Monoclonal Antibody-based Biotin–Avidin ELISA for the Detection of Soybean Mosaic Virus in Soybean Seeds

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SUMMARY

Two monoclonal antibodies (M-Ab) specific for different epitopes on particles of soybean mosaic virus (SMV) were used in a double-antibody sandwich ELISA (M-Ab ELISA). The non-isotopic immunoassay, which used a biotinylated second antibody and an avidin–alkaline phosphatase detection system to detect SMV in soybean seed extracts, was compared with a polyclonal antibody-based solid-phase radioimmunoassay (SPRIA). M-Ab ELISA detected less than 10 ng of SMV/ml and was more sensitive than the SPRIA which detected 25 ng SMV/ml. Furthermore, M-Ab ELISA required less than 36 h for seed sample assays and only 5–5 h for assays involving purified virus, whereas SPRIA required 3 or 4 days. When seeds from 33 field plots, in which 0%, 30%, and 50% of the soybean plants had been inoculated with SMV, were assayed by both systems, results of the two tests correlated for 31 of 33 (94%) seed samples. This suggests that dual-site biotin–avidin M-Ab ELISA systems have potential utility for routine screening of seed samples.

INTRODUCTION

ELISA (Lister, 1978; Hill et al., 1981; Chen et al., 1982) and solid-phase radioimmunoassay (SPRIA) systems (Bryant et al., 1982, 1983; Hill et al., 1984) have been developed for the detection of soybean mosaic virus (SMV) in soybean seeds. Although the sensitivity of the double-sandwich ELISA systems is quite high, their utility for quantitative assays of a large range of naturally occurring SMV strains is limited (Chen et al., 1982) due to putative alterations in the antibody combining site (Koenig, 1978). SPRIA systems using a 3H-labelled detecting antibody are sensitive and possess a broad cross-reactivity for different SMV isolates (Bryant et al., 1983; Hill et al., 1984) but require disposal of radioactive waste liquids and frequent conjugation because of decreasing specific activity of the labelled detecting antibody during storage (J. H. Hill, unpublished).

Monoclonal antibodies (M-Ab) produced by somatic cell hybridization (Köhler & Milstein, 1975) have great potential for plant virus detection (Gugerli & Fries, 1983; Diaco et al., 1984; Hill et al., 1984) as well as serological differentiation (Briand et al., 1982; Diaco et al., 1983; Gugerli & Fries, 1983; Halk et al., 1984; Hsu et al., 1984). The objectives of this research were to prepare a highly sensitive, non-isotopic, M-Ab-based immunoassay that could be used to detect SMV antigen in infected seeds. Unlike the immunoassays utilizing M-AbS developed by other researchers, which use polyclonal antisera to capture virus particles (Gugerli & Fries, 1983; Hsu et al., 1984), our assay relies on two M-AbS specific for different epitopes on the virus particle. The dual M-Ab system (M-Ab ELISA) has the advantage over the mixed polyclonal-M-Ab systems of obviating the need for continued production of polyclonal antisera and provides for the use of standardized conditions in different laboratories.

Because conjugation of antisera with large enzyme molecules can cause alterations in the immunoreactivity of the antibodies, especially to heterologous isolates (Koenig, 1978; R. Diaco, unpublished), we examined the potential of a biotin–avidin detection system. Its small size (mol. wt. 244) and the gentle conditions required for coupling it to proteins (Bayer et al., 1979) make
biotin an attractive marker for immunoglobulin molecules. Biotinylation allows binding of several biotin molecules to a single protein (Hoffmann et al., 1978) and, even after extensive substitution of amino groups in antibody molecules by biotin, antigen-binding capacity is not modified (Guesdon et al., 1979). The assay we describe is capable of detecting 1 to 5 ng of purified SMV per ml and less than 10 ng per ml when in the presence of seed extract.

Field-collected seed samples were assayed by M-Ab ELISA and polyclonal antibody-based SPRIA (Bryant et al., 1983). The results of the two tests correlated by 94%, indicating that M-Ab ELISA may be useful for routine screening assays.

