Purification and Serological Detection of Mushroom Viruslike Particles

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ABSTRACT

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A mixture of polyhedral viruslike particles (VLP's) purified from diseased mushroom tissue was separated during rate-zonal sucrose density gradient centrifugation into intact 25-nm particles, 34-nm capsids, and intact 34-nm particles. Other minor components also were observed in the same gradients and these purified products resisted treatment with chloroform and the detergent NP-40, but lacked known viral particle morphology. Antisera prepared against the different sized VLP's were low in titer and reacted with healthy sporophore extracts. Cross-absorbed antisera, however, had sufficient specificity and were used to detect VLP-containing mushrooms. The constant-feed micro double-diffusion technique provided the sensitivity to detect VLP's in clarified extracts of 50 g of tissue concentrated into 2 ml of buffer. The antisera whose preparation is described here and antisera to a 19 × 50-nm particle are currently being used experimentally for indexing of mushrooms.

Electron microscopy of virus-infected mushrooms [*Agaricus bisporus* Lange (Imbach)] collected from commercial mushroom farms in Pennsylvania has revealed three of the viruslike particles (VLP's) associated with LaFrance disease (4). In addition, it is known that the particles may occur in mushroom tissue singly or in combination (5, and J. W. Moyer and S. H. Smith, *unpublished*). The same particle types have been associated with LaFrance disease and transmitted in cell-free preparations (3, 4, 5). Here they are referred to as viruslike particles (LVP's) because transmission was obtained only by hyphal anastomosis between diseased and healthy mushroom isolates and not as purified particles. LaFrance disease constitutes a serious problem in the Pennsylvania mushroom industry, and the absence of a reliable assay restricts the development of an adequate control program. Sporophore symptoms and hyphal growth rate (3, 5) are currently the basis for diagnosis; thus, latent infections may remain undetected and serve as an inoculum reservoir.

Antiserum is available for serological detection of a 19 × 50-nm particle (8), and we now describe the development of antisera to the other two particles, thus establishing the capability of detecting all three VLP's known to occur in mushrooms in Pennsylvania.

A preliminary report of portions of this work appeared earlier (7).

MATERIALS AND METHODS

Sporophores were chosen as a source of VLP's using a diseased isolate of an *A. bisporus* strain collected from a commercial grower. A healthy isolate of the same strain was obtained from The Pennsylvania State University Mushroom Laboratory and used as the healthy control. The diseased isolate used showed typical sporophore symptomatology, mycelial growth rate on potato dextrose yeast agar (PDYA), and the presence of VLP's as determined by electron microscopy. The isolate contained the 25-nm, 34-nm, and the 19 × 50-nm particles which are associated with LaFrance disease (5). Both the healthy and virus-infected isolates were maintained on PDYA and were transferred every 3 wk until used for spawn production.

Spawning medium (225 g rye grain, 4 g $CaCO_3$, and 275 ml water) was mixed in Erlenmeyer flasks, sterilized by autoclaving, and cooled to room temperature before seeding it with the desired isolate. Healthy spawn was produced by transferring PDYA plugs colonized with healthy mycelium to the spawning medium and allowing 3-4 wk for colonization of the medium. Diseased spawn was made by transferring to spawning medium equal numbers of PDYA plugs of diseased and healthy mycelium of the same strain and allowing 3-4 wk for colonization. Wooden trays (0.6 m × 0.6 m × 0.14 m) were filled with 22.7 kg of pasteurized compost (wet weight) and 120 g of spawn. After colonization (14 days) the compost was overlaid with approximately 3.2 cm of

pasteurized top soil and placed in environmentally controlled rooms maintained at 21 C throughout sporophore production (11).

The two polyhedral particles were purified by a modification of previously reported methods (6, 7). Preliminary processing of tissue was the same as for purification of the 19 × 50-nm particles (8). The 10% polyethylene glycol 6000 (PEG) pellet which contained the polyhedral particles (6) was retained. The precipitate was resuspended overnight in 0.05 M potassium phosphate buffer pH 7.2 containing 0.1% 2-mercaptoethanol. This buffer, unless otherwise indicated, was used throughout the procedure. Insoluble material was removed by low-speed centrifugation (15,000 *g*, 15 min; Sorvall SS-34 rotor). The VLP's were further concentrated and purified by a second incubation of the suspension with 10% PEG for 1 hr. The pellet was collected by centrifugation (20,000 *g*, 20 min) and resuspended overnight in buffer. An equal volume of cold

