Detection of Pathogenic Strains of Soybean Mosaic Virus by Enzyme-Linked Immunosorbent Assay with Polystyrene Plates and Beads as the Solid Phase

Li-Ching Chen, D. P. Durand, and J. H. Hill

Graduate assistant and professor, Department of Microbiology and associate professor, Department of Plant Pathology, Seed and Weed Sciences, Iowa State University, Ames 50011.
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ABSTRACT

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Optimal conditions were determined for an enzyme-linked immunosorbent assay system to detect soybean mosaic virus (SMV) by using polystyrene beads as the solid phase. The bead system was more discriminating for detection of serological differences among SMV isolates than a similar system using polystyrene microtitration plates as the solid phase. Seven SMV strains were classified into two, and possibly three, serological groups that correlated with virulence of the strains.

Additional key words: serology.

Soybean mosaic virus (SMV), a member of the potyvirus group of plant viruses (4,13,14), may cause significant yield losses in the United States (30) and other regions of the world (6,7). Transmission of SMV through infected soybean seed seems to provide the primary source of SMV inoculum in the upper Midwest (16) while secondary spread occurs by aphids that transmit the virus in a nonpersistent manner (1,22). Resistance to SMV has been documented (18,20,29,30), but soybean cultivars resistant to virus strains with different degrees of pathogenicity are presently unavailable in Iowa. Therefore, it seems that currently the most prudent control measure is the use of SMV-free seed.

Sensitive enzyme-linked immunosorbent assay (ELISA) (15,21) and radioimmunoassay (RIA) systems (5) have been developed for the detection of SMV in soybean seed. Results from research (2,19,28,34,35) on other viruses that were studied in several laboratories suggest, however, that a high degree of serological specificity may occur among different isolates of the same virus and their antisera and that mixed antisera might be required to detect a wide range of naturally occurring strains. Before a seed certification system based on an ELISA could be established, it was necessary to study the interaction of homologous and heterologous reactants. No evidence for existence of diverse SMV serotypes was obtained in previous immunological studies (22,32).

The "double antibody sandwich" form of ELISA utilizing polystyrene microtiter plates has been widely used for plant virus diagnosis (19). Recently, polystyrene beads were used as the solid phase in a system to detect staphylococcal enterotoxins (33). Objectives of this study were to develop optimal conditions for detection of SMV by using beads as the solid phase in ELISA. Secondly, serological properties of seven isolates of SMV, which differed in pathogenicity, were studied by using plates and beads as the solid phase in ELISA. This allowed a direct comparison of the ability of the two systems to discriminate between plant virus serotypes.

MATERIALS AND METHODS

Source of virus and purification. One isolate was selected from each of the seven strains (G1–G7) established by Cho and Goodman from analysis of the pathogenic variation in a collection of 98 SMV isolates (8). An Iowa isolate, Ia 75-16-1 (13,14,22) was used as the virus antigen for antibody production. Within the context of this paper, isolate Ia 75-16-1 will be designated as Ia and isolates from strains G1, G2...G7 will be designated as isolates 1, 2...7, respectively.

SMV isolates were purified from infected *Glycine max* (L.) Merr. 'Williams' as described by Hill and Benner (13). To minimize aggregation, 1% (w/v) sodium metaphosphate $(NaPO_3)_{13}$ was incorporated into resuspension buffers and the sodium-potassium phosphate buffer used for dialysis. Virus concentrations were

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calculated for SMV by assuming an $E^{0.1\%}_{260\ nm} = 2.4$ (26).

Immunological procedures. Anti-Ia SMV-IgG was purified from rabbit serum by acid sucrose density-gradient centrifugation as described by McLaughlin et al (24). Titer of the purified anti-SMV-IgG used in these experiments was 1:256 (undiluted IgG concentration = 4.4 mg/ml as determined by $E^{0.1\%}_{260\ nm} = 1.4$) when tested in microprecipitin tests against a homologous antigen beginning with a concentration of 1.0 mg purified SMV per milliliter.

