Studies of soybean mosaic virus strains by enzyme-linked immunosorbent assay

by

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TABLE OF CONTENTS

INTRODUCTION

The enzyme-linked immunosorbent assay (ELISA) technique has been used successfully for the detection of plant virus diseases. The ELISA is regarded as a quantitative assay by virtue of the absolute measurement of enzymic activities. Quantitative results from the assays, however, depend on standardization of the procedures and utilizing a highly purified antibody preparation. These important aspects have been somewhat neglected by many investigators when using ELISA for plant virus detection. The concentration and time dependence of adsorption of protein to a polystyrene solid phase also plays a role in development of effective ELISA systems. Different properties of polystyrene have been demonstrated to affect the detection level. It is probably due to the variations of leakage of adsorbents (proteins) from solid phases. In this research the widely used polystyrene microplates were utilized as solid phase and compared with polystyrene beads for their suitability for rapid and sensitive detection of soybean mosaic virus (SMV).

Several authors have reported that the ELISA technique shows great potential for use in serological distinction of plant virus strains. This specificity of ELISA is believed to be mainly caused by the behavior of the conjugated antibodies. This may be an advantage when it is important to discriminate different virus strains, but more often it is a drawback in routine and large-scale diagnostic work. Strains of SMV have been found to differ in their effects on soybean cultivars; therefore, breeding programs for developing

mosaic resistant varieties should evaluate hybrid progeny against a wide range of SMV isolates. In this respect, rapid identification of virus strains and use of antisera against the whole range of naturally occurring strains are needed before starting large-scale tests. According to many previous investigators, SMV strains were serologically indistinguishable by conventional serological methods. In this investigation, the double-antibody sandwich method of ELISA was utilized to detect seven SMV strains with antisera to a single virus isolate.

LITERATURE REVIEW

Soybean Mosaic Virus and Its Strains

Soybean mosaic is one of the most serious and widespread virus diseases of the soybean [Glycine max (L.) Merr.] and apparently was present in United States soybeans before 1910 (Piper and Morse, 1910). Clinton (1915) first described the symptoms of the disease and the virus nature of the disease was established by Gardner and Kendrick (1921). Conover (1948) verified that mosaic of soybeans could be caused by more than one strain of the virus. The disease has been found wherever soybeans are cultivated. The host range of soybean mosaic virus (SMV) is narrow; among the 30 plant species that have been infected, only two, Chenopodium quinoa and C. album are not in the Leguminosae family (Quantz, 1961; Galvez, 1963).

The virus morphologically and biochemically resembles members of the potyvirus group (Bos, 1972; Hill and Benner, 1980a). The potyvirus group, named after the type member, potato virus Y (PVY), is the largest group of plant viruses known at present. It includes many viruses that cause economically important diseases of crop plants such as bean yellow mosaic, lettuce mosaic and turnip mosaic. These viruses have flexous filamentous particles which are generally 730 to 790 nm long. They can be transmitted experimentally by inoculation with infectious plant sap, and in nature their vectors are aphids. Infected plants usually show mosaic symptoms. Hill and Benner (1980b) reported that the molecular weight of SMV RNA, approximately 3.25×10^6, and the size of protein subunits are in agreement with other potyviruses.

At least 20 aphid species have been found to be capable of transmitting the virus in a nonpersistent manner (Heinze and Köhler, 1940; Conover, 1948; Koshimizu and Iizuka, 1963; De Vasconcelos, 1964; Abney, et al., 1976; Lucas and Hill, 1980). A narrow host range and aphid transmission via a nonpersistent mechanism suggests the probable importance of seed transmission as a source of primary inoculum (Kendrick and Gardner, 1924; Bowers, 1977; Hill, et al., 1980a). Yield reductions caused by SMV vary with cultivar, virus strain, location and time of inoculation (Quiniones, et al., 1971; Ross, 1968, 1969b, 1977). Data from these investigations also provided evidence that SMV infection reduces seed quality and seed germination.

Recent data (Ross, 1977) indicate that SMV may cause significant yield loss in the United States. He reported some susceptible lines may sustain up to 35% yield reduction in the presence of naturally transmitted SMV. SMV also caused significant yield loss in other regions of the world (Akhatova, 1969; Muraveva, 1973). In 1974, a necrotic strain of SMV caused approximately 50% yield reduction in the leading soybean cultivars in Korea. In some cases, the infection caused complete loss of the crop (Cho and Chung, 1976; Cho, et al., 1977). These commercial cultivars were supposed to have been resistant to SMV.

Various isolates of SMV have been shown to cause a variety of symptoms ranging from mild mosaic to severe necrosis (Conover, 1948; Ross, 1969a; Han and Murayama, 1970; Cho and Goodman, 1979). New strains recovered from soybean cultivars immune to other SMV strains always showed severe symptoms (Ross, 1975; Cho and Chung, 1976). SMV

isolates differing in their aphid specificity and transmission efficiency also have been reported (Lucas and Hill, 1980).

Pathogenic variation depends upon interactions between host plant genotype and strains of the pathogen; therefore, classification of virus strains based on pathogenicity to host cultivars and species have been used (Tosic and Ford, 1972; Bald and Tinsley, 1967). Different isolates of SMV varying in their pathogenicity among soybean cultivars have been reported (Ross, 1969a). In this respect, six different strains of SMV were isolated from soybeans in Taiwan by Han and Murayama (1970). In 1979, Cho and Goodman reported that 98 isolates of SMV were classified into seven strains (Table 1) based on virulence in resistant and tolerant soybean cultivars. These isolates of SMV, present in the USDA soybean germplasm collections, were from a world-wide collection. Their results indicated that no apparent patterns of relationships between the origin of the soybean accessions and the virulence of the isolates collected were observed. Their results also showed that the disease symptoms were somewhat consistent with the common genetic background of the soybean varieties tested. The important feature is that virulent strains in most cases caused severe necrotic symptoms in resistant cultivars that are resistant to less virulent strains. The incidence of systemic necrosis caused by virulent strains of SMV is a threat to efforts to improve soybean breeding programs for SMV resistance (Cho and Chung, 1976; Cho, et al., 1977). Therefore, a wide range of SMV strains differing in virulence should be used in soybean breeding programs in which SMV resistance is an objective. Investigations on the mode of inheritance of resistance in soybean cultivars indicate resistance to be controlled

Table 1. Reactions of soybean cultivars to seven soybean mosaic virus (SMV) strains obtained from seeds in the U.S. Department of Agriculture soybean germplasm collections (Cho and Goodman, 1979)

Soybean cultivars	Symptoms caused by SMV strains[a]						
	SMV-G7	SMV-G6	SMV-G5	SMV-G4	SMV-G3	SMV-G2	SMV-G1
Clark	-/M[b]	-/M	-/M	-/M	-/M	-/M	-/M
Rampage	-/M	-/M	-/M	-/M	-/M	-/M	-/M
Davis	-/M	-/M	-/M	-, N/M, N	-/-	-/-	-/-
York	-/M	-/M	-/M	-, N/M, N	-/-	-/-	-/-
Marshall	N/N	N/N	-/-	N/N	N/N	N/N	-/-
Ogden	N/N	-/-	-/-	N/N	N/N	-/-	-/-
Kwanggyo	N/N	N/N	N/N	-/-	-/-	-/-	-/-
Buffalo	N/N	-/-	-/-	-/-	-/-	-/-	-/-

[a]Symbols for symptoms: - = symptomless, no virus was detected in inoculated tissue by indexing on Phaseolus vulgaris L. cv. Top Crop; M = mosaic symptom; N = necrosis.

[b]Format for symptom symbols: (Reactions on inoculated primary leaves)/(reactions on noninoculated trifoliolate leaves).

or antibodies were linked to an insoluble solid phase so that the reactivity of the immunological component was retained. This is the technique known as ELISA (Enzyme-Linked Immunosorbent Assay) pioneered by Engvall and Perlmann (1971 and 1972) and by Van Weemen and Schuurs (1971 and 1972).

In various forms, ELISA is being used increasingly in clinical pathology and immunology (Feldmann, et al., 1976). For plant viruses, the 'double antibody sandwich' form of ELISA has been found to be suitable for detection of viruses either in purified preparations or in crude extracts of infected plants (Voller, et al., 1976a and b; Clark and Adams, 1977). Various materials have been used as the solid phase in ELISA (Schuurs and Van Weemen, 1977). Polystyrene microtiter plates (Voller, et al., 1974) owing to their commercial availability have been used widely for routine diagnosis. Stiffler-Rosenberg and Fey (1978) first used polystyrene beads which made it possible to detect toxins contained in 20 ml of food extract. In the ELISA method, the conjugates usually consist of proteins coupled to enzymes. The coupling has been satisfactorily carried out using glutaraldehyde (Avrameas, 1969) or by intermolecular disulfide bond formation (King, et al., 1978). In ELISA, a variety of enzymes have been used: alkaline phosphatase, horseradish peroxidase, glucose oxidase, β-d-galactosidase and β-lactamase (Avrameas, 1969; Engvall and Perlmann, 1971; Maiolini, et al., 1975; Kato, et al., 1976; Geetha, et al., 1980). Antigens or antibodies are adsorbed to the plastic polymer surfaces by noncovalent, physical forces (Engvall, et al., 1971). Enzyme-labelled antigen or antibody complexes have both immunological and enzymatic activity. Subsequent

degradation of a chromogenic substrate by the enzymes enables accurate and sensitive detection of the presence of enzyme. Using this technique, 1-100 ng per milliliter of antigen could be determined (Engvall, et al., 1971).

ELISA has been used for the detection of different plant viruses, spiroplasmas, fastidious bacteria, and fungal infection in plant diseases (Voller, et al., 1976a; Clark, et al., 1978; Nomé, et al., 1980; Nachmias, et al., 1979). Infectivity tests, electron microscopy and different serological techniques are also suitable for detection of plant viruses. They have certain limitations, however; for example, low virus concentrations in infected plants, presence of virus inactivators in plant extracts and viruses which have not been transmitted mechanically to herbaceous plants. These disadvantages in most cases could be resolved by using ELISA. ELISA is capable of detecting tissue-limited viruses (Bar-Joseph, et al., 1979; Lister and Rochow, 1979), economically important seed-borne viruses in seeds or tubers (Jafarpour, et al., 1979; Lister, 1978; Casper, 1977), and viruses present in their viruliferous vectors (Gera, et al., 1978; Clarke, et al., 1980). ELISA has great potential for use in large-scale surveys and epidemiological investigations involving the many important crops affected by plant viruses.

