Assay for Maize Stripe Virus-Infected Plants by Using Antiserum Produced to a Purified Noncapsid Protein

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ABSTRACT

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The 16,300-dalton (16.3-kd) noncapsid protein found in maize stripe virus (MStpV) infected plants was purified by SDS-polyacrylimide gel electrophoresis and used as an immunogen to produce antiserum for indexing MStpV infected plants. The antiserum gave only weak reactions with MStpV infected plants by immunodiffusion or double-antibody sandwich enzyme-linked immunosorbent assays (ELISA), but reacted strongly with infected plants by indirect ELISA. Sap dilutions from infected plants gave strong reactions even when diluted to 10^{-7}, and purified 16.3-kd protein was detected at concentrations as low as 150 ng/ml. No significant cross reactions were obtained when tested against purified MStpV 32-kd capsid protein. There were no cross reactions with maize plants infected with other maize viruses. MStpV was detected in infected plant species other than *Zea mays*, but no positive reactions were obtained by testing MStpV viruliferous *Peregrinus maidis*.

Additional key word: SDS-PAGE.

Maize stripe (MStp) is a common disease of maize in south Florida and many other subtropical and tropical maize-producing areas of the world (1,4,6,8,12,14,16,17). The causal agent, maize stripe virus (MStpV), has been known to occur in south Florida since 1974 (17), and is a common component of a severe corn disease complex in south Florida (1,14). MStpV is transmitted by the planthopper, *Peregrinus maidis* Ashmead, in a persistent manner (5,18).

There are several, sometimes conflicting, reports concerning the properties of MStpV. Many differently named diseases with symptoms very similar to those of MStp have been reported (5,6,8,16). Recently, it has been demonstrated that large amounts of MStp-specific, noncapsid, viral protein are produced in plants infected with the Florida MStpV isolate, and that this protein could be isolated in a pure state (5). The protein was found to have a molecular weight of ~16,300 daltons (16.3-kd) and was interpreted to be a noncapsid protein that forms crystalline aggregates in infected plants (5).

In our studies of the maize disease complex in south Florida, it was desirable to develop a method whereby plant samples could be rapidly indexed for MStpV. Because the 16.3 kd non-capsid protein occurs in such large amounts in MStpV-infected plants (5), we tested its usefulness in serological assays for MStpV. We report here the use of the indirect enzyme-linked immunosorbent assay (ELISA) and the MStp 16.3-kd protein to detect MStpV-infected plants.

MATERIALS AND METHODS

Maintenance of *Peregrinus maidis* and MStpV. *P. maidis* were reared on maize plants (*Zea mays* L. 'Guardian') in a growth room at 24 C and 16 hr light (18). The MStpV isolate (17) was propagated by giving nonviruliferous adult *P. maidis* a 72-hr acquisition access period on MStpV-infected plants. Groups of planthoppers were then transferred to maize seedlings and held in growth chambers at 24 C and 16 hr of light for 10 days. Planthoppers were then transferred weekly to new maize seedlings. This was continued for 3–4 wk. After the removal of *P. maidis*, seedlings were sprayed with malathion and kept in the greenhouse for 3–4 wk.

Purification of MStpV 16.3-kd protein. The MStpV-associated 16.3-kd protein was concentrated essentially by using the differential pH method of Gingery et al (5). Five to 10 g of fresh tissue were ground in a mortar and pestle in two volumes of phosphate-citrate buffer, pH 5.0, prepared by mixing 0.2 M K_2HPO_4 and 0.1 M citric acid. The grindate was squeezed through cheesecloth and centrifuged in a Beckman JA-20 rotor at 9,000 rpm for 5 min. Pellets were resuspended in 5 ml of phosphate-citrate buffer, pH 7.0, and centrifuged for 30 min at 20,000 rpm. The supernatant was mixed with an equal volume of phosphate-citrate buffer, pH 3.0, and kept overnight at 4 C. The solution was then centrifuged at 10,000 rpm for 5 min. Proteins were resuspended in distilled water and kept at 4 C.

