), 56.3 (q, 3′,5′-OCH_3), 61.0 (q, 4′-OCH_3), 28.9 (t, 4β-succinate), 172.0 (s, 4β-succinate-COOH); FAB-MS, *m*/*z* 643 (M + H^+ 100), 525 (10), 412 (4), 359 (8), 291 (38), 237 (10), 195 (49), 105 (19), 91 (59); HRMS-FAB, *m*/*z* M^+ calcd for $C_{33}H_{38}O_{13}$, 642.6554, found 642.2132.

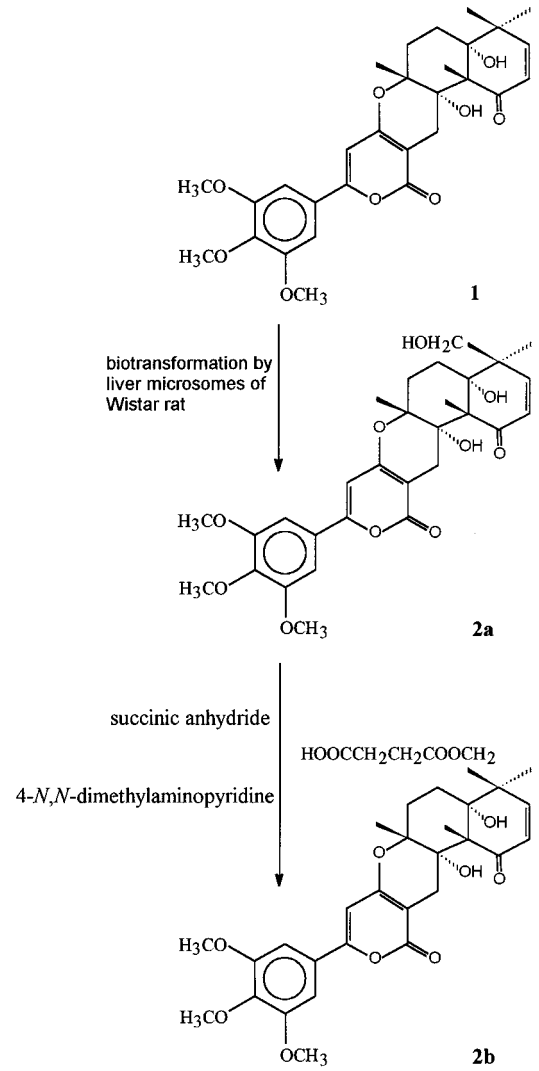

Figure 1. Synthetic pathways used for the preparation of immunizing hapten A (**5**).

Preparation of the Hapten−Protein Conjugate. The synthesized hapten contains a carboxylic group at the end of the spacer arm that can react with free amines of proteins using the mixed anhydride method (*9*).

Preparation of Ethylenediamine-Modified Keyhole Limpet Hemocyanin (EDA-KLH). A 10% aqueous solution of EDA (1 mL) was added to a solution of KLH (300 mg) and EDPC (400 mg) in 15 mL of water, and the mixture was adjusted to pH 5.5 by the addition of ∼2.5 mL of 1 N HCl and then kept at room temperature for 2 h. Another 200 mg of EDPC was added, the pH was again adjusted to 5.5 with 1 N hydrochloric acid, and the mixture was kept at room temperature overnight before being dialyzed against 2 L of distilled water for 3 days (4 °C), changing the distilled water every day. The EDA-KLH was then lyophilized and stored at 4 °C.