Detection and differentiation of plant virus strains using ELISA has been reported recently (Barbara, et al., 1978; Rochow and Carmichael, 1979; Lister and Rochow, 1979; Uyemoto, 1980; Bar-Joseph and Salomon, 1980; Van Regenmortel and Burckard, 1980). Rapid identification of virus strains is particularly important in studying the epidemiology

of plant diseases. It is usually difficult to categorize the strains encountered as they can cause very similar symptoms and are usually indistinguishable by conventional serological techniques. Koenig (1978) reported that for several viruses, e.g., the Andean potato latent virus, the specificity of ELISA was so great that conjugates prepared to one strain failed to detect other serologically closely related strains. This observation emphasizes the importance of a detailed evaluation of the available antisera against the whole range of naturally occurring strains before starting large-scale tests (Barbara, et al., 1978). The great specificity in the heterologous antibody system is mainly caused by the behavior of the free phase (conjugated antibodies) (Koenig, 1978; Bar-Joseph and Salomon, 1980).

For results from different laboratories to be comparable, it is necessary that the reagents and procedures used for ELISA be standardized. Polystyrene was found not optimal for quantitative measurement because the leakage of antigen from the solid phase was too high (Lehtonen and Viljanen, 1980). In this respect, they found cyanogen bromide activated paper with covalent coupling of antigens to the solid phase was superior to polystyrene and nylon. Studies on enzyme rate kinetics for linear dose-response in quantitative ELISA also were reported (Tsang, et al., 1980). The use of highly purified immunoglobulin G for the detection of plant viruses using ELISA also minimized the probable contamination of antibody to healthy host antigen (Hill, et al., 1980b). Double antibody sandwich ELISA using polystyrene beads and microtiter plates as the solid phases were examined in this investigation. Purified SMV strains were then compared in both optimized ELISA systems.

MATERIALS AND METHODS

Virus Isolates and Antisera

Seven strains of soybean mosaic virus (SMV) designated as SMV-G1 to SMV-G7 were received from R. M. Goodman, Department of Plant Pathology, University of Illinois, Urbana. An Iowa isolate of SMV (Ia 75-16-1), which originated from a field collection, was provided by J. H. Hill and served as the only virus antigen used for antibody preparations. All strains were propagated in Glycine max cv. 'Williams' soybean plants at approximately 25°C in a greenhouse. The seeds were planted in steam-sterilized soil. Inoculations were made on Carborundum (600 mesh) dusted primary leaves before the first trifoliolate leaf expanded. The plants were inoculated by rubbing the leaves with inoculum prepared from infected soybean leaves ground in 0.01 M potassium phosphate buffer, pH 7.0.

Antibody preparations specific to Ia-75-16-1 were kindly provided by J. H. Hill. The isolation of virus specific antibody was done by acid sucrose density gradient centrifugation as described by McLaughlin, et al. (1980a). The SMV-specific antibody was lyophilized and stored at - 20°C. Purified specific antibody exhibited electrophoretic properties nearly identical to normal rabbit immunoglobulin G (IgG) (McLaughlin, et al., 1980a) and were used both for enzyme conjugation and coating antibody in ELISA.

Virus Purification

Virus was purified from systemically infected Williams soybeans. The plants were harvested three weeks after inoculation. Infected tissues were homogenized for 1-2 min in a Waring Blender in chilled 0.25 M sodium and potassium phosphate buffer, pH 7.1, containing 1% (v/v) 2-mercaptoethanol (3 ml of buffer per gram of tissue). The extract was strained through two layers of cheesecloth and clarified by addition of butanol-chloroform (1:1, v/v) to 8% (v/v), followed by low-speed centrifugation in a Sorvall refrigerated centrifuge (7,000 rpm for 20 min in a GSA rotor). Clarified extracts were brought to 0.3 M with NaCl before addition of polyethylene glycol, MW 6,000 (PEG-6000) (Union Carbide Co., New York, N.Y. 10017) to 4% (w/v). After 1 hr of incubation at 4°C, the precipitated virus was collected by centrifugation at 8,500 rpm for 20 min in a GSA rotor and resuspended in 0.05 M sodium and potassium phosphate buffer, pH 7.4, containing 1.0 M deionized urea, 1% (w/v) sodium metaphosphate and 0.1% (v/v) 2-mercaptoethanol, hereafter called urea buffer. Deionized urea was prepared by slowly passing an 8.0 M urea solution three times through a column of reactor grade mixed bed resin, 25 to 50 mesh, hydrogen and hydroxide forms (BioRad Laboratories, Richmond, California). The resuspended virus was clarified by low-speed centrifugation at 5,000 rpm for 10 min in an SS-34 rotor, filtered through glass wool, and further concentrated by high-speed centrifugation for 3.5 hr at 21,000 rpm in a Beckman type 21 rotor. Resuspended virus was further clarified by one more cycle of differential sedimentation. Virus was centrifuged for 10 min at 5,000

68504) model D density gradient fractionater coupled to a UA-2 ultraviolet analyzer and external recorder.

Enzyme Conjugation of Immunoglobulin G

Purified anti-Ia-75-16-1 IgG lyophilized in 0.05M borate buffer, pH 7.2, containing 1.4% (w/v) sodium chloride was resuspended in a volume of deionized distilled water which maintained the same buffer and salt concentrations and dialyzed against PBS overnight. Any precipitate forming during dialysis was removed by low-speed centrifugation. Immunoglobulin concentrations were estimated spectrophotometrically ($E_{280}^{0.1\%}$ = 1.4). One to 2.0 ml portions of IgG at 1.0 mg/ml in PBS were mixed with alkaline phosphatase (E. C. No. 3.1.3.1., Sigma Type VII, 5 mg/ml in a suspension of 3.2M ammonium sulfate, pH 7, containing 0.001M magnesium chloride and 0.0001M zinc chloride) at a protein ratio of 2:1 (w/w) enzyme:IgG. Mixtures were dialyzed against PBS overnight at 4°C and 25% aqueous glutaraldehyde was added to a final concentration of 0.2% (v/v) (Engvall, et al., 1971). Preparations were incubated at room temperature for 2 hr, then dialyzed against PBS at 4°C with several changes. Conjugate concentrations were estimated spectrophotometrically ($E_{280}^{1\%}$ = 1.4) and were stored in the dark at 4°C.

The Plate ELISA Procedures

The plate ELISA test procedures closely followed those developed by McLaughlin, et al. (1980b). The assays were performed in polystyrene microtitration plates (No. 1-223-29, Dynatech Laboratories,

Inc., 900 Slaters Lane, Alexandria, VA 22314) with 96 flat bottom wells. Wells were precoated by incubation for 1 hr at 4°C in moist chambers with 200 µl of nonlabelled coating antibody (1 µg/ml in 0.05M sodium carbonate coating buffer, pH 9.6, containing 0.02% sodium azide) per well. Exterior wells were not used because of possible edge effects and 200 µl of water was added instead. Plates were covered using spent plates as lids. Plates then were rinsed three times with PBS, containing 0.05% Tween-20 (PBS-Tween). Plates were first filled with a wash bottle, decanted, refilled, and the liquid was allowed to stand for 3 min between rinses. Plates were then coated with 1% ovalbumin (Grade II, Sigma Chemical Co., St. Louis, MO 63178) (w/v) in sodium carbonate coating buffer (200 µl/well) for 1 hr at 4°C to prevent nonspecific binding of free or conjugated enzyme to sites still available on the wells (Lister, 1978; Hill, et al., 1980b). After washing the plates as before, purified virus (200 µl/well) was added and plates were covered as before and incubated at 4°C for 12-16 hr. After further rinsing, enzyme-conjugated IgG (200 µl/well) diluted in PBS-Tween was added to react with bound antigen during 12 hr of incubation at 4°C. Finally, nonreacted conjugates were rinsed away, and specific antibody-antigen reactions were detected by adding the substrate (p-nitrophenyl phosphate, Sigma Chemical Co., St. Louis, MO 63178) at 1.0 mg/ml (200 µl/well) in 10% (v/v) diethanolamine, pH 9.8. Plates were covered and incubated at room temperature for 2 hr; then, the enzyme-substrate reactions were arrested by adding 50 µl/well of 3.0M sodium hydroxide. Absorbance (A 405 nm) measurements of the resulting yellow nitrophenol hydrolysis products (diluted with distilled water if necessary) were made by trans-

ferring reaction products individually to a microcuvette and reading in a Gilford spectrophotometer. Reactions were assessed as positive when A405 measurements were greater than twice the A405 value of the negative control.

Virus detection was maximized by using an optimized concentration of enzyme-conjugated IgG. Calculation of binding ratios revealed an optimal concentration near the enzyme saturation point. Binding ratios were determined as the ratio of absorbance of conjugated antibody bound in the presence of virus antigen to conjugated antibody bound in the absence of virus antigen (Hill, et al., 1980b). Dose-response curves for each SMV strain were obtained by adding serial dilutions of virus antigen into plates precoated with 1 μg/ml anti-Ia-75-16-1 IgG. The same serial dilutions of homologous Ia-75-16-1 antigens also were incorporated into different wells of the same plate.

The Bead ELISA Procedures

Polystyrene beads (diameter, 6.5 mm) were purchased from Precision Plastic Ball Co., 3000 N. Cicero Ave., Chicago, Ill. 60641. A set of standard conditions for test steps was used throughout the reaction time experiments, with incubation time as a treatment variable for study of a given step in each experiment. Standard conditions were as follows. Twenty polystyrene beads were sensitized in 10 ml of 1 μg/ml IgG in 0.05M sodium carbonate coating buffer, pH 9.6. Beads were incubated in a screw-capped glass vial at room temperature for 6 hr with gentle agitation. After coating, the polystyrene absorbents were washed

three times with PBS-Tween, containing 2% (w/v) polyvinyl pyrrolidone (mol. wt. 40,000, Sigma) and 0.2% (w/v) egg albumin (PBS-Tween-PVP-OVA), by aspirating the liquid and refilling with washing solution (1 ml/bead). The beads were then incubated in the same buffer at room temperature for 1 hr to prevent nonspecific binding of the free antigens or conjugates to the sites still available on the beads. The beads were transferred individually into a Falcon plastic tube which had been precoated with 2.0% (w/v) egg albumin in PBS-Tween for 1 hr at room temperature to saturate the protein binding sites. One ml of purified SMV diluted in PBS-Tween was added and tubes were capped and incubated for 12 hr at room temperature with gentle shaking on a rotator. The virus solution was aspirated and the tubes were refilled with PBS-Tween-PVP-OVA and rinsed three times for three min as before. Each bead was transferred to a new precoated plastic tube to prevent any carry-over reactions and enzyme-conjugated IgG diluted in PBS-Tween was added (0.5 ml/bead). As in the plate ELISA, optimal concentration of labelled IgG was determined by maximal binding ratio. The tubes were again capped and incubated at 37°C for 6 hr. Unreacted enzyme-conjugated IgG was rinsed from the tubes following the rinsing procedures described above. Individual beads were again transferred to a clean glass tube, and 0.5 ml substrate (p-nitrophenylphosphate, 1.0 mg/ml) was pipetted into the tubes, which were incubated for 1 hr at room temperature. Then the enzyme reaction was stopped by the addition of 50 µl of 3.0M NaOH, and the reaction product was determined spectrophotometrically at 405 nm. To demonstrate the heterologous reactivity of each SMV strain, a dose-response curve was determined as described for plate ELISA.

