Antiserum Preparation and Serodiagnosis of Pea Seed-Borne Mosaic Virus

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ABSTRACT

Two antisera, one using intact pea seed-borne mosaic virus (PSbMV) particles as the antigen, and one using D-protein from PSbMV particles, were prepared. These antisera, when used in the microprecipitin test, could be used to identify PSbMV in partially purified virus extracts. The identification of PSbMV in clarified sap from naturally infected pea plants was possible using the antiserum to intact PSbMV in the microprecipitin procedure. Attempts to utilize agar double-diffusion tests with either antiserum were unsuccessful.

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Additional key words: microprecipitin test, agar double-diffusion tests, flocculent precipitate.

The seed-borne nature of the pea seed-borne mosaic virus (PSbMV) makes it a potentially serious problem in pea (*Pisum sativum* L.) production (9). Early detection and identification by seed production specialists are imperative if uncontaminated seed stocks are to be properly maintained. The present system of identification relies on symptom development on a susceptible pea cultivar or the use of an indicator species such as *Chenopodium amaranticolor*. Such techniques have serious limitations of time, quantity, and accuracy. An improved system of identification would be very useful.

The use of antiserum for the serodiagnosis of plant viruses has found wide scale usage, particularly in the potato industry, for the diagnosis of potato virus X, potato virus S, and potato virus Y (11). This study describes methods used to develop an antiserum, specific to PSbMV, so that serodiagnosis and early detection of this virus may be facilitated.

MATERIALS AND METHODS.—*Virus preparations.*—The isolate of PSbMV (ATCC PV85) used in these studies was identified in Wisconsin during 1968 and has since been maintained by periodic transfer to the susceptible pea cultivar 'Dark Skin Perfection' (DSP). The PSbMV used for antiserum preparation was partially purified using the purification schedule previously described (10). Partially purified extracts were highly infectious to pea and contained high concentrations of virus particles.

Preparation of concentrated pea protein extracts.—Healthy DSP pea cultivar seedlings were homogenized in a Waring Blendor with chloroform and a pH 7.5 buffer of 0.5 M sodium borate in a ratio of 1 g fresh wt tissue: 1 ml chloroform: 1 ml buffer, plus 0.02 M 2-mercaptoethanol and 15% w/w Al_2O_3 computed on the basis of fresh tissue weight. The mixture was incubated 2 hr at 4 C and centrifuged 15 min at 3,000 rpm in an International Centrifuge Model UV (GSA rotor). Supernatant solutions were collected through a pad of glass wool and given a single cycle of high-speed ultracentrifugation (3 hr—30,000 rpm) using a Spinco Model L ultracentrifuge and a no. 30 rotor. Pellets were resuspended overnight in 0.05 M sodium borate buffer at pH 7.5 and centrifuged at low speed (15 min—8,000 rpm) using a Sorvall Superspeed Angle Centrifuge Type SS-IA. Supernatant fractions from the final centrifugation were used for sera absorption.

Preparation of clarified sap from infected pea plants.—Pea plants showing vein-clearing and tendril-curl symptoms were triturated with mortar and pestle in 0.05 M Tris-HCl saline buffer, pH 7.5 (1

g tissue/1 ml buffer). The sap was strained through a single layer of cheesecloth and centrifuged twice at 8,000 rpm for 10 min. Final supernatant solutions were free of materials that interfered with reading microprecipitin reactions. Failure to use buffered saline as the diluent in trituration resulted in nonspecific precipitates upon incubation at 24 C in the microprecipitin reaction. Use of phosphate buffer also was unsatisfactory, because of apparently high Ca^{++} ion concentrations in pea sap resulting in copious calcium phosphate precipitates.

Preparation of antisera to intact PSbMV.—Initial efforts at antiserum preparation were directed toward removing contaminating host material from partially purified virus extracts. Gold (2) reported that the use of antihost serum improved the purification of tobacco necrosis virus. Antihost antibodies were used to remove host contaminants from virus extracts. We produced antihost antisera by using pea protein partially purified by Fulton's (1) method of purification from healthy peas 3 wk after planting. New Zealand white rabbits were injected with protein preparations and resulting sera were processed and frozen until use.

