Detection of Four Isometric Plant Viruses in Sodium Dodecyl Sulfate Immunodiffusion Tests

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ABSTRACT

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Antisera were obtained by immunizing rabbits with purified, freeze-dried sodium dodecyl sulfate (SDS)-treated preparations of cowpea mosaic, cucumber mosaic, southern bean mosaic, and squash mosaic (SqMV) viruses. Each antiserum reacted homologously in SDS-immunodiffusion tests against SDS-treated extracts of infected leaves that were heated in a boiling water bath for 4 min prior to use. None of the antisera to the isometric viruses cross-reacted in reciprocal tests. An antiserum to untreated SqMV reacted specifically in SDS-immunodiffusion tests with either aqueous extracts or SDS-treated, nonheated sap, but did not react with heated, SDS-treated extracts. The results provided additional evidence that SDS-immunodiffusion tests are feasible for diagnosis of certain spherical viruses under conditions in which filamentous plant viruses also can readily be detected.

Additional key words: comovirus, cucumovirus, potexvirus, potyvirus, serology, watermelon mosaic virus.

Many filamentous plant viruses diffuse poorly in the agar media used for Ouchterlony double diffusion serological tests (17). To circumvent this problem, sodium dodecyl sulfate (SDS), an anionic detergent, has been used to disrupt tobamoviruses (3), potexviruses (1,3,12,13), carlaviruses (18), potyviruses (1,6,8,12–14), a closterovirus (5), and potyvirus-induced inclusion bodies (1,12) into readily diffusible, antigenic components. In contrast to filamentous viruses, small, isometric plant viruses ordinarily diffuse readily in agar gels without special treatment. Consequently, there has been little incentive to test the efficacy of SDS-immunodiffusion methods with the isometric viruses, although southern bean mosaic virus (1,12) and cowpea mosaic virus (8) have been reported to be detectable by these procedures.

Frequently it is useful to use immunodiffusion tests to identify unknown viruses in infected field samples. Thus, it would be advantageous to have a system that can detect and identify a wide variety of viruses. For this reason, we investigated more fully the potential of SDS-immunodiffusion tests for detecting isometric plant viruses. Isometric viruses have varying degrees of susceptibility to SDS dissociation (2,16). The susceptibility of a virus to SDS would influence the strategy for developing SDS-immunodiffusion procedures for that virus. Using squash mosaic and southern bean mosaic viruses as examples, we have demonstrated that both the treatment of immunogen and of test antigen can influence the results significantly. The methods presented have been used to detect cowpea mosaic, cucumber mosaic, squash mosaic, and southern bean mosaic viruses in crude extracts from infected plants, and for distinguishing these viruses from one another and from several rod-shaped viruses.

MATERIALS AND METHODS

Virus isolates. The American Type Culture Collection (ATCC) isolate PV-36 of squash mosaic virus (SqMV) was the immunogen used for most of the serological tests. Two SqMV isolates obtained from cantaloupe in Florida and a subgroup II isolate (10) obtained from R. Provvidenti also were used as test antigens.

An isolate of cucumber mosaic virus (CMV) obtained from G. Milbrath was used as immunogen. Price's strain 6 of CMV (ATCC isolate PV-30) and the Milbrath isolate were used as test antigens.

A cowpea mosaic virus (CpMV) isolate (the 'Sb' isolate from George Bruening) obtained from R. J. Shepherd was used as the immunogen (8). Another isolate of CpMV-Sb provided by M. McLaughlin and the Bruening isolate were used as test antigens.

A southern bean mosaic virus (SBMV) isolate obtained from J. G. McDonald was used as both immunogen and test antigen.

For various serological tests, the following virus isolates also were used: clover yellow mosaic (ClYMV) (13); the T-4 isolate of citrus tristeza (CTV) (5); white clover mosaic (WClMV) (O. W. Barnett isolate); and watermelon mosaic (WMV), Florida isolates 1 and 2 (14).

Virus purification and antiserum production. In all cases, initial immunizations consisted of purified virus (untreated or treated, as specified for each virus) emulsified with an equal volume of Freund's complete adjuvant. For subsequent injections, incomplete adjuvant was always used.