RESULTS

Virus Purification

All SMV strains tested produced mosaic symptoms on the soybean cultivar 'Williams.' The purification procedures used yielded sufficient virus for routine ELISA testing. Virus yield ranged from 1 to 10 mg virus per kilogram of leaf tissue. Virus aggregation during purification was minimized when 1% (w/v) sodium metaphosphate was incorporated.

Bead ELISA Procedures

Figure 1 depicts the effect of coating IgG concentrations on bead ELISA at 25°C; evidently, at 1 μg/ml, adsorption appeared to have reached equilibrium. In the absence of antigen, nonspecific color development of the substrate was negligible. Figure 2 compares the relative efficiencies of IgG adsorption at 25°C and 4°C. Maximum activity appeared to have been reached within 6 hr incubation at 25°C. The adsorption efficiencies at 4°C were lower than those for 25°C. Antigen incubation for 30 min at 25°C gave a discernible positive reaction (Figure 3), but successively longer incubations resulted in higher reaction levels after 14 hr. As Figure 3 shows, antigen incubation at a lower temperature (4°C) reduced the binding activities. A preliminary experiment also revealed that antigen incubation at room temperature without agitation also slightly reduced the reaction levels (data not shown). Virus antigen was most successfully detected when it was incubated in PBS-Tween (Figure 4). A representative of a typical conjugate IgG preparation (Figure 5) demonstrates that increased conjugate

IgG concentration enhanced the level of virus detection. Nonspecific reactions also were slightly enhanced at higher conjugate concentrations. Calculation of binding ratios revealed an optimum conjugate concentration of 2.5 μg/ml (Table 6, Appendix). The reaction levels of various conjugate incubation intervals (Figure 6) followed a curve similar to that with antigen incubation (Figure 3). The most conjugate binding occurred after 6 hr of incubation at 37°C. Data in Table 7 (Appendix) revealed that further incubation affected binding only insignificantly. Reaction levels at 4°C were considerably lower than those at 37°C. As Figure 7 shows, the detection levels of virus antigen were affected by the size of polystyrene beads used. Detection levels were reduced when beads with smaller (3.2 mm) diameter were used. The minimum amount of purified SMV detectable in bead ELISA was 25 ng/ml when 6.5 mm (dia.) beads and standard conditions (see Materials and Methods) for test steps were followed (Table 10).

Heterologous Reactivities of SMV Strains in ELISA

Dose-response curves for each SMV strain were examined by the plate and bead 'double antibody sandwich' forms of ELISA (Figures 8 and 9). For each system, standard reaction conditions were utilized throughout the investigation. Homologous antigens were incorporated concurrently in parallel experiments. As Figure 8 shows, with the exceptions of SMV strains G6 and G7, virus antigens from various SMV strains exhibited dose-response curves similar to that of the homologous antigen. This indicates a similarity in the nature of bonds that are formed between

coating antibody and homologous and heterologous antigen. SMV-G6 and G7 showed an absorbance higher than the homologous reaction at higher antigen concentrations. Virus aggregation may account for this unexpected result (see below). In contrast, bead ELISA exhibited greater discrimination between different SMV strains (Figure 9). SMV-G1 antigen gave a markedly weaker avidity for antibody to SMV-Ia-75-16-1 (Figure 9a). Similar dose-response curves were obtained with antigens prepared from SMV-Ia-75-16-1 and SMV strains G2, G3 and G4 (Figures 9b, c, and d). As the phenomenon occurred in plate ELISA, antigens prepared from SMV-G5, G6 and G7, gave an increase of absorbance at higher antigen concentrations in bead ELISA (Figures 9e, f, and g). The reasons for this unusual increase are not fully understood. This may have been caused by lateral aggregation of virus particles at the antigen binding sites of the antibody molecules. This putative aggregation will eventually result in an increase of the bound conjugate as well as color development in the substrate. To assess this, the sedimentation characteristics of SMV-Ia-75-16-1, SMV-G3 and G6 were examined simultaneously and repeatedly by density gradient centrifugations (Table 13, Appendix). The results are presented as percent virus particles observed in two UV-absorbing regions of sucrose density gradients, assuming that all virus particles were exclusively sedimented in these two zones. As Table 13 shows, a greater percentage of SMV particles are present in the rapidly sedimenting zone in strain G6 than in strain Ia-75-16-1. The rapidly sedimenting zone has been previously shown to consist of aggregated virus while the zone sedimenting at a slower rate consists of relatively nonaggregated particles (McLaughlin, et al., 1980a).

The UV-absorption profiles in Figure 10, representative of a typical density gradient centrifugation, also demonstrates an apparent increase in the second zone for SMV-G6 as compared to SMV-Ia-75-16-1 and G3.

Data are supplemented in the Appendix in support of the results.

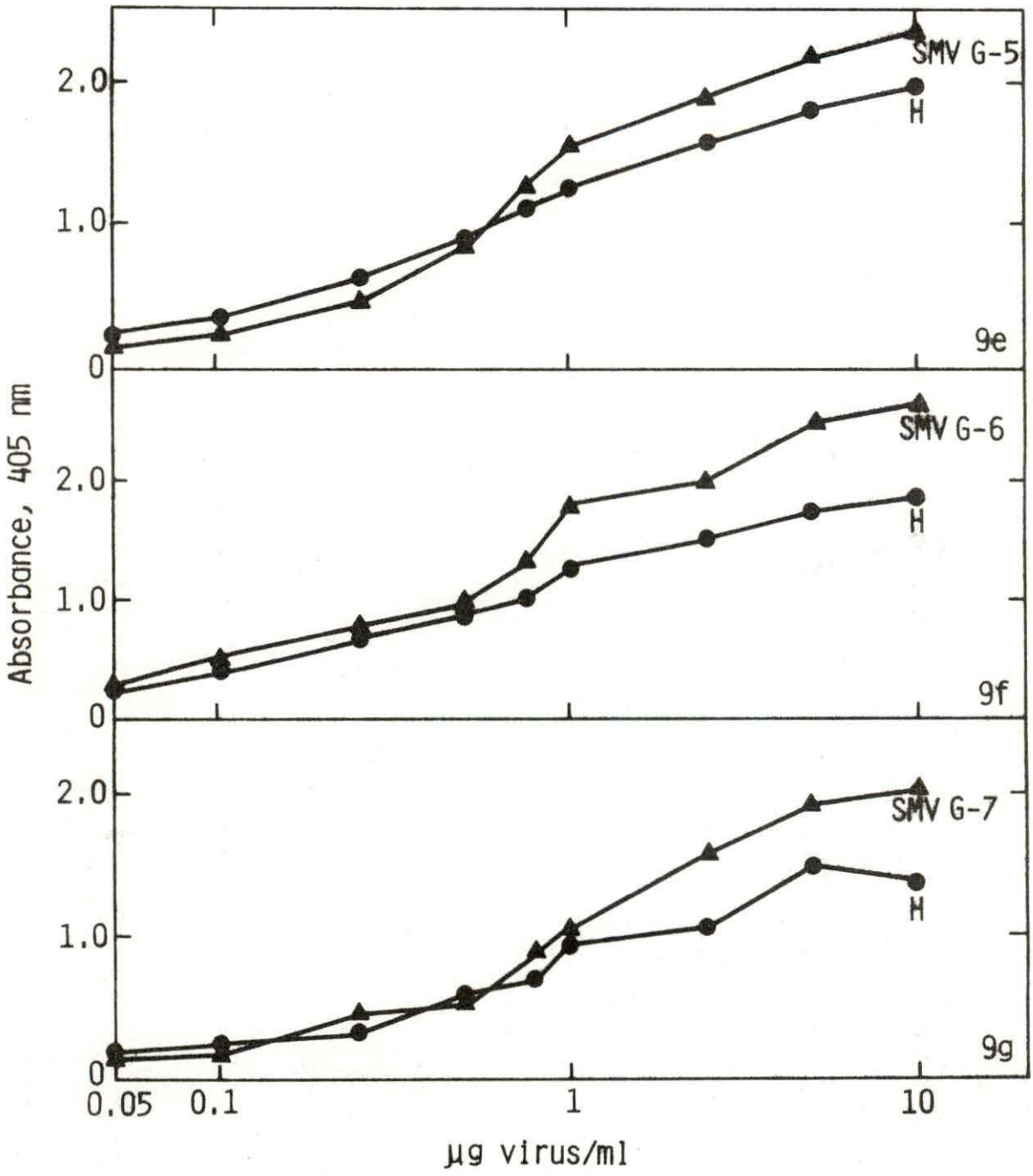


Figure 9 continued.