Purity of the 16.3-kd protein was monitored during the purification process by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by using the system of Laemmli (9). Slab gels of 12% acrylamide were 1.5 mm thick × 12.5 cm wide × 9.5 cm high and had a 1.5-cm 3% acrylamide stacking gel. Protein samples were prepared by reduction and alkylation by using the method of Lane (10), and were labeled with 2-methoxy-2,4-diphenyl-3-(2H)-furanone (MDPF) (obtained from W. E. Scott) in accord with the method of Lane and Cuppels (11). Electrophoresis was for 4.5 hr at 100 V. Proteins were visualized by exposing the gel to ultraviolet (UV) (302 nm) radiation and gels were photographed through a Wratten 9 filter and Polaroid Type 55 positive/negative film.

Equilibrium density gradient centrifugation in cesium chloride gradients with a mean density of 1.27 g/cc was used in attempts to purify the 16.3-kd protein. Gradients were centrifuged 18 hr at 36,000 rpm in the Beckman SW 50.1 rotor. The 16.3-kd protein band was removed, dialyzed against distilled water, and analyzed as described.

The 16.3-kd protein was also purified by using SDS-PAGE. Concentrated proteins prepared by differential pH treatment were incubated with dansyl chloride according to the method of Talbot and Yphantis (15), and separated on 3-mm-thick slab gels. After electrophoresis, proteins were visualized by exposing the gel to UV light (302 nm). The piece of the gel containing the 16.3-kd protein band was excised from the rest of the gel and the protein was extracted from it as previously described (3). In later experiments, proteins were electro-eluted from the gel pieces. The gel pieces were

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placed in dialysis tubing with a small volume of electrophoresis buffer (0.5–1.0 ml). The tubing was placed on a horizontal electrophoresis apparatus so that it was oriented perpendicular to the current. The gel piece was placed in the dialysis tubing so that it was next to the (−) side of the tubing and so that proteins could migrate into the buffer on the (+) side of the tubing. The dialysis bag was barely covered with electrophoresis buffer and then proteins were electro-eluted at 250 V for 2.5 hr. Proteins were collected by removing the buffer from the tubing and precipitating the proteins with 2.5 volumes of methanol containing 0.1 M ammonium acetate at −20 C for 2 hr. The proteins were pelleted by centrifugation at 13,000 *g* for 10 min and resuspended in distilled water. Protein concentrations were determined by using the Bio-Rad micro protein assay.

Purification of the MStpV 32,000-dalton (32-kd) capsid protein. The MStpV nucleoprotein was purified by using the method of Ginery et al (5). After two cycles of differential centrifugation the 32-kd protein was purified by SDS-PAGE electro-elution as for the 16.3-kd protein.

Serological assays for MStpV. Antisera to the 16.3-kd protein was produced by intramuscular injection of New Zealand White rabbits. Approximately 100 μg of SDS-PAGE-purified 16.3-kd protein in 1 ml of water was mixed with an equal volume of Freund's incomplete adjuvant and injected intramuscularly into the hind leg. Three weekly injections were made and bleedings began 2 wk after the last injection. Serum was separated and stored at −20 C.

Immunodiffusion tests were done in 0.6% Ionagar containing 0.02% sodium azide in distilled water, phosphate-citrate, pH 5.5, or phosphate-citrate, pH 7.0. Plates were incubated at room temperature and observed daily for 1 wk.

Double-antibody sandwich (DAS) ELISA tests were done according to the methods of Clark and Adams (2), and indirect ELISA was done according to those of Voller et al (19) and Koenig (7). Immunoglobulins (IgG) purified from antisera to the MStpV 16.3-kd protein and goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, IN 46514) were conjugated separately with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO 63178). DAS ELISA was done by coating polystyrene or Immulon II plates (Dynatech Laboratories, Inc., Alexandria, VA 22314) with purified IgG at 5 μg/ml in coating buffer (0.05 M sodium carbonate, pH 9.6) for 3 hr at 25 C, followed by washing with PBS-Tween (0.02 M phosphate, 0.15 M NaCl, 0.05% Tween-20, pH 7.4). Test antigens were prepared in PBS-Tween containing 2% polyvinylpyrrolidone-40 (PBST-PVP) and incubated in plates overnight at 6 C. Then *p*-nitrophenyl phosphate substrate, at 0.6 mg/ml in diethanolamine buffer (pH 9.8), was added to plates. The reaction was stopped after 0.5–2.0 hr and results were assessed photometrically at 405 nm. Indirect ELISA was performed by coating plates with test antigens in coating buffer overnight at 6 C. Plates were washed and purified anti-16.3-kd protein IgG (1 μg/ml) in PBS-Tween was added to the plates for 3 hr at 25 C. Plates were washed, and conjugated goat anti-rabbit IgG was added at 1/1,000 dilution in PBST-PVP for 2 hr at 25 C. Plates were washed and *p*-nitrophenyl phosphate (0.6 mg/ml) in diethanolamine buffer was added. Results were assessed after 0.5–2 hr.

