

Isolation of Specific Antibody to Plant Viruses by Acid Sucrose Density Gradient Centrifugation

M. R. McLaughlin, G. R. Bryant, J. H. Hill,
H. I. Benner, and D. P. Durand

First author: former graduate research assistant, Department of Plant Pathology, University of Illinois, Urbana 61801. Second and fifth authors: graduate research assistant and professor, Department of Bacteriology, Iowa State University, Ames 50011. Third and fourth authors: associate professor and Laboratory Technician III, Department of Plant Pathology, Seed and Weed Sciences, Iowa State University, Ames.

Present address of senior author: Department of Agricultural Biology, The University of Tennessee, Knoxville 37901.

Address reprint requests to J. H. Hill.

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ABSTRACT

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Antibody preparations specific to southern bean mosaic (cowpea strain) and soybean mosaic viruses were isolated by reaction of virus antigen with immune serum followed by acid dissociation of immune complexes. Dissociated entities were separated by using acid sucrose density gradient

centrifugation to prepare immunologically reactive specific antibody. Antibody to soybean mosaic virus was electrophoretically pure and remained reactive after lyophilization.

Additional key word: serology.

Rapid immunoassay systems such as EIA (enzyme-linked immunosorbent assay) and RIA (radioimmunoassay) require gamma globulin fractions of immune sera. Enhancement of the sensitivity and specificity of these systems may be possible by using only the virus-specific antibodies from the gamma globulin fractions. Immunopurified antibody can be easily prepared by dissociation of immune complexes. Separation of specific immune complexes from unreactive serum elements coupled with washing and careful adjustment of the chemical and physical environments of the antigen-antibody mixture (1,5,7,8,10) is a well known procedure. Van Regenmortel and Hardie (11) used these methods to prepare specific antibody to tobacco mosaic virus (TMV). They used ultracentrifugation to sediment TMV following acid dissociation of immune complexes and recovered 90-100% pure antibody from the supernatant fluid. We report isolation of virus-specific antibody by sucrose density gradient centrifugation as a useful modification of the Van Regenmortel and Hardie technique. We have successfully applied this modified method to the isolation of specific antibody to icosahedral and long flexuous rod-type viruses.

MATERIALS AND METHODS

Virus and antiserum production. In work done at the University of Illinois, the cowpea strain of southern bean mosaic virus (CP-SBMV, kindly provided by J. P. Fulton, University of Arkansas, Fayetteville 72701) was purified as described by McLaughlin (6). Immune serum was produced in a rabbit after a regimen of intramuscular and subcutaneous injections of purified virus emulsified with Freund's incomplete adjuvant. In work done at Iowa State University, the Ia 75-16-1 isolate of soybean mosaic virus (SMV) was purified as described by Hill and Benner (3). Immune sera were produced in New Zealand white rabbits after intramuscular injections of purified virus emulsified with Freund's incomplete adjuvant.

Contamination of purified preparations of flexuous rod-type

plant viruses with healthy host antigens was an occasional problem; therefore, all antisera raised to SMV were routinely cross-absorbed with preparations of lyophilized healthy host tissue. Healthy 'Williams' soybean (*Glycine max* [L.] Merr. 'Williams') tissue, equal in grams to four times the volume in milliliters of hyperimmune serum to be cross-absorbed, was triturated 1:1 (w/v) in 0.05 M sodium borate buffer, pH 7.2 (BB) in a Waring blender. Homogenates were dialyzed against 0.01 M sodium borate, pH 7.2, divided into four equal fractions, and lyophilized. Immune serum was added to the lyophilized preparation from one of the four equal fractions and placed at 4 C on a rotary shaker overnight. The mixture was centrifuged at 27,000 g for 25 min, and the supernatant serum was added to a second fraction and the procedure was repeated. After four cycles of cross absorption, the serum was centrifuged for 1.5 hr at 85,000 g followed by addition of 0.5 volume of saturated (NH₄)₂SO₄ (24 C) solution to the supernatant serum to isolate globulins and albumins. After three salt precipitations, the product was resuspended in 0.05 M sodium borate buffer, pH 7.2, containing 0.85% NaCl (BBS), and dialyzed against BBS.

