Purification, Partial Characterization, and Serological Comparison of Soybean Mosaic Virus and its Coat Protein

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Accepted for publication 16 October 1979.

ABSTRACT

SOONG, M. M., and G. M. MILBRATH. 1980. Purification, partial characterization, and serological comparison of soybean mosaic virus and its coat protein. Phytopathology 70:388-391.

An Illinois isolate of soybean mosaic virus (SMV-IL) was purified from cultivar Kanrich soybean leaves, 16–18 days after inoculation by chloroform-butanol clarification, followed by polyethylene glycol (PEG) precipitation and ultracentrifugation, first through a 30% sucrose solution and then to density equilibrium in cesium chloride. The purified virus absorbed maximally between 258–263 nm and minimally at 244 nm. The ratio of absorption at 260 and 280 nm (A260/A280) of purified virus (not corrected for light scattering) was 0.79. Viewed in the electron microscope, SMV appeared to be homogeneous, unaggregated, and to have a most frequent length of 675–750 nm. Infectious SMV RNA was separated from protein by disruption of virus in 2 M lithium chloride (LiCl). Purified SMV protein migrated as a single band in SDS-polyacrylamide gels (SDS-PAGE) with a molecular weight of 32,150 ± 420 for SMV-IL and 33,075 ± 1,889 for PV-94-ATCC. The antigenic specificity of protein from degraded SMV was not identical to that of intact virus particles. The SMV is identical to SMV-PV-94-ATCC based on serological titer, cross-absorption studies, coat protein molecular weight estimation on SDS-PAGE, and ultraviolet absorption spectra.

A strain of soybean mosaic virus (SMV-IL) that caused severe mosaic symptoms was isolated from cultivar Amsoy soybeans grown in an experimental plot at the Agronomy South Farm of the University of Illinois. The physical properties of SMV-IL isolate were similar to those of a North Carolina isolate (PV-94-ATCC) (3), but we were unable to purify SMV-IL by any of several reported procedures (7,8,11,12). In this paper, we report a new purification procedure which gives a homogeneous preparation of SMV-IL; enables characterization of the whole virus, the purified viral RNA, and protein; and allows preparation of high-titer specific antisera against the virus.

Because studies with potato virus X (PVX) indicated differences in antigenic specificities between whole virus and its coat protein (16), the antigenic specificities of SMV-IL and SMV-PV-94-ATCC and their dissociated protein subunits were compared.

MATERIALS AND METHODS

Virus culture and propagation. An isolate of soybean mosaic virus designated as Illinois severe (SMV-IL) was used in this study. An isolate from North Carolina (PV-94, American Type Culture Collection, Rockville, MD; SMV-ATCC) was used for serological comparisons. Both isolates were maintained in cultivar Kanrich soybean plants at approximately 25 C in a greenhouse and were transferred every 3 wk. The cultivar Kanrich soybean seeds used for propagation were obtained from the Burpee Seed Company, Clinton, IA. The seeds were planted either in steam-sterilized sand or composted soil and the seedlings were selected for size uniformity before inoculation by rubbing the inoculum with a pestle on the primary leaves before the first trifoliolate leaf expanded.

Virus purification. Virus was purified from systemically infected cultivar Kanrich soybean leaves harvested 16–18 days after inoculation. Purification was done at 4 C. Infected tissues were homogenized for 1.5 min in a Waring Blendor in chilled 50 mM sodium phosphate buffer, pH 7.0, containing 10 mM sodium-diethyldithiocarbamate (Na-DIECA) (4 ml of buffer per gram of tissue). The extract was strained through two layers of cheesecloth and clarified by using an 8% final concentration of a 1:1 mixture of *n*-butanol and chloroform, 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 and then 4 g of polyethylene glycol, MW 6,000, (PEG-6000) (Union Carbide Chemical Co., Chicago, IL 60606) were added per 100 ml of extract. After 1-hr of incubation at 4 C, the precipitated virus was collected by centrifugation at 7,000 rpm for 20 min and resuspended in 50 mM sodium phosphate buffer, pH 7.0, containing 500 mM urea and 20 mM Na_2SO_3 (resuspending buffer). The resuspended virus was clarified by low-speed centrifugation and further concentrated by high-speed centrifugation for 3 hr at 27,000 rpm through a 9-ml cushion of 30% sucrose in a Spinco No. 30 rotor. Birefringence was checked by swirling the resuspended virus solution between two crossed polarizing prisms and observed the resulting light patterns.