RESULTS

Purification of the 16.3-kd protein. Approximately 1 mg of protein was obtained per gram of MStpV-infected fresh tissue by using the differential pH protein concentration method. SDS-PAGE analysis of this protein material revealed considerable contamination of the 16.3-kd protein by host proteins and the MStpV 32-kd capsid protein (Fig. 1). Antisera produced by injecting this initially concentrated preparation still reacted significantly with the MStpV capsid protein. It was, therefore, necessary to further purify the 16.3-kd protein before it could be used as an immunogen.

We were unable to obtain pure 16.3-kd protein by incubating the initial preparation at 3 C for 1 wk, during which time many of the contaminating proteins were precipitated, or by cesium chloride

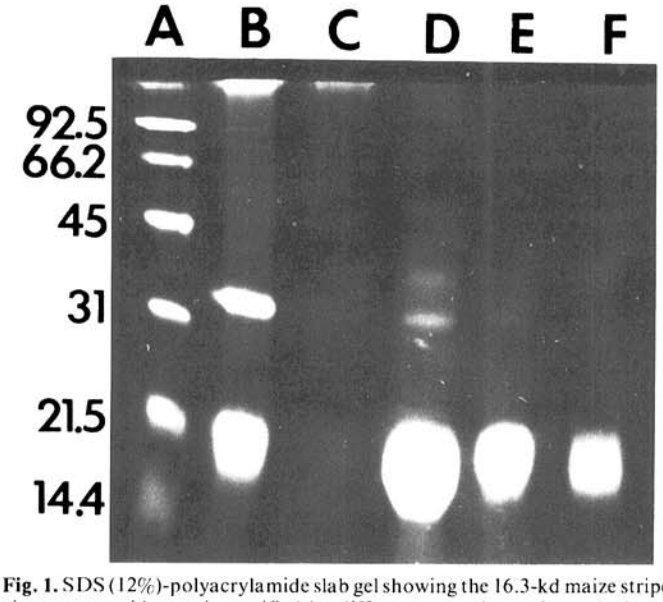

Fig. 1. SDS (12%)-polyacrylamide slab gel showing the 16.3-kd maize stripe virus noncapsid protein purified by different procedures. Lane A shows protein standards (from top to bottom: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme). Lane B is a mixture of the MStpV 32-kd dalton capsid and 16.3-kd noncapsid protein. Lane C and D show healthy and MStpV infected maize extracts, respectively, after differential pH treatment to concentrate the 16.3-kd protein. Lane E is the 16.3-kd protein fraction after equilibrium density gradient centrifugation in CsCl. Lane F shows the 16.3-kd protein extracted from SDS-gels.