The SqMV was propagated in squash (*Cucurbita pepo* L. 'Early Prolific Straightneck' or 'Zucchini') and purified by a slight modification of the method described by Nelson and Knuhtsen (10). Tissue was homogenized in two volumes of 0.2 M sodium acetate, pH 5.0. The homogenate was adjusted to pH 5.0 with 10% acetic acid and stirred for 2 hr at 4 C. The homogenate was centrifuged at 16,000 *g* for 10 min, and virus was precipitated from the supernatant by the addition of polyethylene glycol (Carbowax 6000) to a final concentration of 8%. The precipitated virus was concentrated by slow-speed centrifugation, the pellets were resuspended in 0.1 M potassium phosphate, pH 7.0, and the solution was again clarified by centrifugation at 27,000 *g* for 10 min. The virus was subjected to sucrose density gradient centrifugation on linear gradients (10) for 4 hr at 21,000 rpm in a Beckman 25.1 rotor. The bottom nucleoprotein component (9) was removed and reconcentrated by centrifugation at 86,000 *g* for 3 hr. The resultant pellets were resuspended in water, and a portion was stored at 4 C. The remainder was freeze-dried prior to storage at 4 C. One rabbit was injected intramuscularly with 2 mg of nonfrozen

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bottom component, followed by an injection of 4 mg 4 wks later. A different rabbit was injected with 2 mg of freeze-dried bottom component. The sample was suspended in 1 ml of 1% sodium dodecyl sulfate (SDS) and 20 μl of 2-mercaptoethanol (2-ME), heated in a boiling waterbath for 4 min, cooled to room temperature, emulsified with adjuvant, and injected intramuscularly. This rabbit was reinjected 4 wks later with 2 mg. The antiserum collected from the rabbit injected with untreated bottom component and from the rabbit injected with SDS-treated bottom component are hereafter referred to as SqMV antiserum and SqMV-DT antiserum, respectively. The SqMV antiserum used herein represents pooled samples collected 5, 8, 13, 16, and 24 wk after the first injection. The SqMV-DT antiserum consisted of pooled samples collected on the same dates.

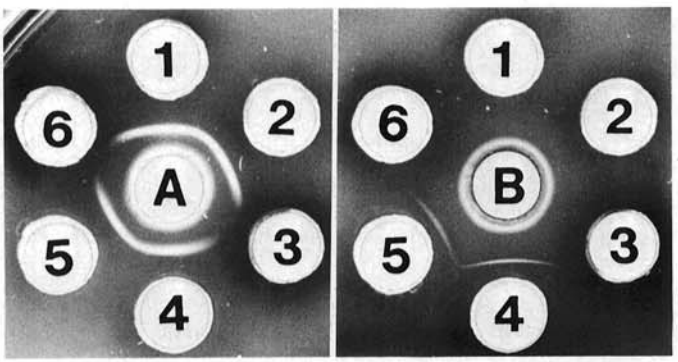

Fig. 1. Effects of antigen preparation and antiserum type on the reactivities of squash mosaic virus (SqMV) isolates in the SDS-double diffusion tests. The center wells contained antiserum to: well A— detergent-treated SqMV, and well B— antiserum to untreated SqMV (B). The peripheral wells all contained SDS-treated extracts from squash plants: well 1—heated extracts from SqMV (PV-36)-infected leaves; well 2—heated extracts from SqMV (Group II)-infected leaves; well 3—heated extracts from healthy leaves; wells 4 and 5—unheated extracts from SqMV (PV-36) and SqMV (Group II)-infected leaves, respectively; and well 6—unheated extracts from healthy leaves.