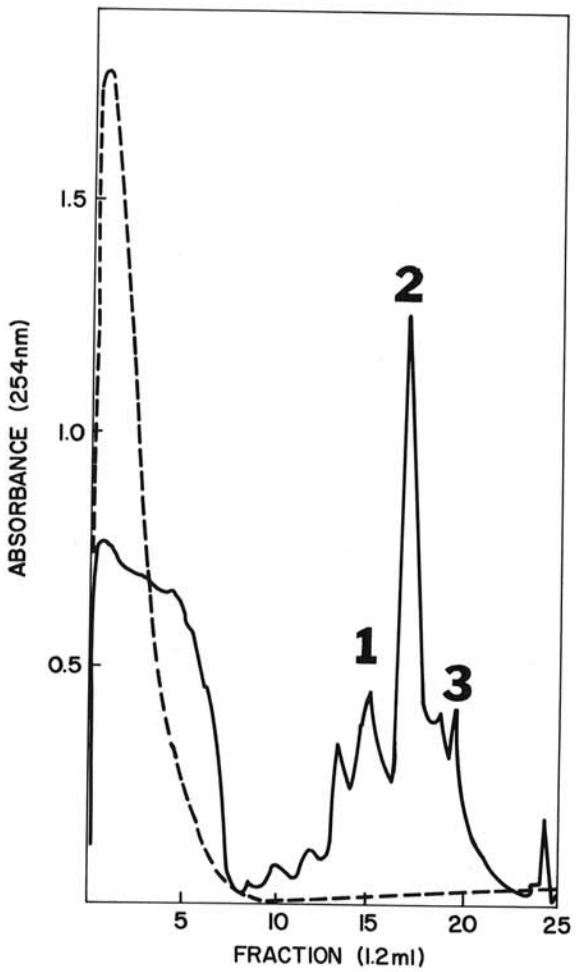


Fig. 1. Ultraviolet absorption profile of polyhedral viruslike particles from mushroom. Peaks 1, 2, and 3 contain 25-nm, 34-nm capsids, and 34-nm particles, respectively. The solid line represents the profile of the diseased preparations and the broken line represents the profile of the healthy preparations. Sedimentation is from left to right.

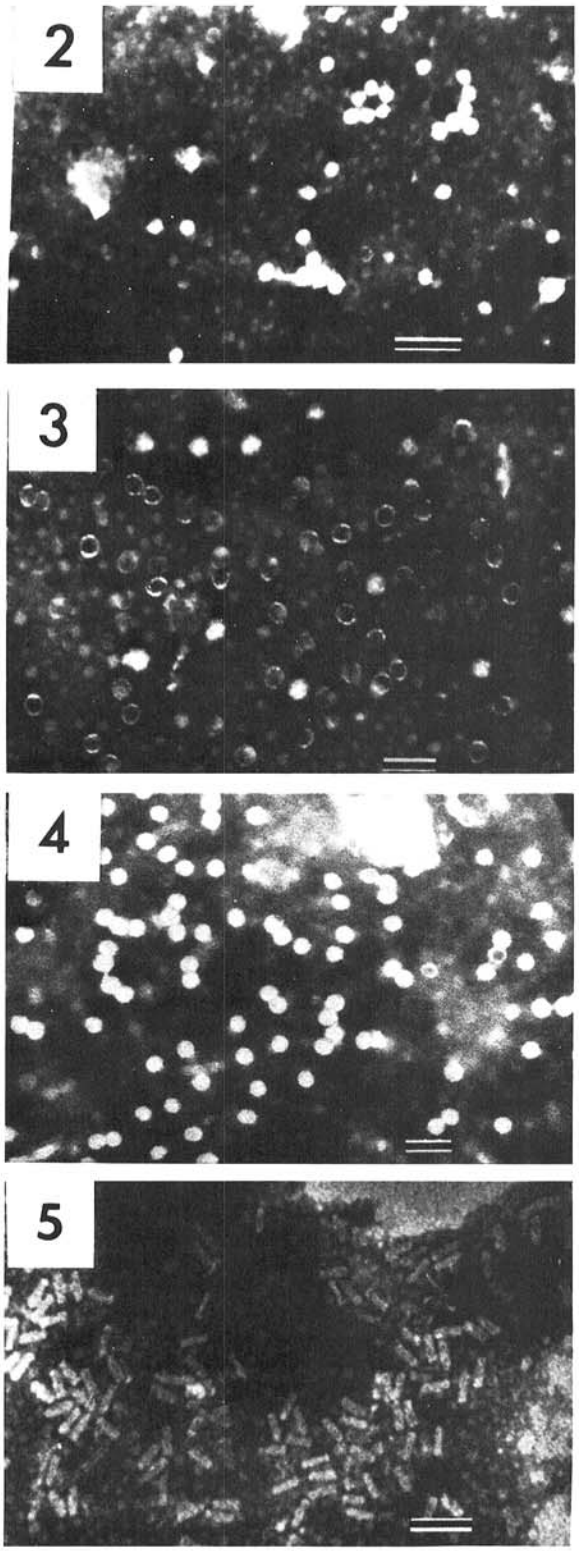


Fig. 2-5. Electron micrographs of purified mushroom virus-like particles negatively stained with 1% phosphotungstic acid pH 7.0. Bar equals 0.1 μm. **2)** Electron micrograph of 25-nm virus particles. **3)** Electron micrograph of 34-nm capsids. **4)** Electron micrograph of intact 34-nm particles. **5)** Electron micrograph of 19 × 50-nm particles.