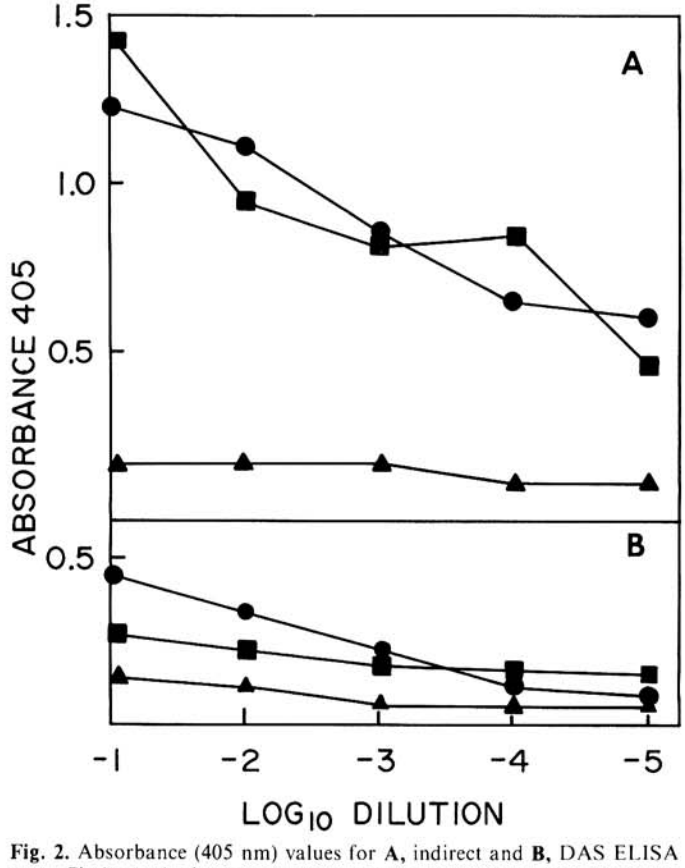

Fig. 2. Absorbance (405 nm) values for **A**, indirect and **B**, DAS ELISA tests. Circles and triangles represent sap dilutions of maize stripe virus-infected and healthy maize plants, respectively. Squares represent ten-fold dilutions of concentrated proteins from MStpV-infected plants prepared by differential pH treatment. The proteins were adjusted to an initial concentration of 400 μg/ml.

equilibrium density gradient centrifugation (Fig. 1). Minute amounts of the MStpV capsid protein were still detected in preparations from the CsCl gradients. Purified 16.3-kd protein was obtained by using the SDS-PAGE purification procedure. The SDS-PAGE-purified 16.3-kd protein was free of any MStpV capsid protein and therefore was suitable as an immunogen.

Serological assays for MStpV. The antiserum obtained from rabbits injected with the SDS-PAGE-purified 16.3-kd protein reacted only very weakly with sap from MStpV-infected plants by immunodiffusion tests. Therefore, it was necessary to increase the sensitivity of the serological test for detecting the MStpV 16.3-kd protein.

The indirect and double antibody sandwich (DAS) ELISA methods were compared for detecting MStpV-infected plants. The 16.3-kd antiserum immunoglobulins gave strong positive reactions with MStpV-infected plants by indirect ELISA tests, however, only weak reactions were obtained by DAS ELISA (Fig. 2). Similar results were obtained with differential pH concentrated protein preparations from MStpV-infected plants.

We wanted to test the sensitivity of the indirect ELISA method for detecting the MStpV 16.3-kd protein and to determine whether it reacted also with the MStpV 32-kd capsid protein. The purified preparations of the MStpV 32-kd and 16.3-kd proteins were tested for purity by SDS-PAGE before being used for indirect ELISA tests. Neither appeared to be contaminated with the other protein (Fig. 3).

When tenfold serial dilutions of the purified proteins, and healthy and infected plant saps were tested, no significant cross reactions were detected for the 32-kd protein, even at the highest concentration of 15 μg/ml (Fig. 4). At the same time, the 16.3-kd protein still reacted strongly at 150 ng/ml, but not at 15 ng/ml. Sap dilutions from MStpV-infected plants still reacted positively at a 10^{-7} dilution while no reaction was ever obtained with sap from healthy maize.

Because MStpV infects plant species other than maize and its insect vector, *P. maidis* (13), we tested whether the 16.3-kd protein could be detected in these other hosts by indirect ELISA. Strong reactions were obtained against MStpV from infected rye (*Secale cereale*, L.), itchgrass (*Rottboellia exaltata*), and oats (*Avena sativa* L.) which also is a new host for MStpV. No positive reactions were ever obtained with healthy controls. The 16.3-kd protein was not, however, detected in viruliferous *P. maidis*. In some experiments, high absorbance values were obtained from both viruliferous and nonviruliferous *P. maidis*, but generally both gave negative reactions. Also, in a single experiment, no 16.3-kd protein was detected from viruliferous *P. maidis* by differential pH concentration and analysis by SDS-PAGE.

DISCUSSION

These data show the usefulness of utilizing the MStpV 16.3-kd non-capsid protein for serological studies on MStpV. The 16.3-kd protein is produced in much higher amounts in infected plants than is the capsid protein (5), and is easier to purify than the MStpV capsid protein because sufficient amounts of immunogen can be obtained from 5–10 g of infected tissue. In addition, ultracentrifugation is not necessary as is required to purify the MStpV nucleoprotein. Gingery et al (5) were also able to purify the 16.3-kd protein by equilibrium density gradient centrifugation. However, in these studies absolute purification of the 16.3-kd protein was achieved only after SDS-PAGE and extraction of the protein from the gels.