Isolation of virus-specific antibody. Antibody specific for CP-SBMV was prepared by adding purified CP-SBMV to 5 ml of anti-CP-SBMV serum. Purified virus was added in 0.9-mg increments to a total of 7.2 mg. After each addition of virus, the resulting precipitate was collected by centrifugation at 10,400 g for 30 min, and more virus was added to the supernatant serum. Precipitates were combined and resuspended in 0.05 M Tris-HCl, pH 7.2, containing 0.85% NaCl (Tris-saline). The precipitate was washed with Tris-saline, resuspended in 2.0 ml of 0.85% NaCl, and adjusted to pH 2.0 (8) with 1 N HCl. After incubation overnight at 20 C, 1-ml fractions were layered onto linear sucrose density gradients.

For isolation of specific anti-SMV antibody, purified SMV was added in 2-mg increments to 5 ml of serum globulin until a total of 8 mg of virus had been added. After each addition, the mixture was incubated at 37 C for 30 min and the precipitate was collected as described. The precipitates were resuspended in BBS, pooled, and washed twice in BBS. The final pellet was resuspended in 2.0 ml of deionized distilled water and adjusted to pH 3.0 with 0.1 N HCl. After incubation at 20 C overnight, 1-ml fractions were layered

onto linear sucrose density gradients.

Sucrose gradients for separation of CP-SBMV specific antibody were prepared in 0.85% NaCl, pH 2.0, by layering 5 ml each of 5, 10, 15, 20, and 25% sucrose over 7.5 ml of 30% sucrose in a 2.5 × 8.9-cm centrifuge tube. Gradients were equilibrated overnight, loaded, and centrifuged for 2.5 hr at 82,500 g. Gradients were fractionated by using an ISCO Model D density gradient fractionator and UV analyzer. Absorption (A_{254}) peaks were collected and dialyzed against Tris-saline. Precipitates remaining in the fractions were removed by centrifugation, and fractions were analyzed spectrophotometrically and by agar-gel immunodiffusion against purified CP-SBMV and anti-CP-SBMV serum.

Sucrose gradients for separating SMV-specific antibody were prepared in deionized distilled water adjusted to pH 3.0, by layering 5, 9, 9, 9, and 5 ml, respectively, of 5, 10, 20, 30, and 40% sucrose in a 2.5 × 8.9-cm centrifuge tube. After overnight diffusion of the sucrose, gradients were loaded, centrifuged, and fractionated as described. After dialysis against BB or BBS, fractions were analyzed by spectrophotometry and electron microscopy. Serological specificity of the fractions was determined by agar-gel immunodiffusion with antisera raised to healthy Williams soybean tissue (homologous titer, 1:256) and pyrrolidine-degraded SMV (p-SMV) (homologous titer, 1:16) (3). For the latter, fractions to be tested were first treated with an equal volume of 5% (v/v) pyrrolidine in BB. Certain fractions also were tested for reaction to healthy host antigen prepared by grinding tissue 1:1 (w/v) in BB and squeezing the homogenate through cheesecloth. Fractions

containing SMV-specific antibody were pooled and dialyzed against BBS. Any precipitate forming during dialysis was removed by centrifugation before addition of 0.66 volume of saturated $(\text{NH}_4)_2\text{SO}_4$ (24 C) solution to the supernatant liquid. Ammonium sulfate precipitates were collected by centrifugation, resuspended in BB containing 1.4% NaCl, and dialyzed against BB containing 1.4% NaCl. Titters were determined by microprecipitin tests and the specific antibody was lyophilized and stored at -20C.

SDS-gel electrophoresis. The SMV-specific antibody was examined in 8% polyacrylamide gels containing sodium dodecyl sulfate (SDS) (3). Sufficient 0.04 M sodium phosphate, pH 7.2, containing 4% SDS and 4% 2-mercaptoethanol was added to antibody preparations to yield a final concentration of 0.01 M sodium phosphate, 1% SDS, and 1% 2-mercaptoethanol. The product, containing 40–60 μg of protein, was heated in a boiling water bath for 1 min before being layered onto gels. Reduced carboxymethylated bovine serum albumin (4) was used as an internal standard. Comparisons were made with normal rabbit IgG (Sigma Chemical Co., St. Louis, MO 63178) treated in the same manner as for specific antibody. Proteins, run at 8 mA per gel until a bromophenol blue tracking dye reached the bottom of the gels, were stained with aniline blue black. Gels were scanned at 620 nm in a Gilford linear transport coupled to a Beckman Model DU monochromator.