Purified anti-SMV-IgG preparations in PBS (0.02 M sodium phosphate, pH 7.2, containing 0.85% NaCl) were conjugated with alkaline phosphatase (Type VII, Sigma Chemical Co., St. Louis, MO 63178) as previously described (15). Conjugate concentrations were estimated spectrophotometrically ($E^{0.1\%}_{280\ nm} = 1.4$) and stored in the dark at 4 C.

ELISA procedures, using flat-bottomed polystyrene microtitration plates (No. 1-223-29, Dynatech Laboratories Inc., Alexandria, VA 22314), were as described previously (15). A dose-response curve for each SMV isolate was obtained by adding serial dilutions of recently purified virus antigen in 0.05 M sodium borate, pH 7.2, to plates precoated with 1 μg/ml of anti-SMV Ia-IgG. The same serial dilutions of homologous antigens also were incorporated into different wells of the same plate. Virus concentrations were determined spectrophotometrically immediately before use.

ELISA tests on polystyrene beads (6.5-mm diameter, Precision Plastic Ball Co., Chicago, IL 60641), were standardized as described below except for certain variables, which are outlined in results. Twenty polystyrene beads were sensitized in 10 ml of 1 μg/ml anti-SMV-IgG in 0.05 M sodium carbonate coating buffer, pH 9.6, in a screw-capped glass vial at 25 C for 6 hr with gentle agitation. After coating, the polystyrene beads were washed three times with PBS-Tween-PVP-OVA (PBS containing 0.05% Tween-20, 2% [w/v] polyvinylpyrrolidone [mol wt = 40,000, Sigma], and 0.2% [w/v] ovalbumin [Grade II, Sigma]) by aspirating the liquid and refilling with washing solution (1 ml/bead). The beads were then incubated in PBS-Tween-PVP-OVA at 25 C for 1 hr to prevent nonspecific binding of the free antigens or conjugates to unoccupied sites on the beads. The beads were transferred individually into a Falcon plastic tube (Falcon Plastics, Los Angeles, CA 90045) that had been precoated with 2.0% (w/v) ovalbumin in PBS-Tween for 1 hr at 25 C to saturate the protein-binding sites. One milliliter of a PBS-Tween solution of purified SMV was added and the tubes were capped and incubated for 12 hr at 25 C with gentle shaking. The virus solution was aspirated, and the tubes were refilled with PBS-Tween-PVP-OVA three times for 3 min as before. Each bead was transferred to a new ovalbumin-coated plastic tube to prevent the carryover of SMV. Enzyme-conjugated IgG at an optimal concentration of 1–5 μg/ml and diluted in PBS-Tween (0.5 ml/bead) was added. The optimal concentration of labeled IgG to use was determined by maximal binding ratio (15). The tubes were capped and incubated at 37 C for 6 hr. Unreacted enzyme-conjugated IgG was rinsed from the tubes by following the rinsing procedures described above. Individual beads were transferred to clean glass tubes, 0.5 ml of substrate (*p*-nitrophenylphosphate, 1.0 mg/ml, in 10% diethanolamine, pH 9.8) was added to each tube, and the tubes were incubated for 1 hr at 25 C. The enzyme reaction was stopped by the addition of 50 μl of 3.0 M NaOH, and the amount of reaction product was determined spectrophotometrically at 405 nm. A dose-response curve was determined in a manner similar to that used for plate ELISA to demonstrate the heterologous reactivity of each SMV isolate.

Density-gradient centrifugation. Sucrose gradients were prepared by layering 5, 9, 9, 9, and 5 ml of 5, 10, 20, 30, and 40% sucrose in 0.05 M sodium borate, pH 7.2, in 2.5 × 8.9-cm centrifuge tubes and stored overnight at 4 C. To analyze the sedimentation properties of each SMV isolate, 1 ml of virus sample was added. Gradients were centrifuged at 25,000 rpm for 1.5–2.0 hr in a Beckman SW 27 rotor and fractionated with an ISCO (Instrumentation Specialities Co., Lincoln, NE 68504) model D density-gradient fractionator coupled to a UA-2 ultraviolet analyzer and external recorder.