*Hapten A, Conjugation of 4β-Hydroxymethyl-4β-demethylterritrem B-Succinate to EDA-KLH (5) (**Figure 2**).* A 1 mg sample of **2b** in 2 mL of dry THF was cooled to −5 °C in an ice/salt bath, then 100 μL of triethylamine in THF (7.5 mg/mL) and 100 μL of isobutylchloroformate in THF (10 mg/mL) were added, and the solution was mixed well, then left at −5 °C for 25 min before the addition of 10 mg of EDA-KLH in 6 mL of 33% pyridine in water at 4 °C. Coupling was carried out for 30 min at 4−6 °C and then overnight at room temperature. The reaction mixture was then passed through Sephadex G-25 using 0.05 M phosphate buffer, pH 7.2, as the eluant. Because the UV spectrum of the conjugate was only slightly different from that of the free protein (peaks at 254 and 330 nm instead of at 255 nm for the free protein and at 330 nm for the free hapten), the molar ratio

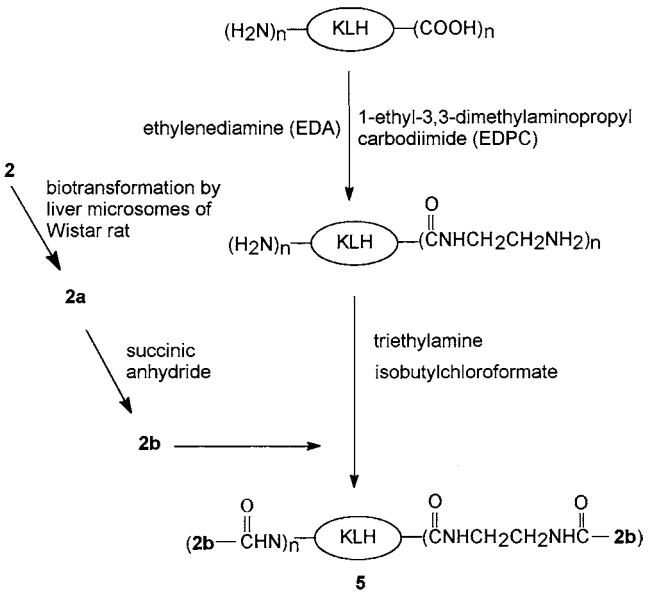

Figure 2. Conjugation of hapten A (**5**) to KLH.

(MR) of hapten to protein was calculated from the absorbance values at 330 and 254 nm by assuming that the molar absorption values for the hapten and protein were the same in the free and conjugated forms. The purified conjugates were stored at −20 °C.

*Hapten B, Conjugation of 4β-Hydroxymethyl-4β-demethylterritrem B-Succinate to EDA-BSA (**6**).* Ten percent aqueous ethylenediamine (1 mL) was added to a solution of BSA (300 mg) and EDPC (400 mg) in 15 mL of water. The mixture was adjusted to pH 5.5 by the addition of ∼2.5 mL of 1 N HCl and kept at room temperature for 2 h. Another 200 mg of EDPC was added, and the pH was again adjusted to 5.5 with 1 N hydrochloric acid. The mixture was kept at room temperature overnight and dialyzed against 2 L of distilled water for 3 days (4 °C), the water being changed every day. The EDA-BSA was lyophilized to dryness and stored at 4 °C before use.

One milligram of **2b** was dissolved in 2 mL of dry THF and the solution cooled to −5 °C in an ice salt bath. One hundred microliters of triethylamine in TEF (7.5 mg/mL) and 100 μL of isobutylchloroformate in THF (10 mg/mL) were added, and the solution was mixed well, left at −5 °C for 25 min, and then added to 10 mg of EDA-BSA in 6 mL of 33% pyridine in water at 4 °C. The coupling reaction was carried out at 4−6 °C for 30 min and then at room temperature overnight. The reaction mixture was passed through Sephadex G-25 using 0.05 M phosphate buffer, pH 7.2, as the eluant, and the conjugate determined spectrophotometrically. The conjugate was only slightly different from that of the free protein (peaks at 278 and 330 nm instead of at 280 nm for the free protein and at 330 nm for the free hapten); the molar ratio (MR) of hapten to protein was calculated from the absorbance values at 330 and 278 nm by assuming that the molar absorption values for the hapten and protein were the same in the free and conjugated forms. The purified conjugate was stored at −20 °C.