RESULTS

Isolation of CP-SBMV-specific antibody. Antibody specific for CP-SBMV was separated from the acid-dissociated antigen-antibody complex by density gradient centrifugation. Antibody remained in a zone near the top of the gradient while the virus sedimented midway down the gradient (Fig. 1A). Lesser absorption peaks observed further down the gradient were presumed to be polymers of virus particles that had not been completely dissociated from reacting antibody. Purified antibody showed a typical IgG absorption spectrum with a maximum at 280 nm, a minimum at 252 nm, a maximum:minimum ratio of 2.35, and a shoulder at 290 nm. The purified antibody reacted strongly with purified CP-SBMV in immunodiffusion tests. Unfractionated CP-SBMV antiserum did not react with the purified antibody fraction, which suggested that antibody recovered after acid dissociation was not contaminated by viral protein.

Stability of SMV under dissociating conditions. Conditions for isolation of anti-SMV antibody were quite different from those for CP-SBMV. Preliminary tests indicated that SMV dissociated into protein and RNA at pH 2.5–2.7 as determined by loss of birefringence, density gradient centrifugation analysis, and electron microscopy of preparations stained with 2% potassium phosphotungstate, pH 6.8. Density gradient analysis of such preparations (Fig. 1B) revealed a UV-absorbing region near the top of the gradient. Material in this zone reacted with antiserum raised to p-SMV (3) and exhibited an absorption spectrum characteristic of protein. The zone midway down the gradient had an absorption spectrum characteristic of nucleic acid and was unreactive with p-SMV antiserum. No fractions reacted with antiserum prepared to healthy Williams soybean or contained virus particles visible by electron microscopy. Virus dissociation could not be obviated by fixation with formaldehyde (2). Similar results were obtained when SMV was adjusted to pH 3.0 and layered onto sucrose gradients prepared in 0.85% NaCl at pH 3.0, but virus adjusted to pH 3.0 and layered onto gradients prepared in distilled water at pH 3.0 maintained stability and showed no UV-absorbing region at the top of the gradient (Fig. 1C). Two of the three distinct UV-absorbing regions midway down distilled water gradients reacted with p-SMV antiserum, and all contained virus particles as observed with the electron microscope. The major peak contained nonaggregated virus particles, and the two minor peaks contained aggregated particles that sometimes appeared "rope-like." No other fractions contained particles or reacted with p-SMV antiserum, and none of the fractions reacted with antiserum to healthy Williams soybean. Similar results were obtained when virus in BB was centrifuged in sucrose gradients prepared in BB (authors', unpublished).