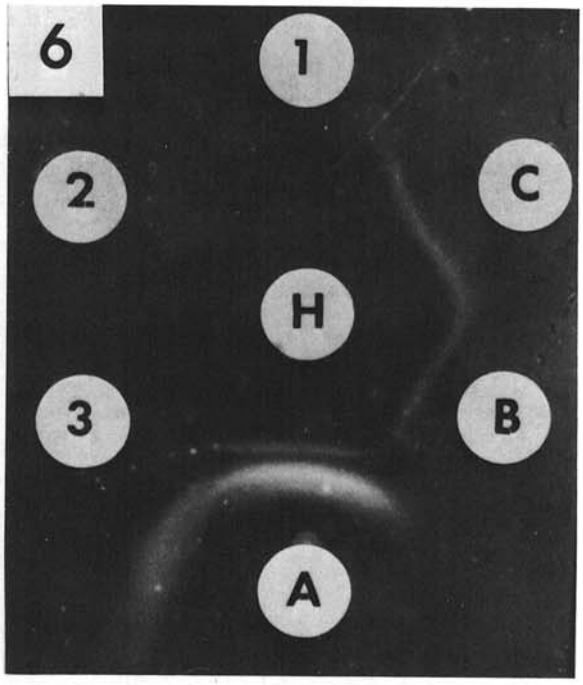
chloroform then was added to the suspension, stirred for 5 min and the aqueous phase was removed after centrifugation (10,000 *g*, 10 min). The remaining debris was removed by low-speed centrifugation. The final separation of VLP's from host material was by rate-zonal sucrose density gradient centrifugation. Approximately 20 optical density (O.D.) units (1.0 O.D. unit = absorbance of 1.0 at 260-nm, 1 cm path length) in 1 ml were layered on a 10-40% linear sucrose gradient and centrifuged for 5 hr at 90,000 *g* in an SW 25.1 rotor. The gradient columns were analyzed using an ISCO density gradient fractionator and ultraviolet absorbance monitor.

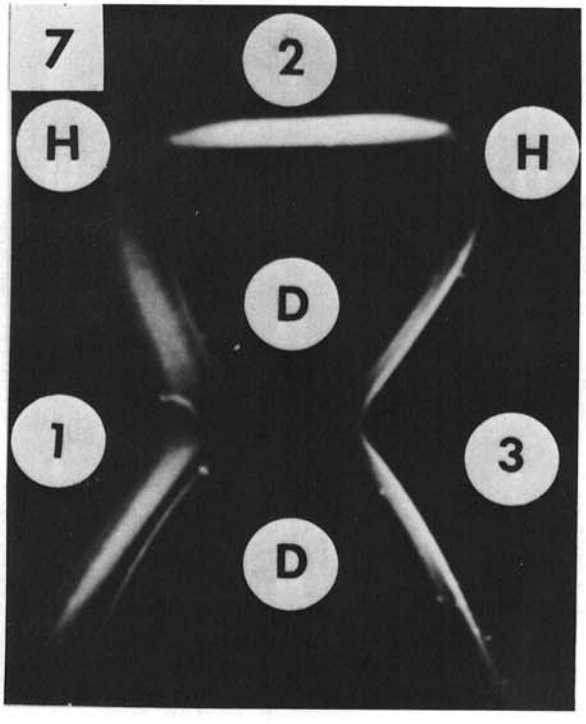
Samples from each ultraviolet-absorbing region were examined on Formvar-coated grids, negatively stained with 1% phosphotungstic acid, pH 7.0, using a Hitachi HU-11E electron microscope.

Fractions containing the 25-nm and 34-nm particles were dialyzed against 0.05 M phosphate buffer, pH 7.2, overnight and stored for antiserum production. Each preparation was combined with an equal volume of complete Freund's adjuvant and administered subcutaneously at weekly intervals for three consecutive weeks followed by a fourth intravenous injection. Serum was collected 1 wk after the final injection. Serological tests were performed by a constant-feed micro double-diffusion technique (2) using 0.75% agarose in 0.14 M NaCl and 0.02 M phosphate buffer at pH 7.2 containing 0.2% sodium azide. Reservoir ports were 1 mm in diameter and on 5 mm centers.