Although the antiserum produced to the 16.3-kd protein was not suitable for immunodiffusion tests, it was suitable for ELISA. Much stronger reactions resulted using indirect ELISA than DAS ELISA. The lack of strong reactions by immunodiffusion tests and DAS ELISA is not necessarily a disadvantage, as it has been noted by others that antisera that do not work well in DAS ELISA may still be suitable for indirect ELISA (7). In our work, we could still detect the MStpV 16.3-kd protein in dilutions of plant sap at 10^{-7} suggesting that indirect ELISA is very sensitive for MStpV-infected plants.

Utilizing the antiserum to the MStpV 16.3-kd protein will serve two purposes. First, we have been able to use it for routine indexing of field collected maize plants by indirect ELISA. In the past 18 mo we have used this method for testing all plant species for MStpV. The method is suitable for fresh and frozen tissues and MStpV has been positively detected in species other than *Zea mays*. Second, it is not known whether the 16.3-kd protein is virus coded, or a host-coded, virus-induced protein. Aggregates of the 16.3-kd protein form needlelike inclusions in MStpV-infected maize plants (5), and we have obtained positive ELISA reactions with MStpV-infected plant species other than maize. We have never obtained positive indirect ELISA reactions with healthy plants or plants infected with other pathogens such as maize dwarf mosaic virus, strain B, maize rayado fino virus, maize mosaic virus, or corn stunt spiroplasma (*unpublished*). However, we were unable to detect the 16.3-kd protein in viruliferous *P. maidis*. MStpV has been shown to multiply in *P. maidis* (13), and if the 16.3-kd protein is virus coded it might be expected to be present in MStpV infected *P. maidis*.

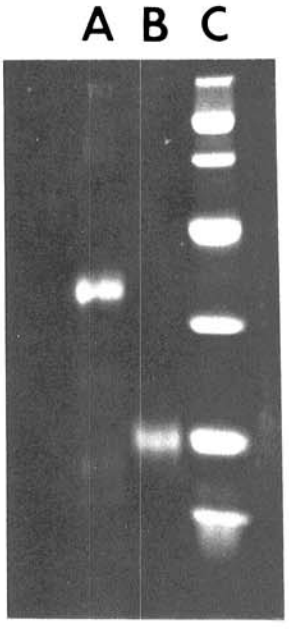

Fig. 3. SDS (12%)-polyacrylamide slab gel showing SDS-PAGE-purified MStpV 32-kd and 16.3-kd proteins. Lane A is the 32-kd protein. Lane B is the 16.3-kd and Lane C contains the protein standards (see Fig. 1).

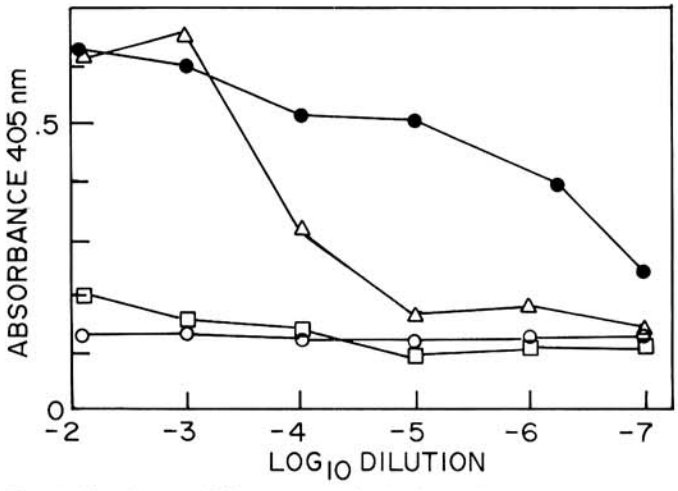

Fig. 4. Absorbance (405-nm) values for indirect ELISA tests using the 16.3-kd antiserum. Closed and open circles are sap dilutions of MStpV infected and healthy *Zea mays*, respectively. Triangles and squares are tenfold dilutions of SDS-PAGE-purified MStpV 16.3-kd and 32-kd proteins beginning at 15 μg/ml.

However, it may be in lower concentrations in *P. maidis* than in infected plants. Utilizing antiserum to the MStpV 16.3-kd protein will also be useful in attempting to answer this question.

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