RESULTS

Optimal conditions for ELISA using polystyrene beads. The anti-SMV-IgG concentration used to coat beads determined as previously described (15), was optimal at 1 μg/ml at 25 C (*unpublished*). In the absence of antigen, nonspecific color development of the substrate was negligible. Adsorption at 25 C was significantly more rapid than at 4 C, up to an incubation time of 2 hr. Maximum absorbance occurred after an incubation time of 6–8 hr (Table 1). Antigen incubation for 0.5 hr at 25 C resulted in a discernible positive reaction, but successively longer incubations resulted in higher absorbance (Table 1). Substrate color development, probably reflecting increased antigen binding, was greater at 25 than at 4 C. Incubation of antigen at room temperature without agitation also slightly reduced the absorbance (*unpublished*). Absorbance was greater when virus was suspended in PBS-Tween rather than in 0.05 M sodium borate, pH 7.2 (*unpublished*). Binding ratios, calculated as the ratio of absorbance of the colored substrate product obtained in the presence of virus antigen to colored product obtained in the absence of virus antigen, revealed that the optimum enzyme-conjugate concentration ranged from 1 to 5 μg/ml. Increased conjugate concentration increased substrate color development, but with a concomitant increase in nonspecific color development. Maximum substrate color development, reflecting maximum conjugate binding, was greater when conjugate was incubated with beads at 37 C than at 4 C (Table 2). Maximum binding reached a plateau at about 6 hr incubation at 37 C (Table 2), and further incubation affected binding insignificantly. The size of polystyrene beads used affected detection sensitivity. Duplicate samples of seven different antigen concentrations ranging from 250 to 1,000 ng/ml were allowed to react with 6.5- or 3.2-mm-diameter beads. Substrate color development was 1.58 ± 0.18 (mean and standard deviation) times greater for the 6.5-mm beads than for the 3.2-mm beads. The minimum detection level of purified SMV was about 50 ng/ml when 6.5-mm beads were used (Table 3).

TABLE 1. Effect of coating antibody and antigen incubation time and temperature on bead ELISA for detection of soybean mosaic virus

Incubation time (hr)	Incubation of coating antibody		Incubation of antigen	
	4 C	25 C	4 C	25 C
0.25	0.55 ± 0.02[a]	0.75 ± 0.04****[b]	0.02 ± 0.01	0.06 ± 0.02*
0.50	0.70 ± 0.07	1.08 ± 0.17***	0.04 ± 0.01	0.26 ± 0.02****
1.00	0.74 ± 0.06	1.26 ± 0.08****	0.09 ± 0.03	0.60 ± 0.03****
2.00	0.93 ± 0.03	1.40 ± 0.02****	0.24 ± 0.07	0.85 ± 0.08***
4.00	1.11 ± 0.09	1.47 ± 0.37	0.34 ± 0.05	1.24 ± 0.13***
6.00	1.26 ± 0.05	1.56 ± 0.30	0.48 ± 0.03	1.64 ± 0.02****
8.00	1.33 ± 0.03	1.57 ± 0.02	0.60 ± 0.06	1.70 ± 0.14****
10.00	1.46 ± 0.09	1.46 ± 0.06	0.61 ± 0.05	1.82 ± 0.02****
14.00	...	...	0.81 ± 0.15	1.86 ± 0.00****

[a] Data are the mean and standard deviation $A_{405\ nm}$ values of three replicates; the experiment, consisting of a single bead for each time and temperature, was replicated on different days. Coating antibody concentration was 1.0 μg/ml; SMV Ia and conjugate were tested at 2.5 μg/ml.

[b] Differences between means at 4 and 25 C are statistically significant $P = 0.1$(*), $P = 0.05$(**), $P = 0.01$(***), or $P = 0.001$(****).