Production of Antisera Using 5. Male New Zealand White rabbits (6 months old) were injected subcutaneously at multiple sites in the back with **5** as described by Chu and Ueno (*7*). Initially, the rabbits received 2 mL of emulsion consisting of 500 μg of **5** in 1 mL of sterile saline and 1 mL of complete Freund's adjuvant (Difco Laboratory, Detroit, MI). After 2 weeks, a booster injection of 2 mL of an emulsion prepared by mixing 1 volume of antigen (500 μg) with 2 volumes of incomplete adjuvant (*13*) was given by hypodermic injection in the thigh muscles. The rabbits were bled via the marginal ear vein at weekly intervals, and the serum was lyophilized and stored at −20 °C until use.

Gel Electrophoresis and Immunoblotting. Ten micrograms of **5** was electrophoresed for 1 h at 125 V on 6% sodium dodecyl sulfate−polyacrylamide gels (*14*), and then the proteins of each gel were transferred to a nitrocellulose membrane (*15*). The membranes were blocked by incubation overnight at 4 °C in blocking buffer [phosphate-buffered saline (PBS), pH 7.4, 5% low-fat milk], immunostained overnight at 4 °C with **5** antiserum diluted 1:1000 in blocking buffer containing 0.5% Tween 20 (*16*), washed with PBS containing 0.5% Tween 20 (PBST), and then incubated for 1 h at 25 °C with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech UK Ltd., U.K.) diluted 1:1000 in PBST. Bound antibody was detected by chemiluminescence using ECL reagent (Amersham Pharmacia Biotech UK Ltd.), and the results were recorded using Kodak X-OMAT film.

Measurement of 5 Antibody Titer. An indirect competitive ELISA procedure similar to that used for aflatoxins (*10*) was used. Ninety-six-well polystyrene ELISA plates were coated by overnight incubation at 4 °C with 10 μg/mL of **5** in 0.1 M sodium carbonate buffer, pH 9.2, and then washed four times with PBST. All subsequent steps were for 1 h at 25 °C with four PBST washes between steps. The wells were incubated sequentially with blocking solution (1% BSA in PBST; 150 μL), a mixture of 95 μL of diluted the antiserum of **5** (1:500 to 1:8000) in 0.1% BSA in PBST, 0.5% Tween 20, 5% fetal bovine serum, 0.5 M NaCl, and 5 μL of 0.18 mM **2** and then 100 μL of a 1/2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (GAR IgG) antibody. After washing, bound peroxidase was measured by adding 100 μL of freshly prepared 2 mg/mL OPD and 0.012% H_2O_2 in 25 mM citrate and 62 mM sodium phosphate buffer, pH 5.4, stopping the reaction after 30 min at room temperature by adding 1 μL of 2.5 M sulfuric acid. The absorbance was then read immediately at 490 nm on an MR-7000 ELISA plate reader (Dynex Technology, Chantilly, VA).

Specificity of 5 Antisera. The specificity of the antisera for **1**−**4** and hyphal or spore chloroform extracts of *A. terreus* 23-1 was tested using essentially the protocol described for the determination of the titer except that a 1:2000 dilution of antiserum was used. **1**, **2**, **3**, or **4** was dissolved in methanol (0.18 mM) and then diluted with 0.1 M PBS, pH 7.4, to concentrations between 1.8×10^{-1} and 1.8×10^{-8} mM.

Growth of the Organism. *A. terreus* 23-1 was maintained and grown in solid culture on PD agar medium (*6*). Each Petri dish contained 9 mL of PD agar medium. After sterilization of the PD agar medium, each Petri dish was inoculated with 6×10^7/mL spores of *A. terreus* 23-1 and incubated at 30 ± 1 °C without shaking.

Preparation of Extracts. Five grams of the hyphae or spores separated by filtration on a stainless filter (300 mesh, pore size 50 μ) was extracted with 20 mL of chloroform, and the chloroform fractions were evaporated to dryness over sodium sulfate. For tests, the material was dissolved in 100 μL of methanol, and then 5 μL was diluted in 0.1 M PBS, pH 7.4. Concentration of **2** in the spore extract was determined from the standard curve of HPLC and expressed in milligrams per kilogram using the following formula: **2** concentration (ng/mL) in sample extract × dilution with buffer × extraction−solvent volume used (mL)/sample weight (g). Concentration of **2** in the hyphal extract was not detected by HPLC analysis.