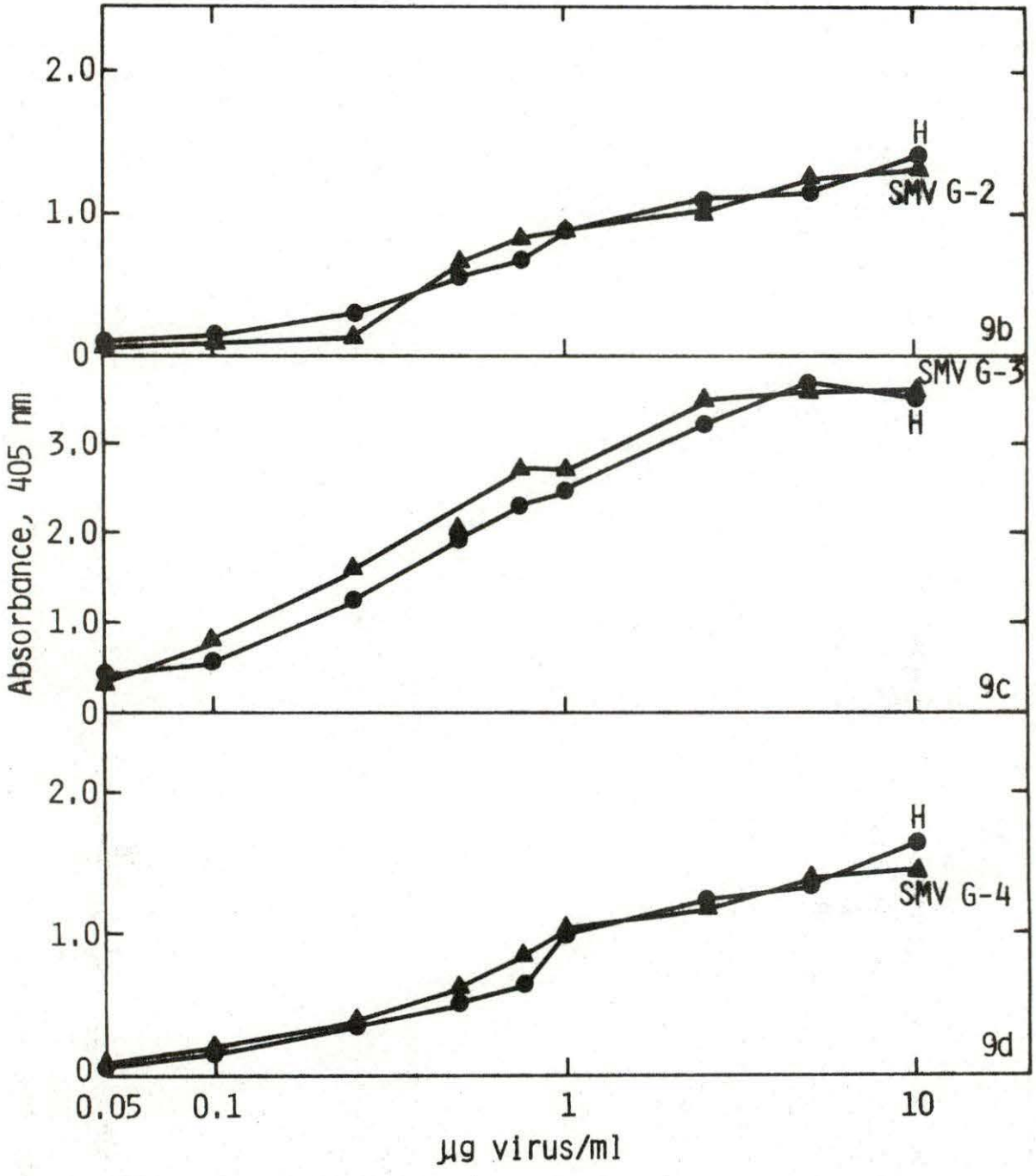
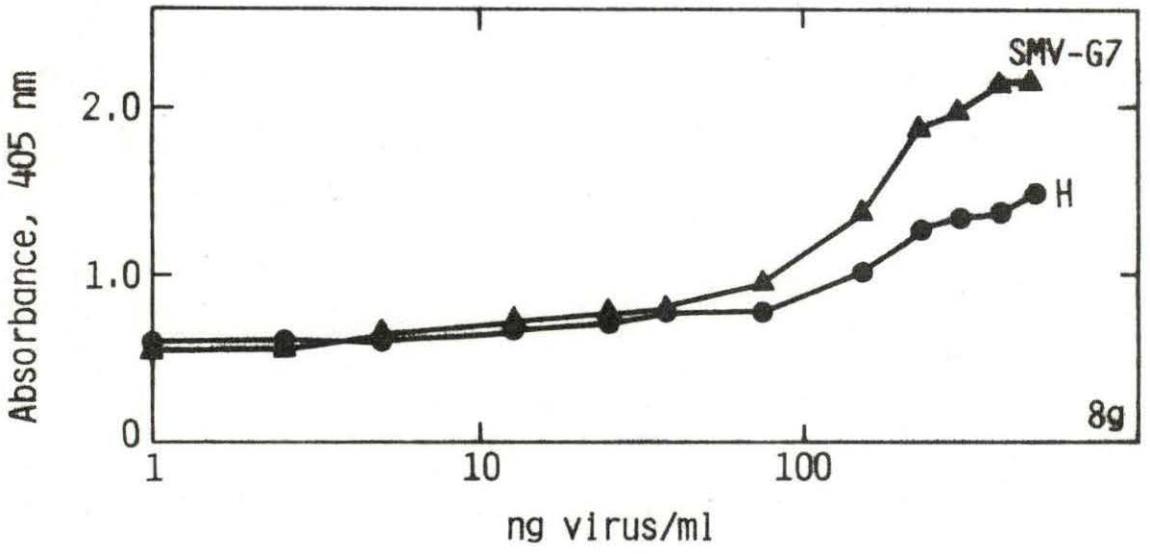


Figure 9 continued.


Absorbance, 405 nm
2.0
1.0
0
1
10
100
ng virus/ml
SMV-G7
H
8g

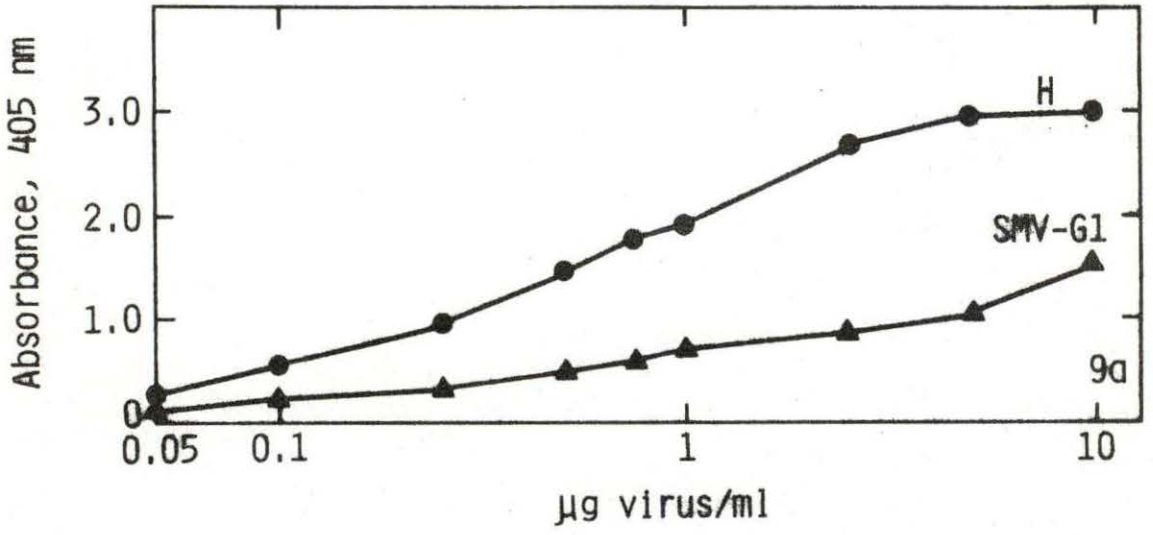

Absorbance, 405 nm
3.0
2.0
1.0
0
0.05
0.1
1
10
µg virus/ml
H
SMV-G1
9a

Figure 8. Continued.

Figure 9. Effect of various concentrations of homologous and heterologous antigens on the A_{405} obtained with 1 μg/ml coating antibody and optimal concentration of conjugate IgG (see text) in bead ELISA, using purified SMV-Ia-75-16-1 (H, ●-●) and heterologous SMV strains (SMV G1-G7, ▲-▲). Data are presented in Table 12 in the Appendix.

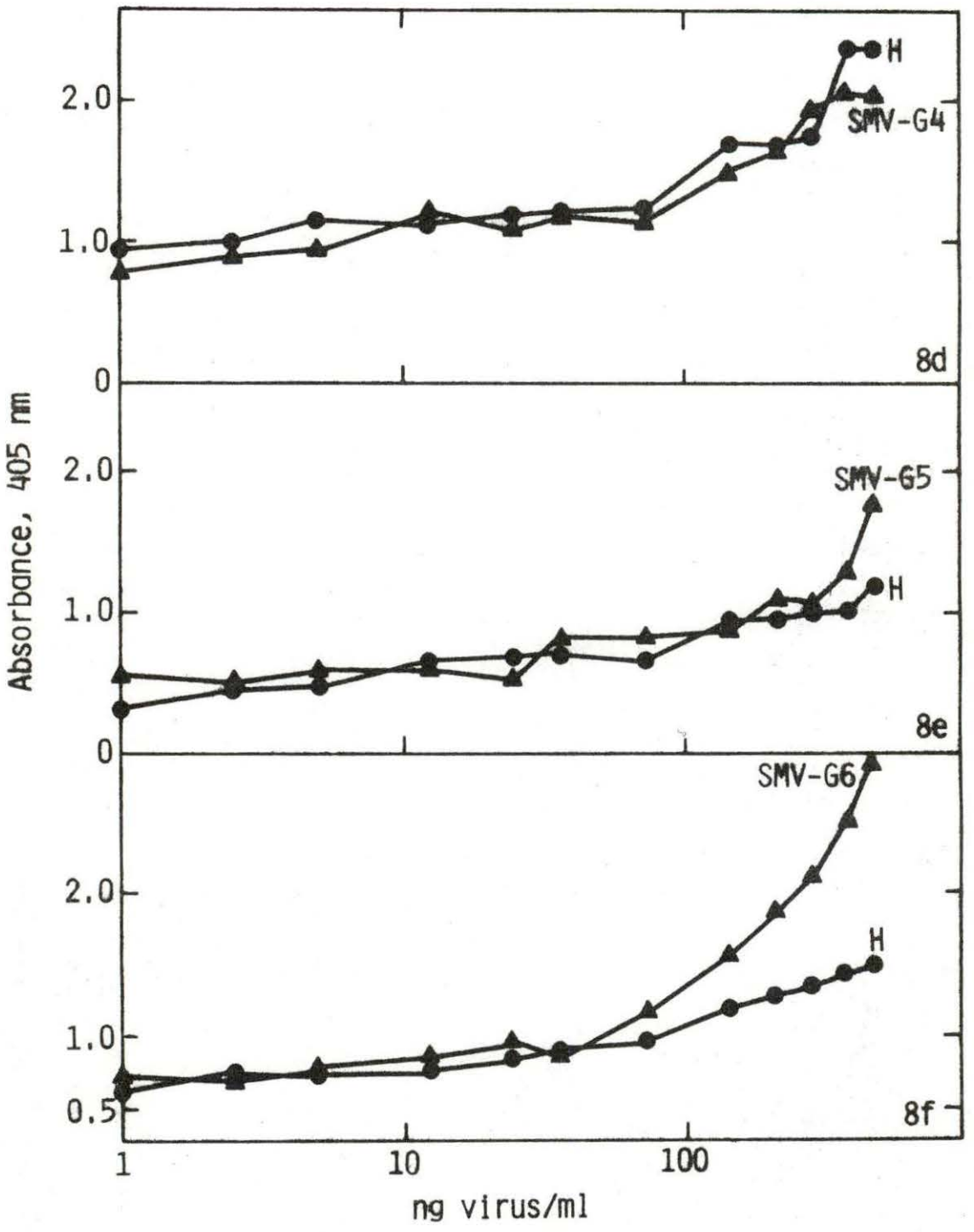


Figure 8 continued.