Other attempts to produce antisera to intact PSbMV were made by using partially purified virus extracts untreated with antihost antiserum as the antigen. The injection schedule of Shepard & Grogan (5) for antiserum preparation against western celery mosaic virus was used. Bleedings were initiated 10 days after the final injection and were made at 1-week intervals for a 6-week period. Booster intramuscular injections of partially purified virus, emulsified in Freund's incomplete adjuvant [1 ml virus (OD_{260} of 40)/1 ml adjuvant], were given on alternate weeks 1 day after bleeding. Sera were collected and absorbed with healthy pea protein supernatant fractions by adding 0.5 ml protein preparation to 2 ml of serum. The mixture was incubated at 24 C for 2 hr, overnight at 4 C, and centrifuged at 8,000 rpm for 10 min. This process was generally repeated twice. Resulting sera were tested in the microprecipitin test with concentrated healthy pea protein preparations. Absence of precipitates in this test was a prerequisite to further processing of sera. Absorbed sera were then dialyzed against several changes of 0.005 M Tris-HCl saline buffer, pH 7.5, to remove traces of borate ions which gave spurious reactions in microprecipitin tests. Sodium azide (0.01 M) was added as a preservative. Sera were diluted with an equal volume of glycerine and stored at −10 C.

Preparation of an antiserum to D-protein of PSbMV.—The recent success of Shepard and coworkers (4, 7, 8) in the preparation of antisera against D-protein of several potato viruses that were usable in double diffusion and radial diffusion tests in agar, prompted us to use this approach using the D-protein of PSbMV. Partially purified extracts of PSbMV were diluted to an OD_{260} of 20. Pyridine was added to a final concentration of 30%. The preparation, which immediately lost its opalescence, was incubated 15 min at 24 C and dialyzed overnight against several changes of 0.05 M borate buffer, pH 8.2, containing 0.37% formaldehyde at 4 C. D-protein concentration was determined using the Folin phenol method. Antigens consisting of 2.7-4.0 mg D-protein/ml emulsified with Freund's incomplete adjuvant (1.8 ml protein/1.8 ml adjuvant) were injected intramuscularly at weekly intervals. Bleedings were initiated after the fourth week of injections, and continued weekly for 1 month. Booster intramuscular injections were given the day after bleeding. Sera were collected and treated in a manner similar to the treatment of the antiserum specific to intact PSbMV except that the protein preparations were treated with 30% pyridine and dialyzed against 0.05 M borate buffer, pH 7.5, containing 0.37% formaldehyde for 24 hr prior to use. Sera absorbed with this preparation were dialyzed against several changes of 0.005 M Tris-HCl saline buffer, pH 7.5 before dilution with an equal volume of glycerine, and storage at −10 C.

Microprecipitin and agar double-diffusion tests.—Microprecipitin reactions were done in plastic petri dishes under mineral oil and incubated at 24 C.

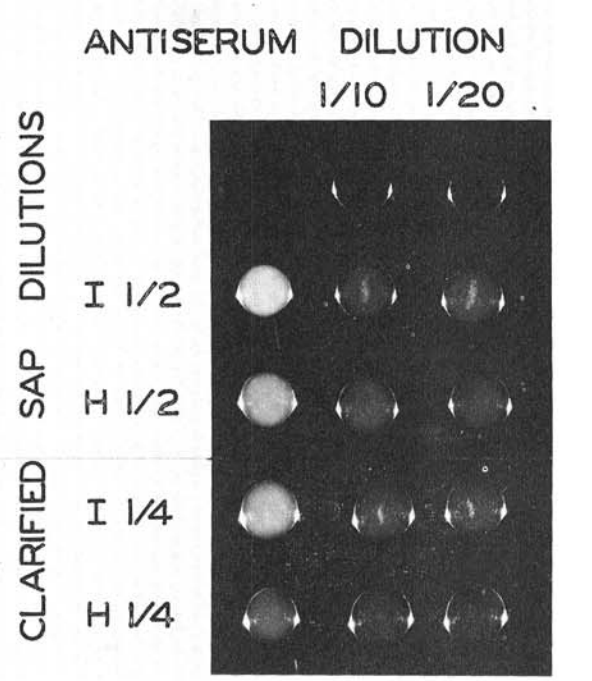

Fig. 1. Microprecipitin test using sap (I = sap from pea seed-borne mosaic virus infected plant; H = sap from healthy plant) clarified by two low-speed centrifugations. All dilutions were made in 0.05 M Tris-HCl saline buffer, pH 7.5. Test was incubated for 6 hr at 24 C and overnight at 4 C. The test was set up in plastic petri dishes and covered with mineral oil. Each dish was tapped lightly prior to viewing to insure maximum visibility of flocculent-type precipitate. The clear drops at the top included two dilutions of antiserum alone, and the column of cloudy drops on the left represents dilutions of clarified sap without antiserum added.