**METHODS**

**Virus and antisera.** SMV (Ia 75-16-1) was purified from infected Glycine max (L.) Merr. cv. Williams as described by Hill & Benner (1980). Virus concentration was estimated spectrophotometrically by using $A_{260}^{1%} = 2.4$ (Puricifull, 1966). The M-Ab S1 and S2 used in this study were generated in mice using purified SMV Ia 75-16-1 as the antigen (Hill et al., 1984).

**Purification and biotinylation of IgG.** M-AbS were purified by affinity chromatography of ascites fluid by using Protein A-Sepharose CL-4B (Pharmacia). IgG was bound from solution in 0.1 M-Na$_2$HPO$_4$, pH 8.0, and eluted with 5% acetic acid in 0.15 M-NaCl pH 3.0. IgG-containing samples were neutralized with 0.5 M-Na$_2$OH, and the concentration was determined using $A_{280}^{1%} = 1.4$. The M-Ab S1 was biotinylated by modification of the method of Bayer et al. (1979). Essentially, biotinyl-$N$-hydroxysuccinimide ester (Sigma), dissolved in dimethylformamide, was added to Protein A-purified S1 IgG in a 1:50 (v/v), 10:1 (mol/mol) ratio. The mixture was rotated for 4 h at room temperature on an end-over-end mixer and then kept overnight at 4 °C. Unconjugated biotin was removed by dialysis against three changes of 0.15 M-NaCl in 0.01 M-sodium phosphate (PBS), pH 8.0. A slight precipitation in the dialysate, presumably caused by degraded immunoglobulin, was removed by centrifugation for 5 min at 8740 g in a Beckman Microfuge B. The clarified preparation was then either stored with 0.02% Na$_3$ at 4 °C or mixed with an equal volume of glycerol and stored at −20 °C.

An indirect ELISA was performed on the biotinylated and unlabelled S1 M-Ab to determine the effect of biotinylation on antibody activity. The indirect ELISA was performed in 96-well Immulon I microtitre plates (Dynatech Laboratories, Alexandria, Va., U.S.A.) by binding 50 μl/well of a 10 μg/ml solution of purified SMV in 0.05 M-carbonate-bicarbonate (coating buffer) pH 9.6 for 4 h at 37 °C. Unattached virus was rinsed off with wash buffer (0.01 M-PBS pH 7.4 containing 0.05% Tween 20), and unbound protein-binding sites were blocked by incubating 300 μl of blocking buffer (wash buffer containing 1% bovine serum albumin) in each well for 45 min at room temperature. After washing the wells twice, 50 μl samples of serial tenfold dilutions of unlabelled and biotinylated S1 M-Ab were added. The plates were incubated for 90 min at 37 °C, washed three times, and then 50 μl of alkaline phosphatase-conjugated rabbit anti-mouse IgG (Sigma, diluted 1:1000 in wash buffer) was added to each well. Following incubation for 90 min at 37 °C, the plates were washed three times, and 50 μl/well of substrate (1 mg/ml p-nitrophenyl phosphate in 10% diethanolamine, pH 9.8) was allowed to react for 90 min at 37 °C. The reaction was terminated by adding 50 μl of 3 M-NaOH per well and the $A_{410}$ was determined using a Dynatech Minireader II.

**Solid-phase radioimmunoassay.** The polyclonal antibody-based SPRIA has been described elsewhere (Bryant et al., 1983). Immunopurified rabbit polyclonal IgG was prepared as described by McLaughlin et al. (1980). Optimum concentrations of capture antibody and $^3$H-labelled IgG, obtained by calculating the binding ratios (Hill et al., 1981), were 2.5 μg available protein per polystyrene bead and 2.5 μg/ml, respectively. The specific activity of the $^3$H-labelled IgG was 675 μCi/mg protein.