Preparation of *A. terreus* 23-1 for Immunoelectron Microscopy. At 2, 4, 6, 8, 10, 12, and 14 days after inoculation, the fungal mycelium was washed with distilled water, dried in an oven at 105 °C for 24 h, and prepared for immunoelectron microscopy (IEM). A sample (1 mm^2) of specimen containing conidia and hyphae was fixed for 24 h at 4 °C with 4% paraformaldehyde/1% glutaraldehyde (*17*). The fixed specimen was then washed three times in 5% sucrose in PBS, postfixed for 1 h in 1% osmium tetraoxide, washed three times in 5% sucrose in PBS, and dehydrated with a graded series of 75, 85, and 95% ethanol for 10 min at 25 °C and then twice in 100% alcohol for 15 min at 25 °C. After being treated for 2 × 15 min at 25 °C in 100% acetone, the sample was embedded for 1 h at 4 °C in Spurr resin (Polysciences Inc., Warrington, PA)/acetone at ratios of 3:1, 1:1, and 1:3 and finally in fresh Spurr resin for 24 h in a 70 °C oven (*18*). Ultrathin sections were cut with a glass knife on a Porter-Blum Ultramicrotome MT-2B (Ivan Sorvall Inc., Norwalk, CT) and mounted on Formvar-coated nickel grids (*19*, *20*). The grids were either contrasted with uranyl acetate and lead citrate and examined using a JEOL 1200 EX transmission electron microscope operating at 80 kV or further prepared for immunocytochemical labeling.

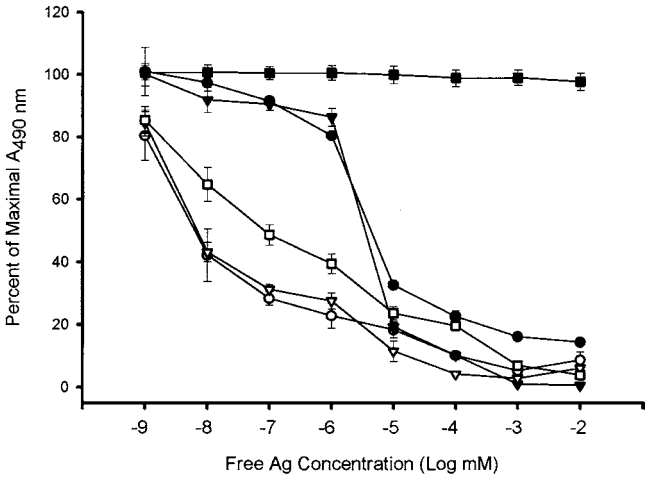

Figure 3. Reactivity of territrems, 3,4,5-trimethoxybenzoic acid, and hyphal and spore extracts with **5** antiserum in a competitive indirect ELISA: (●) territrem A; (○) territrem B; (▼) territrem C; (▽) 3,4,5-trimehtoxybenzoic acid; (■) hyphal extract; (□) spore extract. A 1:2000 dilution of **5** antiserum was used.

Preparation of *A. terreus* 23-1 for Immunochemical Labeling. The method used was similar to those reported by Reijula et al. (*18*) and Sakae et al. (*21*). The grids were gently transferred to wells of microtiter plates (Nunc-Immunoplate, Roskilde, Denmark) and incubated with 5% hydrogen peroxide for 10 min and then with 1% BSA in 0.01 M PBS, pH 7.4, for 30 min at 25 °C. After 3 × 2 min washes in Tris buffer, pH 8.2, containing 0.1% BSA, 200 μL of primary antiserum dilutions was added to the microplate wells. All of the dilutions of test reagents were prepared in PBS−BSA−Tween. An antiserum of **5** was used at a 1:1000 dilution and the colloidal gold (10 nm particle diameter)-conjugated goat anti-rabbit IgG at a 1:200 dilution. After incubation for 1 h at room temperature, the grids were washed three times for 2 min each in Tris buffer, pH 8.2, and then incubated for 1 h at room temperature with 200 μL of colloidal gold-conjugated goat anti-rabbit IgG. The grids were washed in Tris buffer, twice in PBS, and twice in double-distilled water and then air-dried. The grids were examined using a JEOL 1200 EX transmission electron microscope operating at 80 kV.

