Proteinaceous Virus-like Particles from an Isolate of *Aspergillus flavus*

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Virus-like particles were purified from a single nonaflatoxin-producing isolate of *Aspergillus flavus*. The virus-like particles were spherical, measuring 27 to 30 nm in diameter, were electrophoretically homogeneous, and sedimented at approximately 49S. The particles had a buoyant density of 1.28 g/cm³ in CsCl and contained no detectable nucleic acid.

An association between toxin production and the presence of a bacteriophage has been established with the botulinum toxin produced by *Clostridium botulinum* (6) and the diphtheria toxin produced by *Corynebacterium diphtheriae* (8). After a preliminary report by D. W. Mackenzie and J. P. Adler (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 68, 1972) that a nonaflatoxin-producing isolate of *Aspergillus flavus* Link contained virus-like particles (VLP) and a toxin-producing strain did not contain these particles, investigations were made to determine if the VLP influenced the production of aflatoxin. Five nontoxin- and four aflatoxin-producing single-spore isolates of *A. flavus* and *A. parasiticus* Speare, NRRL numbers 1957, 2999, 3161, 3357, A-11611, A-12267, A-12268, A-13668, and A-15648 (courtesy of D. Fennell, USDA Regional Lab., Peoria, Ill.) were assayed for aflatoxin production and the presence of VLP. All isolates exhibited typical morphological and growth characteristics.

Aflatoxin production determinations were made from the broth of 7-day still cultures grown at 25 C on a solution containing 2% yeast extract and 20% sucrose. The aflatoxins were assayed by thin-layer chromatography by using the procedure of Pons et al. (12) with a standard solution of aflatoxins (Supelco Inc., Bellefonte, Pa).

The VLP were isolated from mycelium grown in shake cultures at 27 C for 5 days in Sabouraud broth. The mycelium was either freeze dried and ground in a Wiley Mill (A. H. Thomas Co., Philadelphia, Pa), by using a 60-mesh screen, or was immediately ground with mortar and pestle in the presence of acid-washed sand.

The samples were extracted with 0.1 M, pH 8, potassium phosphate buffer and centrifuged at $7,000 \times g$ for 10 min. The supernatant was then centrifuged at $66,000 \times g$ for 2.5 h in a Spinco type 30 rotor at 5 C. The pellets were resuspended in the same buffer as above and subjected to two additional cycles of low- and high-speed centrifugation. The final sample was layered onto 100- to 400-mg/ml sucrose density gradients (14) and centrifuged for 2 or 3 h at $200,000 \times g$ in a Spinco SW 41 rotor at 5 C. The gradients were fractionated with an ISCO density gradient fractionator and by a UV analyzer (3). Carbon-coated grids with Formvar backing were floated on drops of gradient fractions, drained, and then stained with either 2% potassium phosphotungstate at pH 7 or 1% uranyl acetate. The specimens were examined with a Zeiss EM 9S-2 electron microscope.

Of the nine isolates screened, VLP were found only in the NRRL A-12268 isolate previously reported by D. W. Mackenzie and J. P. Adler (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 69, 1972). The particles singly or in packed arrays measured 27 and 30 nm in diameter, respectively (Fig. 1). When the preparations were further purified by sucrose density gradient electrophoresis (16), the particles migrated as a single electrophoretic species. This fraction was subjected to further analyses. The final yield was approximately 100 to 200 μ g of VLP per g (dry weight) of mycelium.

Rabbit antisera with a titer of 128 was prepared to the VLP as described previously (16). Using Ouchterlony agar double-diffusion tests, according to Ball (1), no serological reaction was observed between *A. flavus* VLP and antisera to the *Penicillium stoloniferum*-f and -s (2), *P. chrysogenum* (15), or *P. brevi-compactum* (16).

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FIG. 1. Electron micrograph of negatively stained VLP from the NRRL A-12268 isolate of *A. flavus*, after sucrose density gradient electrophoresis.

viruses. Concentrated extracts from 11 additional cultures of *A. flavus* and *A. parasiticus* did not react with *A. flavus* VLP antiserum.

The VLP were subjected to CsCl equilibrium centrifugation by using an initial density of 1.25 g/cm^3 in 0.1 M, pH 8, potassium phosphate buffer. The 5-ml gradients were centrifuged at $145,000 \times g$ for 16 h in a Spinco SW 65 rotor at 5 C. The gradients were fractionated with an ISCO gradient fractionator, and the density of 0.25-ml samples was determined by refractometry (13). The buoyant density of the VLP in CsCl was 1.28 g/cm^3 .

In the Beckman model E analytical centrifuge using Schlieren optics, the particles sedimented as a single component at approximately 49S. The ultraviolet absorption spectrum of the particles (Fig. 2) determined in a Cary 15 spectrophotometer gave a maximum absorption at 278 nm and a minimum at 250 nm. Protein determinations (11) followed by diphenylamine and orcinol reactions (5) indicated that the VLP preparations contained no nucleic acid. The level of detection under the experimental conditions was 0.1% nucleic acid.

Freshly harvested and freeze-dried mycelial samples of isolate NRRL A-12268 were extracted in 0.01 to 0.2 M buffers at pH's ranging from 5 to 10. The extracts were analyzed by sucrose density gradient fractionation and examined by electron microscopy. VLP which sedimented faster than 49S, and therefore

FIG. 2. UV absorption spectrum of purified VLP preparation.

might possess encapsulated nucleic acid, were never observed.

Total nucleic acid extractions were made from mycelium of VLP-free and VLP-contain-

ing cultures with either double-phase phenol-SDS (9) or single-phase phenol-SDS (4) procedures. Nucleic acid preparations were fractionated by precipitation in 1 M NaCl at 4 C and CF-11 cellulose column chromatography (7). The fractions were then incubated at 37 C for 30 min with 10 μ g of pancreatic RNase (Schwarz/Mann BioResearch, Orangeburg, N. Y.) per ml of a pH 7.4 buffer containing 0.3 M NaCl, 0.001 M ethylenediamine tetraacetate, and 0.05 M Tris. Preparations prior to and after each fractionation step were analyzed by polyacrylamide gel electrophoresis according to Loening and Ingle (10) and by sucrose density gradient centrifugation (17). Electrophoresis was performed on 2.4% acrylamide gels at 6 mA/tube for 1 to 6 h, and the gels were scanned at 254 nm with a Joyce-Loebl chromoscan. Using these procedures, no double-stranded RNA or unusual single-stranded nucleic acids were detected in the NRRL A-12268 tissue.

Since VLP were found only in the NRRL A-12268 isolate of *A. flavus*, there appears to be no correlation between the presence of VLP and the absence of aflatoxin production. The unusual nature of the observed particles, however, indicates that these data are too superficial for a positive conclusion.

These are the first VLP of fungi in which nucleoprotein forms have not been identified. After exhaustive purification procedures and nucleic acid analysis, it is surmised with reasonable assurance that the genetic coding material is not double-stranded RNA. The data suggest that the VLP protein cistron may exist as nonencapsulated single-stranded nucleic acid similar in molecular weight to host nucleic acids, or it may reside in the host genome. Although the VLP is spore transmitted, attempts to determine if VLP production is inherited as a nuclear or cytoplasmic factor using heterokaryon procedures have not been successful (J. P. Adler and D. W. Mackenzie, unpublished data).

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