**Biotin-avidin ELISA.** Optimum concentration of capture IgG, biotinylated IgG, and alkaline phosphatase-conjugated avidin (Sigma) required for satisfactory assays were determined by calculating the binding ratios (Hill et al., 1981). The capture and biotinylated M-AbS used in this assay were S2 and S1, respectively (Hill et al., 1984). For determination of optimum conditions, or standard curves, purified SMV was diluted in 0.05 M-sodium borate, pH 7.2 with 0.5% sodium metaphosphate [(NaPO$_3$)$_3$] to prevent virus aggregation (Bryant et al., 1983). The optimized M-Ab ELISA for the detection of viral antigen in seed extracts required coating microtitre plates with 100 μl/well of a 2.75 μg/ml solution of purified S2 M-Ab in coating buffer for 90 min at 37 °C. The plates were washed once, unoccupied sites were blocked for 30 min at room temperature, the plates were washed twice more, and then 200 μl samples of purified virus, or seed extracts prepared in borate buffer as described by Bryant et al. (1983), were added to the wells. Tests samples were incubated overnight at 4 °C and washed three times to remove unbound proteins; then, 50 μl/well of a 1-2 μg/ml solution of biotinylated S1 M-Ab was added. Unbound labelled IgG was washed off after 1 h at 37 °C, and 50 μl/well of a 0.25 μg/ml solution of alkaline phosphatase-conjugated avidin, diluted in wash buffer, was added. The enzyme conjugate was reacted for 1 h at 37 °C, and then the plates were washed three times, incubated with 100 μl/well of substrate for 30 min at 37 °C, and the reaction was stopped by adding 50 μl/well 3 M-NaOH. Absorbance values at 410 nm were measured, and the concentration of viral
antigen in seed extracts was determined by interpolation from a standard curve. Reactions were regarded as positive when the $A_{410}$ of the sample was greater than the mean $A_{410}$ of the control (containing no virus antigen) plus three standard deviations; samples with $A_{410}$ values below this level were considered negative.

**Assay of field-collected seed samples.** Thirty-three field plots, each containing 300 plants, were established. Treatments, arranged in randomized complete-block design, were 11 plots each of the soybean cultivar 'Amsoy '71' in which 0%, 30% or 50% of the plants were chosen at random and mechanically inoculated with SMV La 75-16-1 at growth stage V-1 (Fehr & Caviness, 1977). To assay the level of infection in each plot, randomly chosen leaves from 10% of the plants in each plot were indexed for SMV antigen by a polyclonal antibody-based ELISA (Hill *et al.*, 1981) at growth stage R-6 (Fehr & Caviness, 1977). The seeds from each field plot were harvested at maturity, and one sample of 100 seeds from each plot was processed and assayed by both SPRIA and M-Ab ELISA. Seeds were extracted as described by Bryant *et al.* (1983).

**RESULTS**

**Reactivities of biotinylated and non-labelled M- Abs**

Unlabelled and biotinylated M-Ab S1 preparations gave the same dilution endpoint titres in the indirect ELISA (Fig. 1). Furthermore, no difference was seen when using biotinylated M-Ab stored at 4 °C with NaN₃ or mixed 1:1 with glycerol at −20 °C. The small difference in the dilution endpoint absorbance readings (0.75 for unlabelled M-Ab and 0.49 for biotinylated M-Ab at 1 ng/ml virus concentration) may have been caused by slight denaturation of the IgG during labelling or by loss of IgG on the wall of the dialysis membrane. Precipitated (denatured) IgG had been removed by centrifugation before use.

**Biotin–avidin ELISA**

Initial experiments utilizing purified SMV and M-Ab's S1 and S2 defined clear optimum concentrations of S2 capture antibody, biotinylated S1 antibody and alkaline phosphatase-conjugated avidin. In the absence of seed extract, between 1 and 5 ng/ml of purified virus was detected with incubation for 1 h at 37 °C. Known quantities of purified SMV were added to healthy seeds and extracted by standard methods (Bryant *et al.*, 1983). When these extracts were allowed to react in the ELISA for only 1 h at 37 °C, the limit of sensitivity was 25 ng/ml, and the absorbance values were not proportional to virus concentration. However, when the extracts were reacted overnight at 4 °C, the assay detected less than 10 ng/ml of virus antigen, and the relationship between virus concentration and absorbance was proportional between 10 and 1000 ng virus/ml (Fig. 2). The overnight incubation step with seed extracts was therefore adopted as standard protocol.