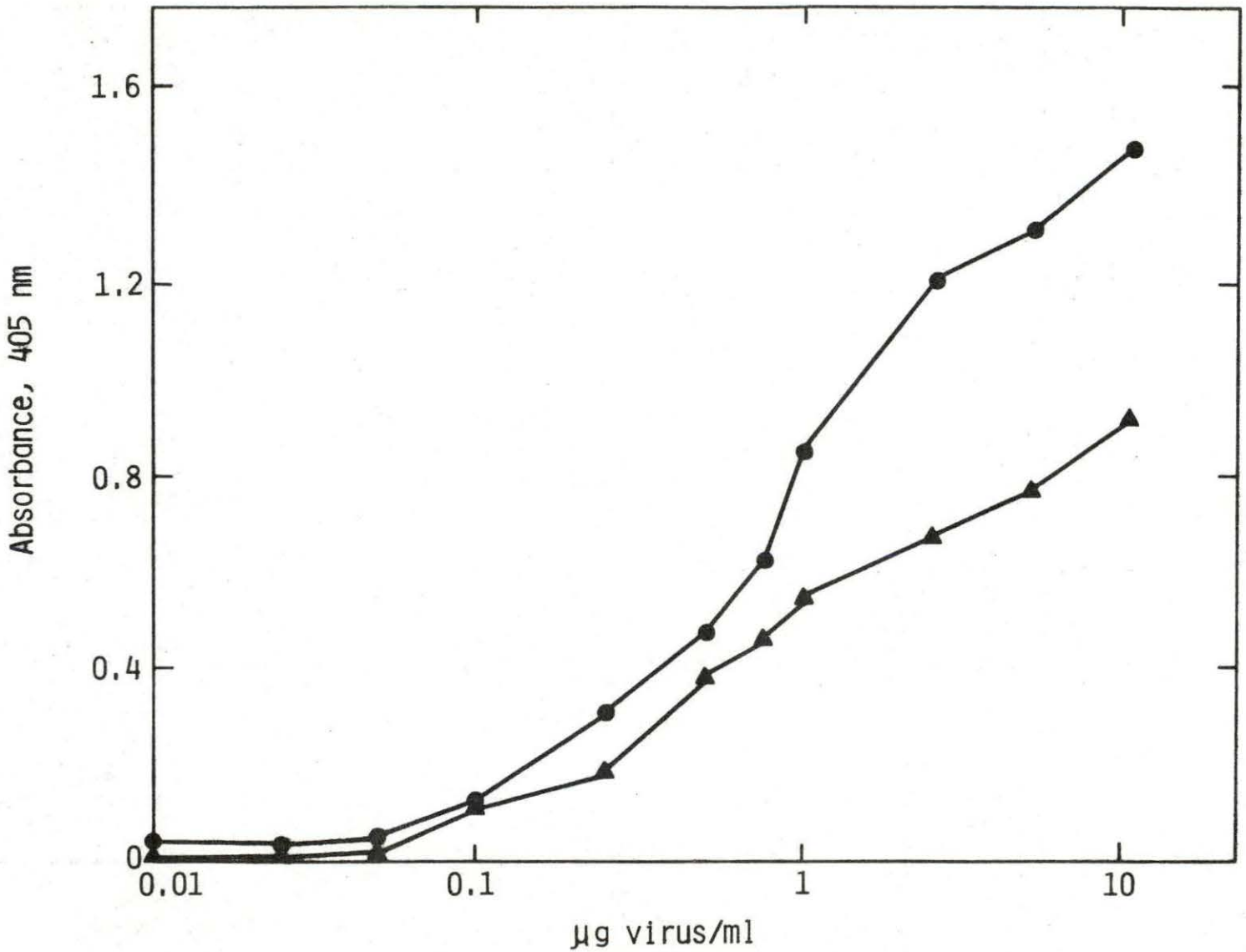

Absorbance, 405 nm
1.6
1.2
0.8
0.4
0
0.01
0.1
1
10
µg virus/ml

Figure 7. Effect of size of polystyrene bead on bead ELISA for detection of SMV. Absorbance means from parallel experiments using 6.5 mm (●-●) and 3.2 mm (▲-▲) beads are shown. Data are presented in Table 9 in the Appendix.

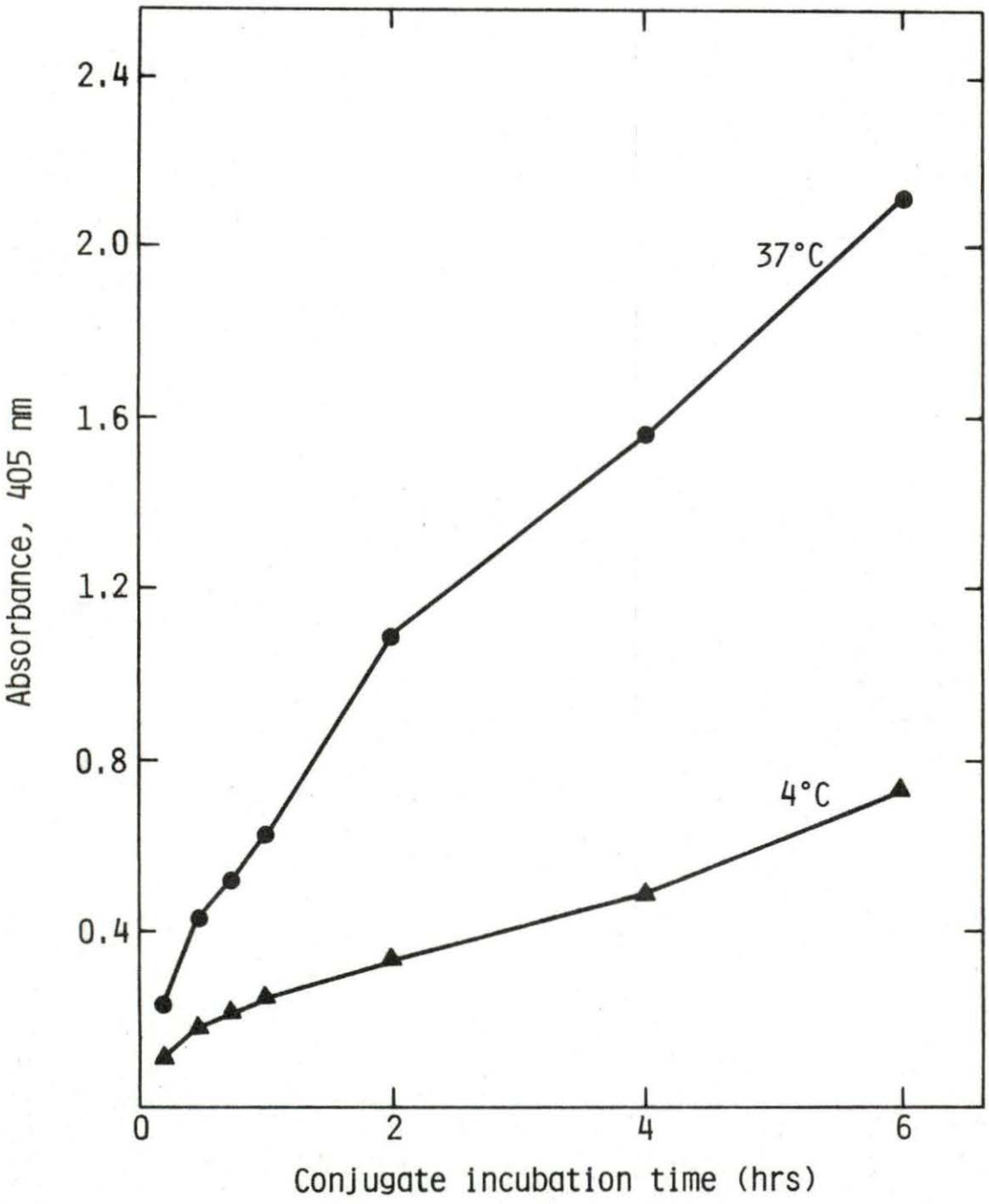

2.4
2.0
1.6
1.2
0.8
0.4
Absorbance, 405 nm
37°C
4°C
0
2
4
6
Conjugate incubation time (hrs)

Figure 6. Effect of conjugate IgG incubation temperature on bead ELISA for detection of purified SMV. Absorbance means from parallel experiments at 37°C (●-●) and 4°C (▲-▲) are shown. Data are presented in Table 8 in the Appendix.