RESULTS AND DISCUSSION

Hapten Design. Mycotoxins are low molecular weight secondary fungal metabolites that are nonimmunogenic; that is, they do not induce an antibody response. To render them immunogenic, they must be conjugated to a protein carrier before immunization. However, conjugation of a mycotoxin to a protein carrier is dependent on the mycotoxin having a reactive group. Although some mycotoxins, such as ochratoxins, patulin, and penicillic acid, do have a suitable reactive group, others, including aflatoxins, trichothecenes, and territrems, do not, and a carboxyl or other reactive group must first be introduced into the toxin molecule. The production of the hemisuccinate of aflatoxin Q_1 (*22*), T-2 toxin (*23*), and deoxyverrucarol (*8*) has been reported. To introduce a carboxylic acid group into **2**, **2** was first transformed by liver microsomes into **2a**, which contains a hydroxyl group that can react with succinic anhydride to give **2b**, which contains a reactive carboxylic acid (**Figure 1**). Höfle and Steglich (*24*) reported that 4-*N*,*N*-dimethylaminopyridine is a better catalyst for acylation of hydroxyl groups than pyridine. This reagent provided a yield of **2b** of up to 80%. The mass spectra of **2b** has a series of common ion fragments such as *m*/*z* 525, 412, 359 291, and 195 in the mass spectrum of **2a**. The hetero-COSY spectrum showed that 4β-succinate-CH_2 (δ 28.9) was coupled to 4β-CH_2 (δ 2.71). These results show that the final product was the 4β-CH_2 succinate of **2a**.

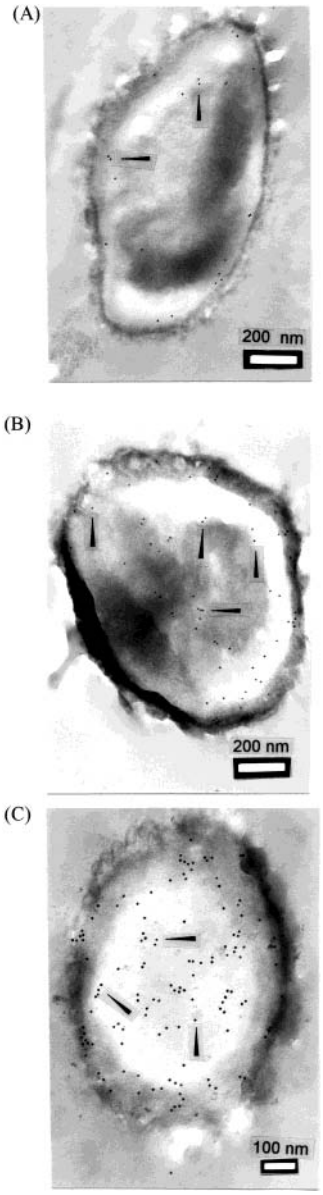

Figure 4. Immunoelectron microscopy of a thin section of *A. terreus* 23-1 conidia incubated with anti-territrem B antiserum. Thin sections of conidia at culture days 4 (**A**), 8 (**B**), and 14 (**C**) were fixed with 4% paraformaldehyde/1% glutaraldehyde, postfixed with 1% osmium tetraoxide, and stained with **5** antiserum and colloidal gold-anti-rabbit IgG; gold particles can be seen in the cytoplasm of the conidia. Arrowheads indicate gold particles.