**Assay of field-collected seed samples**

The M-Ab ELISA was examined for applicability to virus detection in field-grown soybean seed and was compared with a polyclonal antibody-based SPRIA. Thirty-three seed lots were assayed, 11 each from plots in which 50% or 30% or none of the plants had been inoculated. Each seed lot was sampled independently, in duplicate, and the results were averaged to give an estimation of the concentration of SMV in seed from each field plot. As estimated by M-Ab ELISA, the seed samples from 50% inoculated plots contained from 240 to 2000 ng of SMV/ml, with an average of 872 ng/ml; the 30% inoculated plots contained from 20 to 600 ng/ml, with an average of 361 ng/ml; SMV could not be detected (i.e. less than 5 ng/ml) in those from uninoculated plots.

Polyclonal antibody-based ELISA of leaf samples estimated that between 20 and 73% of the plants in the 50% inoculated plots were infected, 10 to 50% of the plants in the 30% inoculated plots were infected, and 0 to 20% of the plants in the 0% inoculated plots contained virus.

Results from the SPRIA correlated well with those of M-Ab ELISA. Twenty of the 22 field samples found positive by M-Ab ELISA also gave positive results in the SPRIA. Eleven of the field samples were negative by both SPRIA and M-Ab ELISA. The limit of sensitivity of the SPRIA was found to be about 25 ng/ml. Estimates of SMV content in the seed samples with use of SPRIA were 124 to 436 ng/ml, with an average of 224 ng/ml for the 50% inoculated plots, and 0 to 302 ng/ml, with an average of 147 ng/ml, for the 30% inoculated plots. No virus was detected in seed from the plots not inoculated.
Indirect ELISA comparing the reactivities of unlabelled M-Ab S1 (○), biotinylated S1 stored with NaN₃ at 4 °C (△) and biotinylated S1 mixed 1:1 (v/v) with glycerol and stored at -20 °C (★). Dilutions of the three M-Ab preparations were added to wells of microtitre plates coated with 0.5 μg/well soybean mosaic virus. The absorbance at 410 nm for bound antibody was determined, after washing, by reaction with alkaline phosphatase-conjugated rabbit anti-mouse IgG followed by addition of substrate. The absorbance values for control samples plus three standard deviations were consistently less than 0.08.

Fig. 2. Relationship between absorbance at 410 nm in M-Ab ELISA and concentration of purified soybean mosaic virus (SMV) in extracts of healthy seed prepared in 0.05 M-sodium borate pH 7.2. Capture S2 M-Ab and biotinylated S1 M-Ab concentrations were 2.75 μg/ml and 1.2 μg/ml respectively. Extracts were incubated with capture S2 M-Ab for 15 h at 4 °C. The absorbance values for control samples plus three standard deviations were consistently less than 0.08.

The two seed lots that gave contrasting results by SPRIA and M-Ab ELISA were from plots in which 30% of the plants had been mechanically inoculated with SMV. When indexed at growth stage R-6 with the polyclonal antibody-based ELISA, it was estimated that 27% and 23% of the plants in these field plots contained SMV. Furthermore, seed from these field plots were estimated to contain 100 and 450 ng of SMV/ml, respectively, by M-Ab ELISA.

Storage of ELISA plates

To determine the effect of storage on capture antibody-coated plates, M-Ab ELISA was performed using freshly prepared ELISA plates and plates that had been coated with capture antibody, blocked, washed and stored dry at 4 °C for 6 weeks. In one such experiment, the mean difference in absorbance values between freshly prepared and aged plates was 0.014 A₄₁₀ units. This suggests that plates can be coated well in advance of seed preparation with no loss of sensitivity or discrimination in the assay.
Sensitivity and reliable immunoassays for plant viruses are important for routine screening or indexing systems, especially when materials may be exported to foreign countries that prohibit importation of virus-infected germplasm. The SPRIA developed in our laboratories requires 3 to 4 days for completion (Bryant et al., 1983). We attempted to shorten the assay time considerably with M-Ab ELISA. Binding of capture IgG to microtitre plates was optimized for a 90 min incubation at 37 °C. It is feasible to use higher concentrations of immunoglobulins when using M-Ab because further production of specific IgG is greatly simplified, whereas the immunopurified polyclonal antiserum used in SPRIA (McLaughlin et al., 1980) is more difficult to produce.