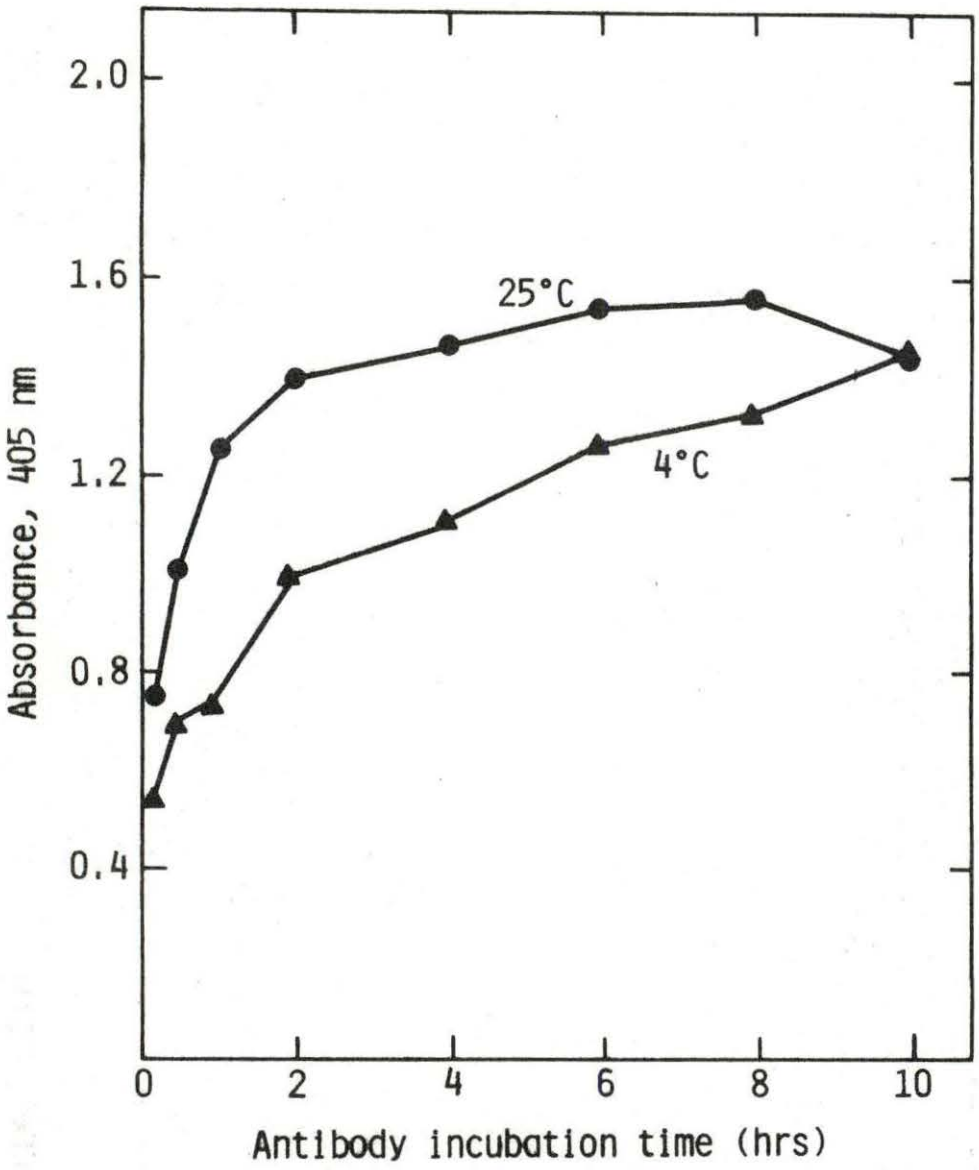

2.0
1.6
1.2
0.8
0.4
Absorbance, 405 nm
25°C
4°C
0
2
4
6
8
10
Antibody incubation time (hrs)

Figure 2. Effect of antibody incubation time and temperature on bead ELISA for detection of purified SMV. Absorbance means from parallel experiments at 25°C (●-●) and 4°C (▲-▲) are shown. Data are presented in Table 3 in the Appendix.

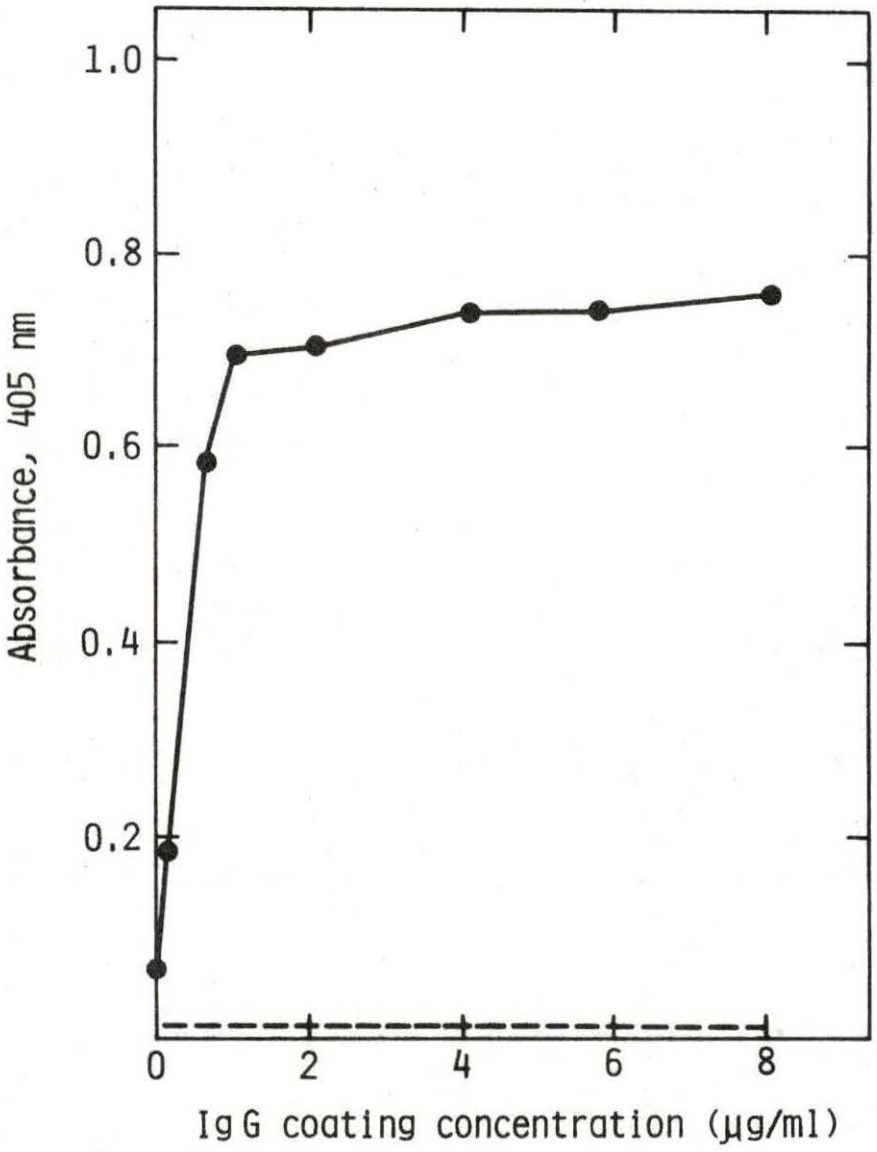

1.0
0.8
0.6
0.4
0.2
Absorbance, 405 nm
0
2
4
6
8
IgG coating concentration (µg/ml)

Figure 1. Effect of coating antibody concentration on bead ELISA for purified SMV (o-o). Dotted line indicates absorbance level for PBS-Tween controls. Data are presented in Table 2 in the Appendix.

Table 13. Percent virus particles observed in the two UV-absorbing regions of sucrose density gradients for Ia-75-16-1, SMV-G3 and G6[a]

Virus isolate	Percent virus particles in[b] Zone[c] A	Zone B
Ia-75-16-1	51%	49%
SMV-G3	60%	40%
SMV-G6	44%	56%

[a]The procedures for running density gradient centrifugation were as described in the text.

[b]Average percentage from five sucrose density gradients for each virus isolate. Virus concentrations of each isolate were spectrophotometrically ($A_{260\ nm}$) the same. Three SMV isolates were centrifugated concurrently in Beckman SW 27 rotor.

[c]See legends of Figure 10.

Table 12. Effect of various concentrations of homologous and heterologous antigens on the A_{405} in bead ELISA[a]

	Virus (μg/ml)								
	10	5	2.5	1	0.75	0.5	0.25	0.1	0.05
H	2.91[b]	2.96	2.61	2.00	1.79	1.44	0.97	0.58	0.28
SMV-G1	1.49	1.07	0.82	0.62	0.54	0.44	0.34	0.21	0.13
H	1.46[c]	1.18	1.11	0.88	0.68	0.58	0.29	0.13	0.09
SMV-G2	1.35	1.28	1.07	0.89	0.85	0.68	0.11	0.07	0.07
H	3.61[c]	3.78	3.32	2.50	2.37	1.91	1.30	0.58	0.39
SMV-G3	3.73	3.73	3.61	2.79	2.82	2.20	1.61	0.84	0.38
H	1.68[c]	1.37	1.23	0.96	0.66	0.50	0.35	0.14	0.07
SMV-G4	1.49	1.41	1.22	1.03	0.85	0.64	0.35	0.16	0.08
H	1.97[b]	1.79	1.59	1.24	1.10	0.89	0.63	0.34	0.22
SMV-G5	2.38	2.19	1.89	1.55	1.27	0.85	0.47	0.21	0.11
H	1.89[d]	1.76	1.54	1.30	1.04	0.90	0.68	0.42	0.27
SMV-G6	2.71	2.51	2.04	1.79	1.32	1.08	0.80	0.49	0.32
H	1.40[c]	1.49	1.06	0.91	0.70	0.56	0.30	0.21	0.16
SMV-G7	2.06	1.92	1.59	1.03	0.89	0.54	0.45	0.16	0.13

[a]Test conditions as described in the text.

[b]Average $A_{405\ nm}$ values of six replications from three parallel experiments. H: SMV-Ia-75-16-1.

[c]Average $A_{405\ nm}$ values of four replications from two parallel experiments.

[d]Average $A_{405\ nm}$ values of ten replications from five parallel experiments.