Heterologous reactivities of SMV strains in ELISA. Dose-response curves for each SMV isolate demonstrated somewhat different levels of discrimination, depending on whether plates or beads were used as the solid phase. In ELISA with beads as the solid phase, SMV isolate 1 antigen had a markedly lower reactivity with antibody to isolate Ia than did the homologous antigen (Fig. 1A). Dose-response curves for antigens prepared from SMV isolates Ia, 2, 3, and 4 were similar (Fig. 1B). Unexpectedly isolates 5, 6, and 7 produced absorbance values higher than the homologous reaction at higher antigen concentrations (Fig. 1C). With plate ELISA, virus isolates 1 through 5 exhibited dosage-response curves similar to that of the homologous antigen. Isolates 6 and 7 produced absorbance values higher than the homologous reaction at higher antigen concentrations (Fig. 2).

This unusual increase may have been caused by lateral aggregation of virus particles at the antigen binding sites of the antibody molecules. This putative aggregation would allow an increase in conjugate bound to the antigen with a concomitant increase in substrate reaction. To test this hypothesis, an SMV isolate that did not exhibit this phenomenon (isolate 3) was compared with one that did (isolate 6) and with Ia in sucrose density gradient experiments. Sedimentation patterns showed a rapidly sedimenting zone previously shown to consist of aggregated virus particles and a zone sedimenting at a lower rate consisting of relatively nonaggregated particles (24). Assuming that all virus particles were exclusively sedimented in these two zones, the data showed that for isolates Ia, 3, and 6, 51 and 49%, 60 and 40%, and 44 and 56% of the particles were in the gradient zones containing nonaggregated and aggregated virus particles, respectively.

TABLE 2. Effect of conjugate IgG incubation time and temperature on bead ELISA for detection of soybean mosaic virus (SMV)

Incubation time (hr)	SMV[a]		PBS-Tween[a]
	4 C	37 C	37 C
0.25	0.12 ± 0.01[a]	0.14 ± 0.01*[b]	0.00 ± 0.00
0.50	0.19 ± 0.01	0.25 ± 0.01***	0.00 ± 0.00
0.75	0.21 ± 0.02	0.30 ± 0.02**	0.01 ± 0.01
1.00	0.24 ± 0.01	0.40 ± 0.02***	0.00 ± 0.00
2.00	0.33 ± 0.02	0.59 ± 0.02***	0.00 ± 0.00
4.00	0.50 ± 0.01	0.84 ± 0.03***	0.01 ± 0.00
6.00	0.75 ± 0.02	1.01 ± 0.03***	0.02 ± 0.00
8.50	···	0.96 ± 0.06	0.01 ± 0.00
10.00	···	1.12 ± 0.15	0.02 ± 0.00

[a] Data are the mean and standard deviation $A_{405\ nm}$ values of three replicates; the experiment, consisting of a single bead for each time and temperature, was replicated on different days. Coating antibody concentration was 1.0 μg/ml, SMV Ia and conjugate were tested at 2.5 μg/ml. PBS-Tween was used as a control.

[b] Differences between means at 4 and 37 C are statistically significant $P = 0.10$(*), $P = 0.01$ (**), or $P = 0.001$ (***) by Student's t-test.

TABLE 3. Sensitivity of bead ELISA for detection of purified soybean mosaic virus (SMV)[a]

SMV (μg/ml)	A_{405}[b]
25,000	1.69 ± 0.21
10,000	1.68 ± 0.20
5,000	1.37 ± 0.08
2,500	1.23 ± 0.08
1,000	0.96 ± 0.12
750	0.66 ± 0.05
500	0.49 ± 0.05
250	0.35 ± 0.03
100	0.14 ± 0.03
50	0.07 ± 0.02
25	0.05 ± 0.02
10	0.05 ± 0.02
5	0.04 ± 0.02
1	0.03 ± 0.01
0	0.02 ± 0.02

[a] Coating antibody concentration was at 1 μg/ml and conjugate IgG was at 2.5 μg/ml.