With purified virus in the absence of seed extract, it was possible to perform a sensitive and proportional assay by incubating the antigen with capture antibody-coated plates for 1 h at 37 °C. However, when purified virus was mixed with seed extract and reacted only 1 h at 37 °C, the assay was less sensitive and displayed unsatisfactory proportionality. This is similar to results obtained previously with both polyclonal and monoclonal antisera in SPRIA (Bryant et al., 1983; Hill et al., 1984) and is probably due to inhibition of antibody diffusion and binding caused by the high concentrations of oils, host tissues and proteins present in soybean seed extracts. To circumvent this problem and achieve satisfactory levels of sensitivity and proportionality, it was necessary to extend the incubation time with seed extracts to 15 h (overnight) at 4 °C. Even with this prolonged incubation, a given antigen concentration gave absorbance values that were about 50% of those obtained with purified virus alone. Biotinylated S1 M-Ab was optimized to react with immobilized antigen for 1 h at 37 °C and the incubations of enzyme conjugate and substrate for 1 h and 30 min, respectively. Therefore, the entire assay can be reliably performed in less than 36 h. Under these conditions, the assay can detect less than 10 ng SMV/ml of seed extract, and the reaction is proportional between 10 and 1000 ng/ml. For laboratory work with purified or partially purified preparations of SMV, M-Ab ELISA can be performed in 5-5 h or in only 3-5 h if plates are coated with capture antibody and blocking buffer in advance. Plates prepared in this way can be stored for up to 6 weeks with no loss in assay sensitivity or discrimination. This is extremely useful for routine monitoring of virus purification procedures or when large numbers of seed lots will be monitored over a period of several weeks.

The discrepancy observed between M-Ab ELISA and SPRIA with two seed lots may be due to differences in sensitivity or possibly the form of SMV antigen in the seeds. The M-Ab ELISA may recognize viral subunits or degraded virus epitopes not recognized by the polyclonal antibody-based SPRIA. Alkaline phosphatase-conjugated polyclonal antibodies specific for barley yellow dwarf virus (BYDV) do not recognize dissociated BYDV (R. Diaco, unpublished). The presence of incomplete or dissociated SMV antigen in the seeds may therefore be responsible for the positive responses by M-Ab ELISA. This would also explain why M-Ab ELISA gave higher estimations of viral content in all the positive seed lots than did SPRIA (data not shown).

Virus antigen was found in plants from six of the 11 uninoculated field plots. The virus presumably had been introduced into these plots by aphid spread. Between 2 and 20% of the plants in these plots were estimated to be virus-infected; however, when tested by M-Ab ELISA and SPRIA, none of the seed lots from these plants was found to contain detectable levels of SMV. This is probably because secondary spread by aphids (Abney et al., 1976; Lucas & Hill, 1980) did not occur until late in plant development. Seeds from plants infected late in development often contain low concentrations of virus (Bowers & Goodman, 1979). These concentrations were seemingly undetectable by our assays.

When SMV-specific M-Abs S1 and S2 were reacted against SMV isolates G1 to G5, no differences were detected (Hill et al., 1984). Although S1 and S2 distinguish different epitopes on SMV particles, these epitopes are common to all SMV strains tested (Hill et al., 1984). Our results demonstrate that an effective M-Ab ELISA can be developed for detection of SMV antigen in soybean seeds. The assay requires less time and is more sensitive than a SPRIA using
rabbit polyclonal antiserum. We also demonstrate the utility of using a biotin–avidin detection system for plant virus detection. The ease of use and high sensitivity obtained with this system will allow application to a wide variety of assays.


REFERENCES


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