A_{405}							
H	SMV-G4	H	SMV-G5	H	SMV-G6	H	SMV-G7
2.36[c]	2.02	1.13[c]	1.70	1.41[c]	2.85	1.49[c]	2.10
2.38	2.05	0.93	1.20	1.35	2.43	1.34	2.12
1.69	1.88	0.94	0.98	1.27	2.03	1.29	1.95
1.60	1.60	0.87	1.02	1.20	1.80	1.26	1.88
1.63	1.41	0.85	0.80	1.09	1.47	0.99	1.36
1.18	1.08	0.58	0.76	0.86	1.04	0.73	0.92
1.15	1.16	0.67	0.77	0.80	0.78	0.78	0.79
1.16	1.04	0.67	0.47	0.74	0.84	0.69	0.74
1.10	1.16	0.59	0.59	0.69	0.76	0.68	0.70
1.13	0.93	0.45	0.54	0.66	0.69	0.60	0.65
0.99	0.90	0.43	0.47	0.65	0.63	0.60	0.58
0.94	0.78	0.34	0.59	0.54	0.63	0.60	0.59

Table 11. Effect of various concentrations of homologous and heterologous antigens on the A_{405} in plate ELISA[a]

Virus (ng/ml)	A_{405}					
	H	SMV-G1	H	SMV-G2	H	SMV-G3
500	1.14[b]	1.48	1.69[c]	1.59	1.98[c]	2.38
400	0.98	1.35	1.49	1.24	1.89	1.76
300	0.90	1.25	1.23	1.04	1.71	1.76
225	0.74	0.97	0.76	0.75	1.51	1.72
150	0.68	0.69	0.61	0.65	1.42	1.25
75	0.47	0.46	0.48	0.51	1.23	1.19
37.5	0.36	0.35	0.41	0.38	1.17	1.13
25	0.26	0.30				
12.5	0.21	0.26				
5	0.21	0.22				
2.5	0.17	0.23				
0.5	0.16	0.19				

[a]Test conditions were as d

[b]Average $A_{405\ nm}$ values of experiments; H: homologous SMV

[c]Average $A_{405\ nm}$ values of experiments.

Table 10. Sensitivity of bead ELISA for detection of purified SMV[a]

SMV (μg/ml)	$A_{405\ nm}$[b]
25	1.687
10	1.678
5	1.366
2.5	1.230
1	0.959
0.75	0.655
0.5	0.495
0.25	0.347
0.1	0.141
0.05	0.068
→ 0.025	0.049
0.01	0.047
0.005	0.038
0.001	0.028
0.00	0.025

[a]Coating antibody concentration at 1 μg/ml; conjugate IgG tested at 2.5 μg/ml.

[b]Average of four replications; absorbance value greater than twice that of a negative control is assessed as positive. The arrow indicates the limit of sensitivity. Polystyrene beads with 6.5 mm diameter were used.

Table 8. Effect of conjugate IgG incubation temperature on bead ELISA

Incubation at	Conjugate IgG incubation time (hr)						
	6	4	2	1	3/4	1/2	1/4
37°C	2.12[a]	1.58	1.10	0.63	0.54	0.44	0.26
4°C	0.75	0.50	0.33	0.24	0.21	0.19	0.12

[a]Average $A_{405\ nm}$ values of three replications; coating antibody concentration at 1 μg/ml; SMV-Ia-75-16-1 antigen and conjugate IgG tested at 2.5 μg/ml.

Table 9. Effect of size of polystyrene bead on bead ELISA

SMV (μg/ml)	Bead size (diameter)	
	6.5 mm	3.2 mm
10	1.51[a]	0.94
5	1.32	0.77
2.5	1.21	0.68
1	0.86	0.55
0.75	0.64	0.46
0.5	0.49	0.38
0.25	0.32	0.20
0.1	0.12	0.11
0.05	0.05	0.03
0.025	0.03	0
0.01	0.03	0

[a]Average $A_{405\ nm}$ values of duplicate samples; coating antibody concentration at 1 μg/ml; conjugate IgG tested at 2.5 μg/ml.

Table 6. Determination of optimal conjugate IgG concentration for bead ELISA

	Conjugate IgG concentration (μg/ml)								
	30	20	15	5	2.5	1	0.5	0.25	0.1
SMV (A)	17.6[a]	12.76	10.12	3.85	2.16	1.03	0.49	0.25	0.12
PBS-Tween (B)	0.66	0.50	0.36	0.12	0.05	0	0	0	0
Binding ratio[b] (A/B)	26.67	25.52	28.11	32.08	43.2	–	–	–	–

[a]Average $A_{405\ nm}$ values of duplicate samples; coating antibody concentration at 8 μg/ml; SMV-Ia-75-16-1 antigen tested at 3 μg/ml; PBS-Tween was used for controls.

[b]Definition of binding ratio described in the text.

Table 7. Effect of conjugate IgG incubation time on bead ELISA

	Conjugate IgG incubation time (hr)								
	10	8.5	6	4	2	1	3/4	1/2	1/4
SMV	1.12[a]	0.96	1.01	0.84	0.59	0.40	0.30	0.25	0.14
PBS-Tween	0.02	0.01	0.02	0.01	0	0	0.01	0	0

[a]Average $A_{405\ nm}$ values of three replications; coating antibody concentration at 1 μg/ml; SMV-Ia-75-16-1 antigen and conjugate IgG tested at 2.5 μg/ml; PBS-Tween was used for controls.

Table 4. Effect of antigen incubation time and temperature on bead ELISA

Incubation at	Antigen incubation time (hr)								
	14	10	8	6	4	2	1	0.5	0.25
25°C	1.86[a]	1.82	1.70	1.64	1.24	0.85	0.60	0.26	0.06
4°C	0.81	0.61	0.60	0.48	0.34	0.24	0.09	0.08	0.02

[a]Average $A_{405\ nm}$ values of three replications; coating antibody concentration at 1 μg/ml; SMV-Ia-75-16-1 antigen and conjugate IgG tested at 2.5 μg/ml.

Table 5. Effect of antigen incubation buffer on bead ELISA

Buffer	Virus concentration (μg/ml)							
	10	5	2.5	1	0.75	0.5	0.25	0.1
PBS-Tween	0.85[a]	0.76	0.66	0.58	0.41	0.34	0.26	0.18
Borate	0.17	0.14	0.14	0.13	0.12	0.12	0.14	0.12

[a]Average $A_{405\ nm}$ values of duplicate samples; coating antibody concentration at 1 μg/ml; conjugate IgG tested at 2.5 μg/ml.

Table 2. Effect of coating antibody concentration on bead ELISA

	Antibody concentration (μg/ml)							
	8	6	4	2	1	0.5	0.1	0
SMV[a]	0.78[b]	0.76	0.75	0.72	0.70	0.59	0.19	0.08
PBS-Tween	0.00	0.00	0.001	0.00	0.003	0.008	0.005	0.003

[a]Purified SMV-Ia-75-16-1 concentration at 2.0 μg/ml; conjugate IgG tested at 1:1000 dilution.

[b]Average A_{405} nm values of duplicate samples; PBS-Tween was used for controls.

Table 3. Effect of antibody incubation time and temperature on bead ELISA

	Antibody incubation time (hr)							
Incubation at	10	8	6	4	2	1	0.5	0.25
25°C	1.46[a]	1.57	1.56	1.47	1.4	1.26	1.08	0.75
4°C	1.46	1.33	1.26	1.11	0.93	0.74	0.70	0.55

[a]Average A_{405} nm values of three replications; coating antibody concentration at 1 μg/ml; SMV-Ia-75-16-1 and conjugate IgG tested at 2.5 μg/ml.

APPENDIX

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SUMMARY

The suitability of two different polystyrene solid phases for detecting soybean mosaic virus (SMV) in double-antibody sandwich ELISA systems has been determined. SMV isolate Ia-75-16-1 and its homologous antibody were used to test effects of time and temperature on bead ELISA. Optimum assay conditions, component concentrations, reaction intervals, and the effect of bead size are described. The bead ELISA could detect purified virus at concentrations as low as 25 to 50 ng/ml when optimum test conditions were employed. The detection limit of plate ELISA for SMV is 2.5 ng/ml as reported by other investigators. Shorter operating time and less quantity of enzyme conjugate were employed in bead ELISA than in plate ELISA for detection of SMV at optimal conditions.

Comparisons of these two ELISA procedures for their suitabilities in detecting different strains of SMV by means of a single labelled antibody preparation also are demonstrated. The bead ELISA procedure shows greater discrimination between SMV strain than does the plate ELISA. Among seven SMV strains obtained from the USDA soybean germplasm collection, at least two and possibly three serologically distinct types were identified. SMV strain G1 reacted faintly with antiserum prepared from SMV-Ia-75-16-1 in bead ELISA. SMV strains G2, G3, and G4 were closely related to SMV-Ia-75-16-1. SMV strains G5, G6, and G7 show an unexpected increase of reaction levels at higher heterologous antigen concentrations in bead ELISA. Some possible explanations of this unusual phenomenon are discussed.

distinguished by host range or specific aphid vectors also showed serologic heterogeneity.

The double antibody sandwich bead ELISA showed greater discrimination between SMV strains than plate ELISA. Shorter operating time and less quantity of enzyme conjugate used also favor the use of bead ELISA for virus detection. Other alternative methods for detecting serologically distinct strains of plant viruses have been reported. An indirect ELISA method which uses an antiglobulin enzyme conjugate has been found capable of differentiating between a wide spectrum of TMV strains (Van Regenmortel and Burckard, 1980). Use of heterologous combinations of coating and conjugated antibodies also has been reported to increase the specificity of ELISA (Koenig, 1978; Bar-Joseph and Salomon, 1980).

The specificity of ELISA in differentiating closely related SMV strains, however, limits its use for field detection of SMV. This investigation emphasizes that antiserum prepared against a mixture of serotypes is recommended to insure that both serologically close or distantly related virus strains are reliably detected in routine field tests.

The serological relationships between SMV-Ia-75-16-1 and SMV-G5, SMV-G6, and SMV-G7 cannot be clearly deduced because of the unexpected increase in reaction levels at higher heterologous antigen concentration. The reasons for this unusual increase are not fully understood. Virus aggregation may have caused the increased A_{405} values of the heterologous strain as compared to the homologous reaction as demonstrated (represented by SMV-G6) in this research. It is possible that substantial serological distinction between the homologous and heterologous antigens has caused this contradiction. To verify this, reciprocal tests should be performed when antisera to all seven SMV strains are available. These three particular strains might possess more highly reactive, common, and characteristic antigen determinants for the SMV group than does SMV-Ia-75-16-1. Bar-Joseph and Salomon (1980) have indicated this possibility for the type TMV strain and TMV-A, which caused different immunological responses of rabbits. Further investigations on the base ratios of viral RNAs, amino acid compositions of the coat proteins, and the tryptic peptide maps might detect difference between these SMV isolates. SMV strains G5, G6, and G7 are highly virulent strains, causing systemic necrosis in resistant soybean cultivars (Table 1).