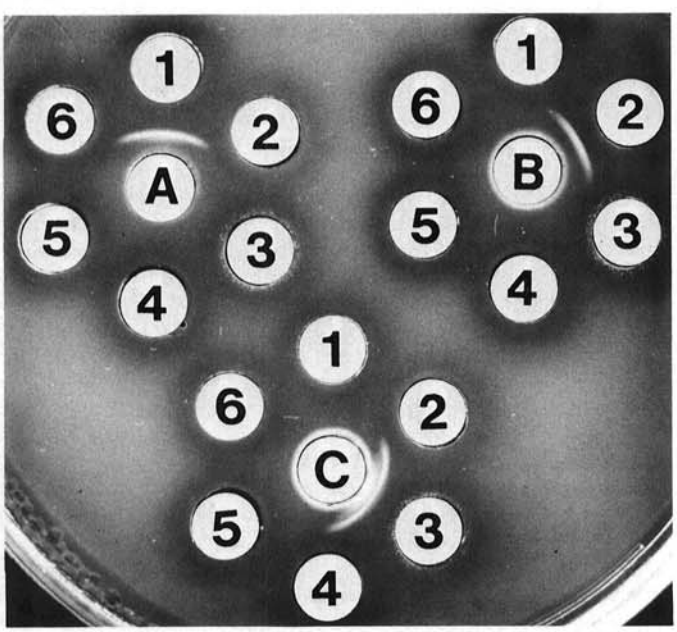

Fig. 2. Use of SDS-immunodiffusion to distinguish three isometric viruses. The center wells contained antisera to SDS-treated virus: well A—southern bean mosaic; well B—squash mosaic; and well C—cowpea mosaic. Heated, SDS-treated extracts from leaves were added to the peripheral wells of each pattern: well 1—southern bean mosaic virus in bean; well 2—squash mosaic virus in squash; well 3—cowpea mosaic virus in cowpea; well 4—healthy bean; well 5—healthy cowpea; and well 6—healthy squash.

Southern bean mosaic virus (SBMV) was purified from bean (*Phaseolus vulgaris* L. 'Bountiful') by using a modification of the chloroform-butanol clarification procedure of Batchelor (1). Following clarification of the homogenate by low-speed centrifugation and concentration of the virus by precipitation with polyethylene glycol (10%), the virus was purified by a cycle of differential centrifugation followed by equilibrium centrifugation in CsCl. The virus recovered from CsCl gradients was concentrated by high-speed centrifugation and the final pellets were resuspended in water. The preparation was divided among several vials, some of which were stored at 4 C. The others were freeze-dried prior to storage at 4 C. Antisera to SDS-treated SBMV (SBMV-DT antiserum) and untreated SBMV (SBMV antiserum) were prepared as described for SqMV except that the interval between the injections was 9 wk, and that 2 mg were used for all injections.

The CMV isolate was purified from infected leaves of *Nicotiana* × *edwardsonii* Christie and D. W. Hall by clarification of citrate-buffered sap with chloroform-butanol, followed by polyethylene-glycol precipitation and equilibrium centrifugation in Cs_2SO_4 (E. Hiebert, *unpublished*). Two milligrams of freeze-dried virus was resuspended in 1 ml of 0.15% SDS in 0.025 M Tris-HCl, pH 7.2, at room temperature. After a few minutes, the sample was emulsified with adjuvant and injected into a toe pad (21) and the thigh muscle of a rabbit. A second, similar injection was administered after 1 mo. The antiserum (hereafter designated CMV-DT) used in this study was collected 3 mo after the first injection.

The CpMV-DT antiserum used in this study was prepared as previously described (8), by using boiled, SDS- and 2-ME-treated virus as immunogen.

The WClMV immunogen consisted of 2-mg samples of lyophilized virus that were suspended in 1 ml of 0.06% SDS and heated to 50 C for 5 min. The rabbit was injected three times, at 2-wk intervals, in the toe pads and thighs. Sera were collected from 3–4 mo after the first injection. The antisera to CTV protein (5), to ClYMV protein (1,13), and to WMV-1 and WMV-2 (14), were prepared as described in the references indicated.

Serological tests. The SDS-immunodiffusion tests (12) were conducted in a medium consisting of 0.8% Noble agar (Difco), 1.0% sodium azide (Sigma), and 0.5% SDS (Sigma) (SDS-medium). Unless stated otherwise, test antigens were prepared by grinding tissues in water (1 ml/g tissue), followed by addition of 1 ml of 3% SDS per gram of tissue. The triturate was squeezed through cheesecloth and placed in tubes which were incubated in a boiling water bath for 4 min. In certain tests, the SDS-treated antigens were not heated, or sometimes the tissue extracts were prepared in water (not heated). Except for CTV, freeze-dried antigens were prepared in SDS as described previously (13). They were resuspended and heated in a boiling water bath for 4 min just prior to use. Freeze-dried extracts from citrus tissues infected with CTV (5) were suspended in 0.5% SDS for testing.