Further purification was done either by equilibrium density gradient centrifugation in CsCl or by sucrose rate-zonal density gradient centrifugation. CsCl density gradients prepared by mixing 1.8 ml of saturated CsCl (4 C) in resuspending buffer with 3.2 ml of virus solution were centrifuged at 40,000 rpm for 20–24 hr in a Beckman SW 50.1 rotor. The gradients were scanned at 254 nm and fractionated with an ISCO density gradient fractionator. Each fraction was concentrated by high-speed centrifugation and assayed for infectivity on detached cultivar Top Crop bean leaves (10). For routine purification, the virus band was removed from the tube and dialyzed at 4 C for 24 hr against resuspending buffer.

Virus characterization. The purified virus was prepared for electron microscopy by staining with 2% phosphotungstic acid, pH 6.9, and viewed with a JEOL 100 C electron microscope. Analytical ultracentrifugation with a Beckman Model E ultracentrifuge was done at 12,590 rpm and 20 C and photgraphed at 4-min intervals. The ultraviolet absorption spectrum of the purified SMV was measured in a Beckman DBG spectrophotometer. The virus concentration was estimated with the instrument set for the extinction coefficient reported for tobacco etch virus of 2.4 cm^2mg^2 at 261 nm (5).

Isolation of SMV-RNA and protein. SMV-nucleic acid and protein were prepared by the lithium chloride (LiCl) method as described by Francki and McLean (6). The precipitated RNA collected by centrifugation at 5,000 rpm for 15 min in a Sorvall

0031-949X/80/05038804/$03.00/0

refrigerated centrifuge was washed twice with 2M LiCl and dissolved in 10 mM NN-*bis*-(2-hydroxy-ethyl)-glycine, pH 6.8. The RNA concentration was determined at an extinction coefficient of 25 cm^2mg^2 at 260 nm. The Orcinol reagent was prepared according to Shatkin (14). The reagent for the diphenylamine test was prepared according to Burton (4).

The SMV-protein, which remained in solution in 2 M LiCl, was dialyzed overnight in 50 mM borate buffer, pH 8.2, and then centrifuged at 40,000 rpm in a Beckman Ti 50 rotor for 2 hr to remove any remaining virus particles. The protein concentration was determined using an extinction coefficient of 1.0 cm^2mg^2 at 280 nm. The molecular weight of SMV-protein was estimated on 7.5% gel columns according to the method of Weber and Osborn (18). Electrophoresis was carried out at 6 mA/gel for 4 hr. Gels were stained in 0.25% Coomassie brilliant blue in 45% glacial acetic acid with 10% methanol and destained in 7% acetic acid with 5% methanol. The destained gels were scanned at 600 nm with a CGA McPherson Model EU scanner.

Production of antisera and serology. New Zealand white rabbits were used for antisera production. Preimmune serum was collected from each rabbit. Purified SMV in 50 mM borate buffer, pH 8.2 and SMV-protein stabilized by dialysis against 50 mM borate buffer, pH 8.2, containing 0.37% formaldehyde were emulsified 1:1 (v/v) with Freund's complete adjuvant (Difco) and 1 ml of the antigens was injected intramuscularly or intrascapularly into different rabbits weekly for 4 wk. To minimize variability, antigen from the same preparation was injected into the rabbits at each date. Antisera were collected starting 2 wk after the first injection. Serological tests were performed using the precipitin ring and Ouchterlony double diffusion tests (2). Chemically dissociated SMV protein was prepared by treatment of virus suspension with pyridine and SDS (15,16).

RESULTS

Virus purification. The purification procedure described here yielded highly infectious and homogeneous SMV. Generally, infected leaves were chilled for 1 hr at 4 C before purification. Storage of the harvested leaves at 4 C for 9 days or freezing of the infected tissues caused a severe loss of virus. Only about 10% of virus was recovered after the final step of purification.

Precipitation of SMV by PEG (6000) was effective and convenient for the initial concentration of virus from clarified extracts. The concentration of PEG-6000, the presence of NaCl, and the pH of the clarified extract affect the efficacy of virus precipitation. The combination of 4% PEG and 0.3 M NaCl gave the greatest infectivity and cleanest virus preparation as assessed by CsCl equilibrium centrifugation and SDS-polyacrylamide gel electrophoresis. About the same amount of virus was recovered from clarified leaf extract kept at pH 6.0 or pH 6.8. Virtually no virus was recovered when the clarified leaf extract was kept at pH 8.0. One hour of incubation of the extract at 4 C was enough to precipitate most of the virus. Longer incubation, especially overnight, also precipitated some host protein, which remained with the virus preparation even after CsCl equilibrium centrifugation.