If the resultant three bead ELISA patterns imply three distinct SMV serotypes, a pattern consistent with the virulence of the seven SMV strains (Cho and Goodman, 1979) will be established. Similar observations for watermelon mosaic virus isolates and barley yellow dwarf virus strains also have been reported (Purcifull and Hiebert, 1979; Rochow and Carmichael, 1979). In these reports, virus isolates

SMV (Hill, et al., personal communication, Plant Pathology Dept., Iowa State University), but is not quite equal to the detection limit of plate ELISA, which is 2.5 ng/ml (Hill, et al., 1980b). This is probably due to the fact that less quantity of conjugate was used in bead ELISA. Increased conjugate concentration will apparently increase the sensitivity. However, the potential of enhanced nonspecific reactions at higher conjugate concentrations, as pointed out above, should not be overlooked. By using the optimum test conditions, the bead ELISA can be accomplished within two days, compared to three days for plate ELISA (McLaughlin, et al., 1980b). Incubation temperature (room temperature and 37°C used in bead ELISA and 4°C used in plate ELISA) probably accounts for this difference, since Yang and Kennedy (1979) have reported that the ELISA method could be accomplished at shorter periods when the reagents were incubated at higher temperatures.

Studies of heterologous reactivities of SMV strains by the ELISA technique have shown that there are at least two and possibly three serologically distinct types of SMV. This is the first observation of differences in serological relationships among SMV strains. SMV strain G1 is a distinct serotype which reacted faintly with antiserum prepared from SMV-Ia-75-16-1 in bead ELISA. SMV-G1 represents a group of SMV isolates that are least virulent and can only infect the susceptible cultivars, Clark and Rampage (Table 1, Cho and Goodman, 1979). These results also indicate a very close serological relationship exists between SMV-Ia-75-16-1 and SMV-G2, SMV-G3, and SMV-G4. The virulence of these three strains as described by Cho and Goodman is intermediate.

conjugate IgG (1-5 μg/ml) was required in bead ELISA than in plate ELISA (25-60 μg/ml) (Hill, et al., 1980b) for detection of SMV. The reasons for this disagreement are not fully understood. It is probable that transferring individual beads into new tubes for each test step and the spherical geometry of the bead have certain effects in reducing nonspecific reactions.

The kinetics of conjugate binding followed similar curves as described by other workers (Engvall, et al., 1971; Engvall and Perlmann, 1972; McLaughlin, et al., 1980b). Maximum binding occurred after 6 hr of incubation at 37°C (Table 7). The result also verified that incubation at 37°C as used by Clark and Adams (1977) and many investigators was more efficient than at 4°C for effective conjugate IgG and antibody binding. It has been proposed that conjugation with enzyme causes spatial impairment or steric hindrance in the combining sites and, as a result, the avidity of the antibodies decreases (Engvall, et al., 1971, Koenig, 1978). Use of conjugate with highly specific antibody therefore is favored to achieve greater sensitivity. Phillips, et al. (1980) has recently reported an effective treatment for quantitation of conjugate-specific activities for use in immunoassay. Use of different sizes of beads affected the reaction levels in bead ELISA (Figure 7). The result indicates that increasing the diameter of beads by a factor of two almost concurrently doubled the reaction levels (Table 9).

The bead ELISA is sensitive, enabling assay of the viruses at concentrations as low as 25 to 50 ng/ml in purified preparations (Table 10). This sensitivity approaches that of RIA for detecting

styrene plates over a 6-8 hr incubation period without noticeable decline of reaction levels.

Kinetic studies of antigen binding to beads revealed that binding increased linearly until a saturation level was reached (Figure 3). This result correlated with those of previous workers (Hollinger, et al., 1971; Pesce, et al., 1977; Hertl and Odstrchel, 1979; McLaughlin, et al., 1980b). The reactions probably follow the same kinetics as described by Hertl and Odstrchel (1979), where the rate is proportional to the amount of immobilized antibody and to the instantaneous concentration of available unbound antigens. PBS-Tween was found superior to borate buffer for antigen incubation (Figure 4). It also has been demonstrated to be sufficient for inhibiting nonspecific adsorption of immunoreagents (Bullock and Walls, 1977).

Binding of conjugate is thought to be restricted to conditions that favor antibody-antigen interactions. The practical variables at this stage in most cases are the concentration of conjugate and the time of incubation. Use of an optimum conjugate concentration has been suggested (Hill, et al., 1980b; Ruitenberg, et al., 1976). At the optimum concentration, the absorbance values of negative conjugate controls are negligible whereas the reaction levels are still appreciable. Calculation of binding ratios (Table 6) clearly selected an optimum concentration for use in ELISA. The enhanced nonspecific reaction at higher conjugate IgG concentrations was negligible in bead ELISA (Figure 5), compared to that effect on plate ELISA (Hill, et al., 1980b). Therefore, from the calculations of binding ratios, less

DISCUSSION

Virus specific antibody prepared by acid sucrose density gradient centrifugation provides enhanced specificity in both bead and plate ELISA. Nonspecific reactions were minimized when purified virus and antibody as well as a secondary ovalbumin coat were employed. The optimum concentration of antibody coating was 1 μg/ml for both bead and plate ELISA. Use of higher concentrations did not increase the level of final color yield in bead ELISA (Figure 1), since at these concentrations, the protein apparently saturated the polystyrene.

Adsorption of antibody to polystyrene beads was relatively rapid and followed a curve (Figure 2) similar to that reported by Hollinger, et al. (1971) for antibody to Australian antigen. No significant increase in binding of antibody occurred following a 2 to 6 hr period of adsorption. This observation is not in accordance with the reports of McLaughlin, et al. (1980b) using microtitration plates and Pesce, et al. (1977) using Falcon polystyrene tubes as the solid phase. They reported that the ELISA technique has an optimal time (1 hr and 2.25 hr incubation time, respectively) during which the adsorbed IgG is immunoreactive and this reactivity decreases with increasing incubation time. This observed difference may be due to variations in leakage of antibody from the polystyrene during incubation as pointed out by Lehtonen and Viljanen (1980). They also reported that variation of leakage may be due to the differences in the properties of the polystyrene used. In this respect, polystyrene beads seem to be capable of binding antibody more firmly than poly-

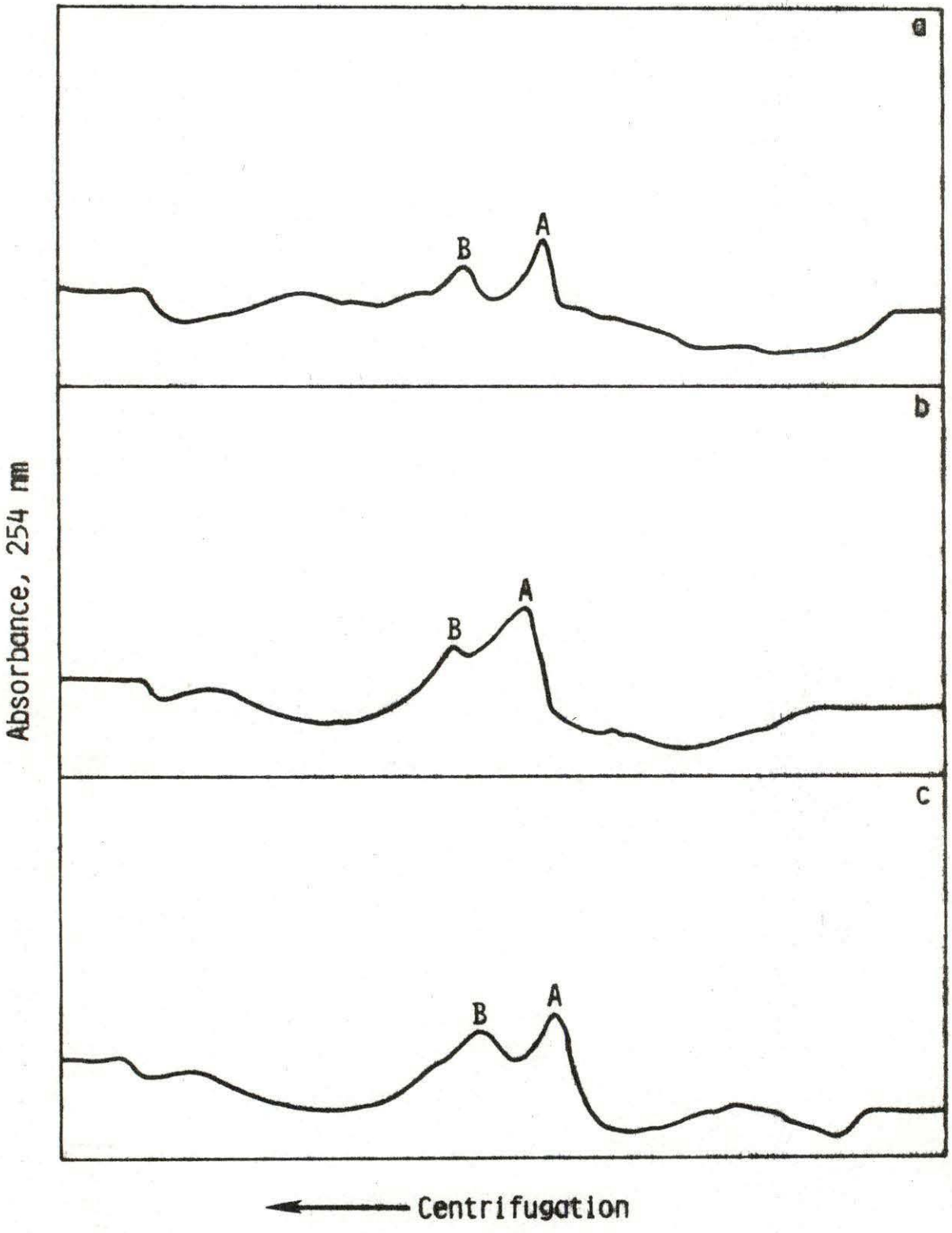
a
b
c
A
B
A
B
A
B
Absorbance, 254 nm
Centrifugation

Figure 10. Sedimentation characteristics of three SMV strains demonstrated by absorption profiles of linear 5-40% sucrose density gradients. Virus concentrations were adjusted spectrophotometrically (A_{260}) to the same (200 μg/ml). Profiles of (a) an Ia-75-16-1, (b) a SMV-G3, and (c) a SMV-G6 preparation. A: zone containing nonaggregated virus particles; B: zone containing aggregated virus particles.