Antiserum titers were determined with antisera that were diluted in normal serum (12).

To compare the sensitivities of SDS-immunodiffusion tests with conventional immunodiffusion tests for detecting SqMV, the following tests were performed. Extracts from infected tissue were prepared in SDS and diluted 1/20, 1/40, 1/80, 1/160, 1/320, and 1/640 in SDS-treated sap from healthy squash leaves. These samples were then boiled and tested against SqMV-DT antiserum (undiluted and twofold dilutions to 1/16) in the SDS medium. For comparison, extracts from infected tissues were prepared in water, diluted with water-treated extracts from healthy tissue, and tested against SqMV antiserum (at dilutions ranging from 1/32 to 1/4,096) in a medium consisting of 0.7% Noble agar, 0.85% NaCl, 0.03% NaN_3, and 0.05 M tris-HCl, pH 7.2 (non-SDS medium). The sensitivities of the two systems for detecting known amounts of SqMV also were determined. Purified virus was added to SDS-treated sap from healthy plants to give final virus concentrations of 10 μg/ml, 1 μg/ml, 0.1 μg/ml, and 0.01 μg/ml; the samples were heated and tested against dilutions of SqMV-DT antiserum in the SDS-medium. Purified virus also was prepared at identical dilutions in water extracts from healthy leaves and tested against SqMV antiserum in the non-SDS medium.

RESULTS

Specificity and reactivity of antisera. The results with SqMV were affected markedly by the type of serum used and by certain means of antigen preparation. When tested against heated, SDS-treated leaf extracts, the SqMV-DT antiserum gave strong precipitin lines which were slightly curved around the antiserum wells (Figs. 1 and 2). The reactions were weaker either with unheated, SDS-treated extracts (Fig. 1), or with extracts prepared in water (heated, or unheated). The SqMV-DT antiserum had a titer of 1/16 against heated, SDS-treated antigens in crude sap. The SqMV-DT antiserum did not react with sap from healthy plants (Fig. 1).

The antiserum to untreated SqMV reacted either with unheated, SDS-treated extracts (Fig. 1) or with unheated extracts prepared in water to give precipitin bands which were arced around the antigen wells. Sharp precipitin lines resulted when leaf extracts prepared in water (1 g of tissue to 2 ml of water) were tested against a 1/32 dilution of the SqMV antiserum. Against aqueous extracts, the serum titer was 1/2,048. The SqMV antiserum failed to react with boiled, SDS-treated extracts from SqMV-infected plants or with extracts from uninfected squash plants (Fig. 1).

The non-SDS system was more sensitive for detecting SqMV antigen than the SDS system. Purified virus diluted in water extracts from healthy leaves was detected at 1 μg/ml but not at 0.1 μg/ml, whereas purified virus diluted in SDS-treated extracts from healthy leaves was detected at 10 μg/ml but not at 1 μg/ml. When extracts from infected plants were diluted with healthy sap, a dilution of 1/160 gave positive results in the non-SDS system, whereas virus was detected at a dilution of 1/80, but not 1/160, in the SDS system.

The SqMV-DT antiserum reacted specifically with sap from SqMV-infected cantaloupe (*Cucumis melo* L. 'Hales Best Jumbo'), pumpkin (*Cucurbita pepo* L. 'Small Sugar'), and squash (*C. pepo* 'Zucchini' and 'Early Prolific Straightneck') leaves. This serum also reacted with extracts from infected squash roots and corollas, and with freeze-dried extracts from infected cantaloupe or squash leaves. The SqMV ATCC PV-36 and Arizona Type II isolates gave reactions of serological identity (no spur formation) against the SqMV-DT antiserum (Fig. 1). Two viruses isolated from naturally infected cantaloupe in Florida were identified as SqMV by their reaction with both the SqMV-DT and SqMV antisera. No cross-reactions were observed when SqMV and SqMV-DT antisera were tested reciprocally with CMV, WMV-1, WMV-2, CpMV (Fig. 2), CTV, or SBMV (Fig. 2).