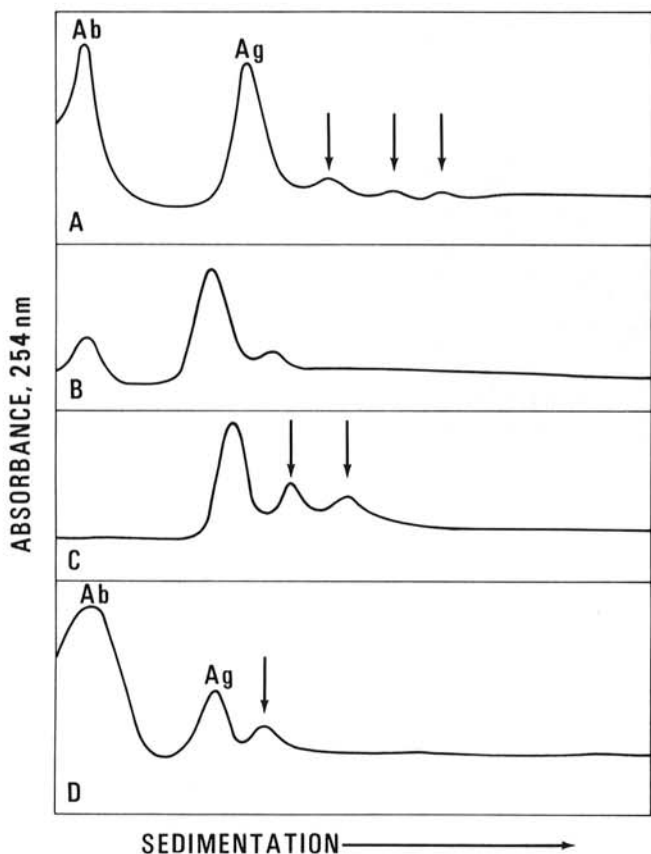


Fig. 1. Absorption profiles of linear (A) 5–30% and (B,C,D) 5–40% sucrose density gradients. Gradients in (A) were prepared in 0.85% NaCl, pH 2.0, and in (B,C,D) in distilled water, pH 3.0. **A,** Separation of acid-dissociated CP-SBMV antigen (Ag) and anti-CP-SBMV antibody (Ab). Arrows mark UV-absorbing peaks containing presumed incompletely dissociated immune complexes. **B,** Profile of an SMV preparation which was adjusted to pH 2.5 before density gradient centrifugation. **C,** Profile of an SMV preparation adjusted to pH 3.0 before density gradient centrifugation. **D,** Separation of acid dissociated SMV Ag and anti-SMV Ab. Arrows in (C) and (D) mark UV absorption peaks containing highly aggregated virus particles. Centrifugation was for 2.5 hr at 25,000 rpm in a Spinco SW27 rotor.

Isolation of SMV antibody. Isolation of the specific antibody was possible because the antigen-antibody complex of SMV could be dissociated at pH 3.0. Antigen-antibody complex dissociated by overnight incubation at pH 3.0 and centrifuged through pH 3.0 sucrose gradients in distilled water yielded a UV-absorbing region at the top of the gradient (Fig. 1D). This fraction, after dialysis against BBS and removal of a small amount of precipitate by centrifugation, reacted with SMV in microprecipitin tests, ring precipitin tests, and gel diffusion tests after treatment of SMV with 5% pyrrolidine. No reaction of this fraction was obtained with p-SMV antiserum, antiserum to healthy Williams soybean, or healthy host antigen, and no virus particles were observed, which indicated that the fraction contained only antibody. Virus particles were observed in the two UV-absorbing regions midway down the gradient. These zones reacted with p-SMV antiserum and were unreactive to healthy host antiserum or healthy host antigen. The major zone contained nonaggregated virus particles, and the minor zone contained aggregated virus particles. These zones had UV spectra characteristic of SMV, while the zone at the top of the gradient had a UV spectrum characteristic of protein.

After concentration of the gradient fraction containing antibody protein by $(\text{NH}_4)_2\text{SO}_4$ precipitation, maximum solubilization was achieved by increasing the NaCl concentration in BB to 1.4%. Titer of the isolated antibody reached 1:1,024 (undiluted IgG

concentration = 2.6 mg/ml) when tested against an antigen concentration of 0.5 mg/SMV ml in microprecipitin tests. The purified IgG was lyophilized, stored at -20°C , and reconstituted at intervals up to 6 mo with no reduction in titer as determined by the microprecipitin test. Preparations routinely have been found to be free of SMV or antihost antibody as determined by serological tests conducted with p-SMV antiserum and healthy host antigen preparations.

SDS-gel electrophoresis. Analysis of purified SMV antibody by SDS-gel electrophoresis (Fig. 2) showed two major bands presumed to be the H (heavy) and L (light) chains of the antibody (9). Relative to bovine serum albumin, the two bands had electrophoretic mobilities similar to those calculated for normal rabbit IgG ($H_{\text{anti-SMV}} = 1.37$, $H_{\text{IgG}} = 1.36$; $L_{\text{anti-SMV}} = 2.25$, $L_{\text{IgG}} = 2.27$). Minor bands above the H chains of anti-SMV antibody were interpreted to be dimer and trimer forms of the H chain on the basis of migration distances and presumed molecular weights.