[b] Data are the mean and standard deviation $A_{405\ nm}$ value of four replicates; the experiment, constituting a single bead for each antigen concentration, was replicated on different days. Absorbance value greater than twice that of a negative control is assessed as positive. Polystyrene beads with 6.5-mm diameter were used.

DISCUSSION

Investigation of polystyrene beads as the solid phase in ELISAs for plant virus detection has revealed properties similar to and different from similar tests using microtitration plates (15) as the solid phase. Although conditions for the bead and plate ELISA tests were somewhat different, we feel comparisons were valid

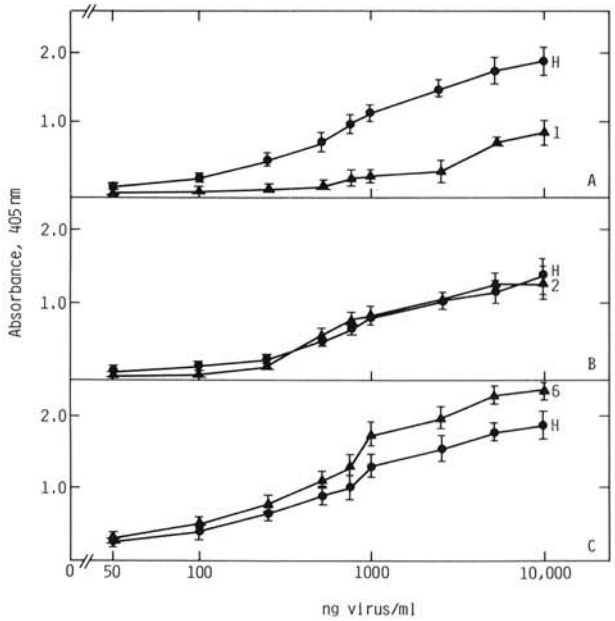

Fig. 1. Dose-response curves of homologous SMV isolate (H) Ia (●——●) and heterologous isolates **A,** 1 (▲——▲), **B,** 2 (▲——▲), and **C,** 6 (▲——▲) in ELISA with polystyrene beads used as the solid phase. Isolates 3 and 4 revealed patterns similar to 2 and isolates 5 and 7 were similar to 6. Data are the mean and standard deviation (vertical bars) of four (A and B) and 10 replicates (C). Each replicate was performed on a different day. Coating antibody and conjugate concentrations were 1.0 μg/ml and 2.5 μg/ml, respectively.

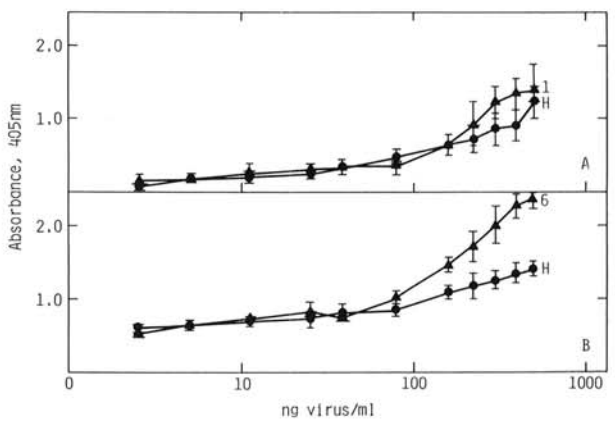

Fig. 2. Dose-response curves of homologous SMV isolate (H) Ia (●——●) and heterologous isolates **A,** 1 (▲——▲), and **B,** 6 (▲——▲) in ELISA tests using plates as the solid phase. Isolates 2,3,4, and 5 revealed a pattern similar to 1 and isolate 7 revealed a pattern similar to 6. Data are the mean and standard deviation (vertical bars) of six (A) and four (B) replicates. Each replicate was performed on a different day. Coating antibody and conjugate concentrations were 1.0 μg/ml and 2.5 μg/ml, respectively.

because tests with each system were made under the optimal conditions established for each assay. An antibody coating concentration of 1 μg/ml appeared optimal for both bead and plate ELISA (15). Use of higher concentrations did not significantly increase the level of final color yield in bead ELISA.