Pyrrolidine-treated extracts (17) from SqMV-infected leaves reacted specifically with the SqMV-DT antiserum in media consisting of 0.7% Ionagar 2, 0.85% NaCl, 0.03% NaN_3, and 0.05 M tris-HCl, pH 7.2.

The CpMV-DT antiserum had a titer of 1/8 against heated, SDS-treated antigens from CpMV-infected cowpea (*Vigna unguiculata* (L.) Walp. 'Knuckle Purple Hull') leaves. Undiluted antiserum reacted strongly with CpMV-infected cowpea (Fig. 2) or with freshly prepared (Fig. 3) or lyophilized extracts from *Nicotiana benthamiana* Domin, but not with extracts from noninfected plants (Figs. 2 and 3). No cross-reactivity was observed in reciprocal tests between CpMV and SBMV (Fig. 2), SqMV (Fig. 2), CMV (Fig. 3), WMV-2 (Fig. 3), ClYMV (Fig. 3), or WClMV.

The CMV-DT antiserum (titer = 1/8) reacted specifically with extracts from leaves of CMV-infected *N. benthamiana*, *N. edwardsonii*, *Capsicum annuum* L., *Cucumis sativus* L., and *C. pepo*. Freeze-dried extracts from *N. edwardsonii* also gave positive reactions. Reactions of serological identity resulted when the Milne isolate was compared directly to Price's yellow strain or to ATCC PV-30. Although heating (in SDS) was not necessary to achieve satisfactory results with CMV antigens in crude extracts, the extracts usually were heated when making direct comparisons with other viruses. No cross-reactions were obtained between CMV and CpMV, WMV-2, ClYMV (all shown in Fig. 3), SBMV, SqMV, and WMV-1.

Heated, SDS-treated extracts from SBMV-infected bean leaves reacted strongly with the SBMV-DT antiserum, which had a titer of 1/16. Extracts from SBMV-infected bean roots and resuspended, freeze-dried extracts from SBMV-infected leaves also were positive. No precipitin bands were observed when the SBMV-DT antiserum was tested against sap from healthy bean leaves or roots. Reciprocal tests between SBMV and CMV, CpMV (Fig. 2), SqMV (Fig. 2), and WClMV gave negative results. Collections of the antiserum to untreated SBMV taken from 3 wk to 4 mo after immunization reacted with unheated sap extracts to give a precipitin band arced around the antigen well (as shown in Fig. 1 of Ref. 12). Heated, SDS-treated extracts from SBMV-infected leaves did not react with the antiserum to untreated SBMV collected 3–6 wk after immunization; some later bleedings of that serum showed a weak reaction.

Detection of isometric viruses in mixed infections. The feasibility of using SDS-immunodiffusion tests to detect specific viruses in doubly-infected plants was examined. Plants were inoculated simultaneously or successively with selected viruses, and systemically infected leaves were collected 2–4 wk after inoculation for testing. The following virus pairs were detected: CpMV and WClMV in cowpea; SqMV and WMV-1 in squash (Fig. 4); SBMV and WClMV in bean; SqMV and WMV-2 in squash; and CpMV and CMV in *N. benthamiana*. In leaves from squash plants which were inoculated with WMV-1, SqMV, and WMV-2, all three viruses were detected, although reactions with WMV-2 were faint.

DISCUSSION

The SDS-immunodiffusion procedures described herein are useful for detecting and distinguishing several isometric and anisometric viruses. These findings, along with prior studies (1,8,12), broaden applicability of this diagnostic test. The techniques given illustrate potential options, but the procedures used for a given situation would depend on the antiserum types available and on the natures of the particular viruses to be detected.