DISCUSSION

Acid dissociation of virus-antibody immune complexes and subsequent isolation of specific antibody by density gradient centrifugation provides a practical and relatively simple method of rapid purification of virus-specific antibody. The specificity of the antibody recovered should be as good as that of the antigen used in its preparation and isolation.

Factors limiting the success of the technique seem to be: obtaining immune sera devoid of healthy-host antibody and determining conditions for dissociation of the antigen-antibody complex. If the first is a problem, it can be obviated by cross absorption with preparations of lyophilized healthy host tissue. Results with SMV antisera indicate that sera may be cross absorbed in this manner at least 10 times with no increase in volume or reduction in titer to SMV (J. H. Hill, *unpublished*).

Conditions for dissociation of the immune complex must be established for each antigen employed. Conditions useful for CP-SBMV, a relatively stable virus, were established readily. Under the same conditions, SMV degraded into coat protein subunits which remained at the top of sucrose gradients and nucleic acid which sedimented midway down sucrose gradients. Suspension of the SMV immune complex in water with subsequent adjustment to pH 3.0 yielded an anti-SMV antibody that was electrophoretically pure, exhibited electrophoretic properties nearly identical to normal rabbit IgG, and which could be lyophilized for storage with no loss in titer. These results demonstrate that specific antibody to plant viruses can be prepared with relative ease by using techniques and equipment commonly available in plant virology laboratories.

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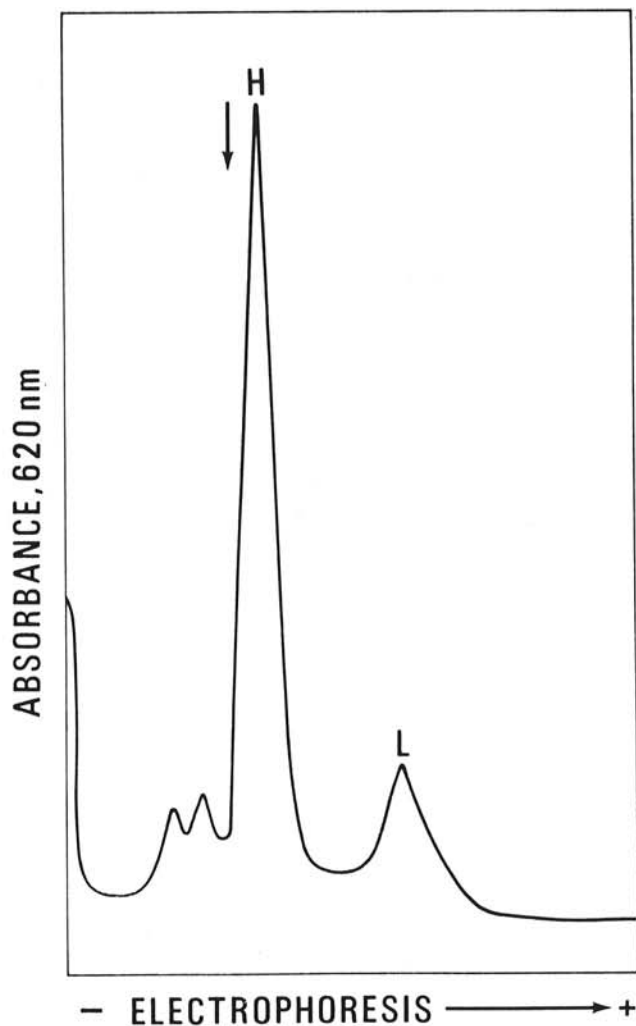


Fig. 2. Absorption profile of anti-SMV antibody in 8% polyacrylamide gels containing SDS. Letters H and L represent the heavy and light chains of the antibody molecule. Absorption peaks trailing the H chain are apparent dimer and trimer of the H chain. The arrow marks the position of reduced carboxymethylated bovine serum albumin included in some gels. Electrophoresis was at 8 mA/gel until a bromophenol blue tracking dye reached the bottom of the gel.

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