Hapten−Protein Conjugates. To improve the efficiency of conjugation of aflatoxin B_1 to BSA, EDA has been used as a linker. Immunization of rabbits with EDA−BSA−aflatoxin B_1-oxime has been reported and found to be more effective than immunization with BSA−aflatoxin B_1-oxime (*9*). Ohtani et al. (*25*) reported the binding of T-2 hemisuccinate (T-2 HS) to different proteins and found that the highest molar ratio was seen with KLH, followed by BSA and then ovalbumin. In this study, **2b**, which contains a carboxylic group, was conjugated to KLH or BSA by the mixed anhydride method to give products with an MR of **2b** to KLH or BSA of 86−2483 or 15, respectively. Because the MR was higher for **5**, it was used as immunogen to produce a polyclonal **5** antiserum.

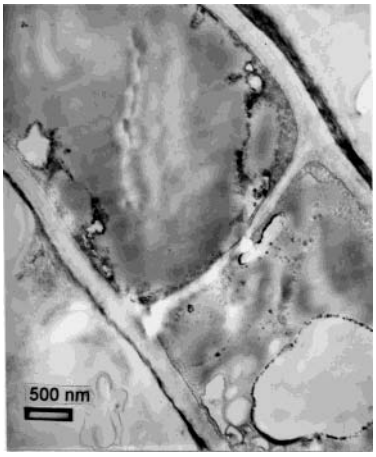

Figure 5. Immunoelectron microscopy of a thin section of *A. terreus* 23-1 hyphae incubated with **5** antiserum.

Production of 5 Antiserum. Antibody levels in rabbits immunized with **5** conjugate were monitored by immunoblotting using **5** as the immobilized antigen, and the blood sample taken at 8 weeks after boosting was chosen for use in tests. The results for KLH, preimmune of sera, 8-week sera indicated that only 8-week sera had the protein levels of the antiserum of **5** (data not shown here).

Determination of Antibody Titers by Indirect Competitive ELISA. Antisera from rabbit immunized with **5** produced the highest titers with optical densities at 490 nm (OD_{490S}) of ~2.0 dilutions of 1:2000 with its corresponding KLH conjugate coating of binding **2**, giving 45% inhibition (data not show).

Specificity of 5 Antiserum. Figure 3 shows the relative binding of **1**−**4** and extracts of hyphae or spores to the antiserum of **5** in an indirect competitive ELISA in which the plates were coated with **5**. The concentrations of the free antigen giving 50% inhibition of antibody binding were 0.9 nM for **2** and **4**, 1 nM of **2** from the spore extract, and 900 nM for **1** and **3**, the hyphal extract causing negligible inhibition. The results for the hyphal and spore extracts showed that **2** biosynthesis in *A. terreus* 23-1 was associated with spore formation.

Location of 2 in *A. terreus* 23-1 Spores. Immunogold labeling electron microscopy has been used to determine the localization of antigens in microorganisms (*17*, *21*, *26*−*31*). Binding of immunogold particles was seen in the cytoplasm of the conidia of *A. terreus* 23-1 grown for 4, but not 2, days in PD medium (**Figure 4A**), and the number of bound immunogold particles increased with time through days 8 and 14 of culture (**Figure 4B**,**C**). However, territrem B was not seen in hyphae at any period of culture, even after 14 days of culture (**Figure 5**).

In the present study, we successfully raised an antiserum against **2** and developed an immunoelectron microscopic method by which the localization of **2** in the conidia of *A. terreus* 23-1 can be precisely determined; we also confirmed previous observations that **2** biosynthesis in *A. terreus* 23-1 is associated with fungal sporulation.

ABBREVIATIONS USED

EDA, ethylenediamine; EDA-KLH, ethylenediamine-modified keyhole limpet hemocyanin; EDPC, 1-ethyl-3,3-dimethylaminopropylcarbiimide; IEM, immunoelectron microscopy; ip, intraperitoneally; KLH, keyhole limpet hemocyanin; MR, molar ratio; OPD, *p*-nitrophenyl diamine; PD, potato dextrose; PBS, phosphate-buffered saline.

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