Adsorption of antibody to polystyrene beads was relatively rapid and was similar to that reported by Hollinger et al (17) for antibody to Australian antigen. Antigen binding was similar to previous reports (12,17,23,25) and probably follows the kinetics described by Hertl and Odstrchel (12) in which rate is proportional to the amount of immobilized antibody and the instantaneous concentration of available unbound antigens.

Binding of antigen with conjugate is thought to be restricted to conditions that favor antibody-antigen reactions. The practical variables at this stage are, in most instances, the concentration of conjugate and the time of incubation. Use of an optimum conjugate concentration has been suggested (15,31). At the optimum concentration, the absorbance values in the absence of antigen are negligible, and the reaction levels in the presence of antigen are appreciable. Binding ratios indicated less conjugate IgG (1–5 μg/ml) was required in bead ELISA than in plate ELISA (25–60 μg/ml) (15) for detection of SMV. The reasons for this difference are not fully understood.

Conjugate binding measured by substrate color development, was similar to previous reports (10,11,23). Maximum substrate product occurred after 6 hr of incubation at 37 C. These results agree with Clark and Adams (9) and many other investigators that incubation at 37 C was more efficient than at 4 C for effective conjugate IgG and antibody binding. The data differ, however, from the optimum of 5 C reported by McLaughlin et al (23) for plate ELISA and which we adopted for our studies using plates as the solid phase (this report, and 15).

Use of different sized beads affected the sensitivity of bead ELISA. Results indicated that increasing the surface area of beads by a factor of 4 increased reaction levels about 1.6 times.

These studies of SMV isolates by ELISA are the first report suggesting differences in serological properties of SMV isolates. Establishment of serotypes, however, must await preparation of the antisera which will make reciprocal tests possible. Isolate 1 reacted weakly with antiserum prepared from the Ia isolate in bead ELISA. A close serological relationship existed between isolates Ia and 2, 3, and 4.

The serological relationships between isolates Ia and 5, 6, and 7 cannot be clearly deduced because of the unusual increase in absorbance values at higher heterologous antigen concentrations. The reasons for these unusual increases are not fully understood. Greater virus aggregation, as represented by density gradient experiments with isolate 6, may have caused the increased absorbance values. Alternatively, it is possible that substantial serological distinction between the homologous and heterologous antigens may exist. To verify this, reciprocal tests should be performed. These three isolates may possess more highly reactive antigenic determinants than the Ia isolate. Bar-Joseph and Salomon (3) recently suggested this possibility in studies of two strains of tobacco mosaic virus. Further investigation of amino acid composition and tryptic peptide maps may detect significant differences between these SMV isolates.

The results reported here do not necessarily represent the range of antigenic variation that may occur among SMV isolates. It is possible that isolates within the strains established by Cho and Goodman (8) because of pathogenic properties may show variant serologic properties, and any correlation between serological properties and pathogenicity may be fortuitous. However, if the results we report imply three groups of SMV consisting of isolates, within G1; G2, G3, and G4; and G5, G6, and G7; a distinct similarity exists between virulence and serological grouping of the isolates utilized in this study. As reported by Cho and Goodman (8), G1 represents a group of isolates that are least virulent; G2, G3, and G4 represent isolates of intermediate virulence; and G5, G6, and G7 are highly virulent. Similar observations have been reported for watermelon mosaic virus and barley yellow dwarf virus isolates (27,28). In these reports, virus isolates distinguished by host range or specific aphid vectors showed serological heterogeneity.

The double antibody sandwich bead ELISA showed greater discrimination between SMV isolates than did bead ELISA. Potential for various levels of discrimination in different immunosorbent assays does not allow extrapolation of results from one system to another. Application for routine virus detection suggests that a system with less discrimination would be desirable. Other applications may require a system with a high level of discrimination.

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