Antigenic disparity between intact virions and their dissociated

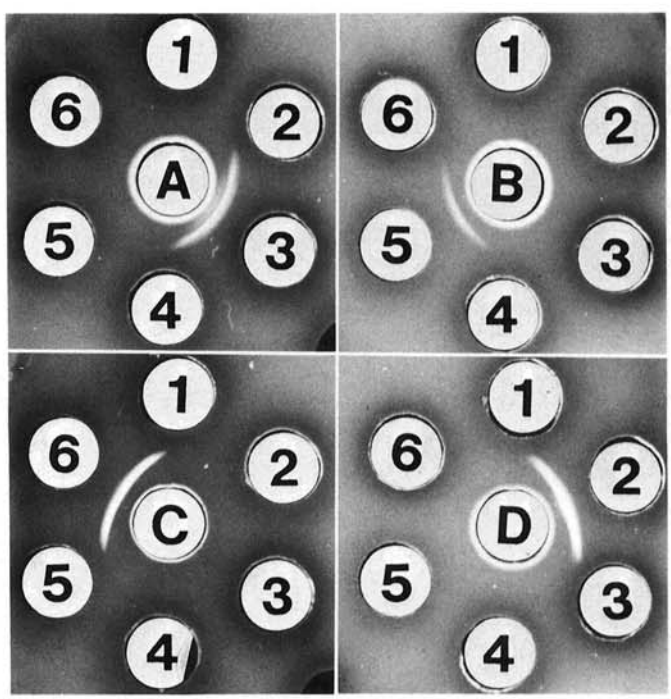

Fig. 3. Serological detection and distinction of a comovirus, a cucumovirus, a potexvirus, and a potyvirus by SDS-immunodiffusion tests. The center wells of the patterns contain antisera to: well A—SDS-treated cowpea mosaic virus; well B—SDS-treated cucumber mosaic virus; well C—SDS-treated clover yellow mosaic virus; and well D—watermelon mosaic virus-2. The peripheral wells contained heated, SDS-treated sap from healthy *Nicotiana benthamiana* (wells 1 and 4) or sap from *N. benthamiana* infected with watermelon mosaic virus-2 (well 2), cowpea mosaic virus (well 3), cucumber mosaic virus (well 5), or clover yellow mosaic virus (well 6).

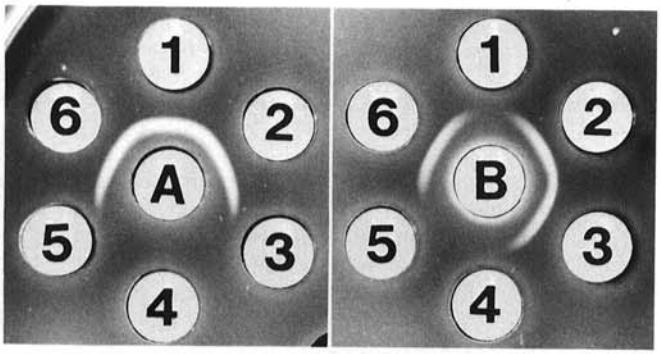

Fig. 4. Detection of a potyvirus and a comovirus in doubly infected plants by SDS-immunodiffusion tests. The central wells contained: well A—antiserum to watermelon mosaic virus-1, and well B—antiserum to SDS-treated squash mosaic virus. The peripheral wells contain heated SDS-treated extracts from leaves as follows: well 1—WMV-1-infected squash; wells 2 and 6—squash infected with both WMV-1 and SqMV; well 3—SqMV-infected squash; and wells 4 and 5—healthy squash.

capsid proteins has been demonstrated for isometric viruses such as turnip yellow mosaic (15), brome mosaic (19), and CMV (21). The published results with SBMV (12) and the results presented herein with SqMV also suggest the possibility of antigenic disparity between the untreated nucleoproteins of these viruses and their SDS-denaturation products. Evidently the protein shell of SqMV consists of two proteins (20; E. Hiebert and D. E. Purcifull, *unpublished*). Perhaps the use of polyacrylamide gel electrophoresis-purified proteins as immunogens (4,5) would be more efficient than the technique used herein to obtain antisera to the SqMV proteins.

There are alternative procedures which are capable of detecting both isometric and anisometric viruses by immunodiffusion. Langenberg and Ball (7) detected SBMV and CpMV, in addition to several rod-shaped viruses, using high pH-ammonia agar plates. Pyrrolidine has been used for SBMV (1), CMV (11), and SqMV (this report), as well as for many filamentous viruses (eg, 1,17). In most cases, the comparative effectiveness of these procedures and the SDS method have not been determined for isometric viruses.

Analyses of SDS-treated potyviral antigens have proved informative for studies on virus relationships (1,12,14), for investigating the composition of virus-induced inclusions (1,12), and for assessment of in vitro translation products (4). Similar applications might be made of serological tests with SDS-treated isometric viruses and their genome products.

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