Readings were taken after 4-6 hr and again after 16-24 hr. Antigen and antiserum dilutions were made in 0.05 M Tris-HCl saline buffer at pH 7.5.

Agar double-diffusion tests were made in plastic petri dishes using 0.5 or 0.8% Oxoid Ionagar (no. 2) or Difco agar dissolved either in saline (0.85% NaCl) alone, or 0.05 M Tris-HCl saline buffer, pH 7.5.

RESULTS.—Addition of antihost antiserum to partially purified extracts of PSbMV resulted in large aggregates of virus that could not be separated from precipitated host material. Sufficient virus material did not remain in supernatant fractions after low speed centrifugation of antiserum-virus preparation mixtures to elicit a measurable antibody response when injected into an unsensitized rabbit.

Antiserum to partially purified virus preparations untreated with antihost antiserum was tested against partially purified PSbMV preparations in the microprecipitin test, and had a titer of greater than 1:8,000. There was no visible reaction against partially purified healthy protein preparations. Precipitates were of the flocculent or H type. This antiserum was also tested against clarified sap from infected pea plants. Dilutions of antiserum at 1/10 or 1/20 and infected sap dilutions of 1/2 or 1/4 in 0.05 M Tris-HCl saline buffer, pH 7.5, gave easily visible flocculent precipitates within 4 hr after the test was initiated (Fig. 1). To avoid nonspecific precipitates which frequently appeared after prolonged incubation at 24 C in spite of the Tris-HCl saline buffer, microprecipitin tests using clarified sap were incubated at 24 C for 6-8 hr followed by overnight incubation at 4 C.

Several attempts were made to use this latter antiserum in agar double-diffusion tests. Both partially purified PSbMV preparations and infected sap were treated with a number of degrading agents prior to testing against this antiserum. The degrading agents, as described by Shepard (4) and Shepard & Grogan (6), included 1 and 2% sodium dibutylnaphthalenesulfonate (Leonil SA); 0.3 M ethanolamine, pH 10.5; 30% pyridine; and 2.5% pyrrolidine. Also tested, was the incorporation of either 0.1, 0.5, 1.0% SDS or 0.1, 0.5, 1.0% Leonil SA within the agar medium according to Gooding & Bing (3). In all tests, regardless of antiserum dilution, antigen dilution, time of antiserum addition, agar concentration, or virus degrading agent, no bands were detected.

The PSbMV D-protein antiserum, when tested against dialyzed, pyridine-degraded, PSbMV preparations in the microprecipitin test, had a titer greater than 1:512. When used in agar double diffusion tests with partially purified virus and virus-infected sap treated with the degrading agents listed above, bands were again not detected. As before, variation in the concentration of antiserum, antigen, or agar did not result in band formation.

DISCUSSION.—The addition of serum to partially purified preparations of PSbMV resulted in a serious aggregation problem. The high degree of aggregation presumably covered sufficient antigenic sites to prevent the development of a high titer antiserum.

The absorption of antiserum to PSbMV and host contaminants with concentrated host protein was a relatively fast and easy method of processing serum. The resulting high-titer antiserum specific to intact PSbMV was successfully adapted for use in large scale screening of infected plants provided that infected sap was adequately clarified and diluted in Tris-HCl saline buffer. The flocculent precipitates in the microprecipitin tests were easy to read and appeared within a few hours after the tests were initiated.

The reason for the failure to detect bands in agar double-diffusion tests was presumably due to the failure of the virus antigen to migrate in agar. Attempts to permit diffusion by reducing the size of the migrating antigen with degrading agents, by varying the concentration of the agar medium, or by varying the concentrations of antigen and antibody were unsuccessful.

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