Tissue extracts used in serological assays of healthy and diseased sporophores were prepared as follows. Fifty grams of sporophores were homogenized in five volumes of buffer and centrifuged (15,000 *g*, 15 min). The precipitate was homogenized again in buffer and centrifuged. The resulting supernatant liquids were pooled and incubated for 1 hr with 10% PEG and 0.6 M NaCl to precipitate all three particle types. The precipitate was collected by centrifugation, resuspended in 2 ml of buffer and centrifuged again to remove remaining insoluble material.

The 19 × 50-nm particles (Fig. 5) were purified and antiserum was prepared as previously described (8).





RESULTS

Several ultraviolet-absorbing regions were observed following rate-zonal sucrose density gradient centrifugation of the partially purified preparations made from diseased tissue, but no corresponding regions were observed for healthy tissue in columns identically prepared (Fig. 1). Electron microscopic examination of samples from the gradient bands revealed the presence of 25-nm particles (Fig. 2), empty capsids (Fig. 3) of the 34-nm particles, and intact 34-nm particles (Fig. 4) in peaks 1, 2, and 3, respectively. Examination of samples from the

Fig. 6-7. 6). A constant-feed micro double-diffusion agar gel slide of the reaction of unabsorbed and absorbed mushroom viruslike particle (VLP) antisera with concentrated healthy mushroom extract (H). Positions 1, 2, and 3 correspond to absorbed antisera and A, B, and C correspond to position of unabsorbed antisera made against the 25-nm, 34-nm, and 19 × 50-nm particles, respectively. 7) A constant-feed micro double-diffusion slide of the reaction of mushroom VLP antisera absorbed with concentrated extracts of healthy sporophores with healthy and diseased sporophore extracts. Positions 1, 2, and 3 correspond to absorbed antisera made against the 25-nm, 34-nm, and the 19 × 50-nm particles, respectively. Legend: H indicates concentrated healthy mushroom extract and D indicates concentrated extract of diseased mushroom sporophores.

other peaks indicated the presence of unidentified amorphous material. Several attempts were made to remove this unidentified material. Increasing the time of chloroform treatment resulted in a reduced yield of VLP's. Also, incubation of partially purified preparations with increasing concentrations of a detergent (NP-40, Shell Chemical Co.) prior to rate-zonal centrifugation was unsuccessful. VLP preparations were incubated with NP-40 for 10 min prior to centrifugation using concentrations in the reaction mixture from 0.1% (v/v), however, this treatment also was detrimental to the virus particles.

All of the antisera to the different size classes of VLP's were of low titer ranging from 1/8 to 1/32 as measured by double-diffusion tests in petri plates (depots 5 mm in diameter on 10 mm centers). Initial tests with antisera to the 25-nm and 34-nm particles contained antibodies which reacted with extracts from healthy sporophores (Fig. 6). The host antibodies were absorbed from the antisera by incubating with an equal amount of healthy sporophore extract. When absorbed antisera to the VLP's were tested against concentrated extracts from healthy and infected sporophores, positive reactions were observed with infected tissue and there was no reaction with the healhy extract (Fig. 7).

Precipitin lines observed in preliminary tests performed in petri plates were diffuse. In addition, large amounts of tissue (100-200 g) were used to ensure detection of infected lots. To circumvent this problem, improve the sensitivity of this assay, and reduce the volume of antisera used in screening mushroom isolates, we used the constant-feed micro double-diffusion adaptation of Ouchterlony's procedure (9). Only one or two sporophores exhibiting severe symptoms of infection are necessary for detection. However, 50 g of tissue is used routinely owing to the variability of VLP titer we have observed.

DISCUSSION

The nature of the unidentified VLP related peaks observed in the absorption profile of the gradient (Fig. 1) remains obscure. However, their sensitivity to chloroform and NP-40 treatments suggest that they are similar to the VLP's. Further investigations may reveal a multicomponent system such as that found with VLP's of *Penicillium stoloniferum* (1) and *Aspergillus foetidus* (10).

Antisera against the three VLP's can be used to detect diseased mushroom isolates. The serological test is more specific than either examination with an electron microscope for VLP's or the comparative culturing technique of indexing. In addition, use of the mixed antiserum to the three particles ensures a reliable test for the mushroom VLP's known to occur in Pennsylvania.

The serological relationship of the three particles remains unclear and warrants further investigation. Preliminary studies demonstrated antigenic differences as well as similarities between the 19 × 50-nm particle and the two polyhedral particles (7). These differences may be sufficient to serve as an aid in associating particle types with specific symptoms. The antisera whose preparations are described here currently are being used experimentally for indexing mushrooms.

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