Further concentration of virus through a cushion of 30% sucrose solution yielded an amber pellet that was easily resuspended. Birefringence, which is detectable at 100 μg/ml, always was observed at this step. Attempts to further concentrate SMV by a second PEG precipitation did not result in greater purity or higher yield. The partially purified virus gave a single band located 2.2 cm below the meniscus after 24 hr of centrifugation in CsCl.

Virus loss was assessed during each step of purification by infectivity (Table 1). About 40% of the original infectivity was lost at the PEG precipitation step. No infectivity was found in the supernatant liquid following PEG precipitation. Re-extraction of PEG-6000 precipitates recovered some virus. High speed centrifugation caused 20–70% loss of infectivity. The loss of infectivity in CsCl equilibrium centrifugation varied with different preparations and ranged from 20 to 60%. By the end of CsCl equilibrium centrifugation, approximately 80% of the infectivity contained in the starting sample had been lost. Part of the infectivity loss may be due to the presence of urea in the resuspending buffer. The yield of virus was 7–16 mg virus per kilogram of leaf tissue. Purified SMV in urea buffer could be stored for months at 4 C without breakdown or aggregation of virus particles as determined by periodic checking of its ultraviolet absorption spectrum and by electron microscopy.

With slight modifications in the clarification procedure (ie, using 7% *n*-butanol) the purification scheme outlined in Materials and Methods was satisfactory for the PV-94 ATCC isolate. The yield of this isolate, however, was consistently lower than that of the Illinois severe isolate.

Virus characterization. Purified virus preparation gave a typical nucleoprotein spectrum with a maximum absorption between 258–263 nm, a minimum absorption at 244 nm, and a tryptophan shoulder near 290 nm (Fig. 1). The 280/260 nm ratio was 0.74 to 0.83 with an average of 0.79 from 23 experiments. This ratio suggests a nucleic acid content of approximately 6.6% (9). The Schlieren pattern of purified SMV showed a single symmetrical peak. In SDS-polyacrylamide gels, purified SMV consistantly showed a single protein band (Fig. 2). Treatment of the purified SMV with 0.01 μg/ml ribonuclease for 30 min at 37 C caused a complete loss of infectivity.

Electron micrographs of purified SMV in 50 mM sodium phosphate buffer, pH 7.0, showed flexuous rod-shaped particles with very little aggregation. Of 261 particles measured, 42% were between 675–750 nm. A small central hole sometimes was observed in the negatively stained virus particles. At higher magnification, a helical arrangement of protein subunits with a distance of 370 nm (37Å) between the turns of the helix could be seen.

Viral nucleic acid and protein. The nucleic acid prepared by the LiCl method was infectious (five-to-ten lesions per half leaf) and sensitive to ribonuclease. The infectivity was lost after treatment with 0.01 μg/ml RNase for 30 min at 37 C. The nucleic acid preparation gave a positive Orcinol reaction and negative diphenylamine reaction indicating that SMV contains ribonucleic acid. No viruslike particles were observed when the nucleic acid preparations were examined under the electron microscope. The UV absorption spectrum of SMV-nucleic acid preparation had a maximum absorption at 256 nm and a minimum absorption at 228 nm (Fig. 1). The 260/280 nm ratio averaged 2.02 with a range of 1.7–2.3. The yield ranged 63–96% and averaged 74%.

The purified SMV-protein preparation absorbed maximum at 276 nm and minimum at 249 nm with a tryptophan shoulder at 288 nm (Fig. 1). The 280/250 and 280/260 nm ratios ranged 2.13–2.19 and 1.42–1.97 and averaged 2.49 and 1.62, respectively. The protein yield ranged 50–84% and averaged 71.4%. The protein preparation was homogeneous as indicated by a regular subunit structure in the electron microscope. The mean molecular weight of SMV-protein estimated by SDS-polyacrylamide gel electrophoresis from four separate experiments were 32,150 ± 420 for SMV-IL and 33,075 ± 1,889 for PV-94-ATCC.

TABLE 1. Assay of an Illinois isolate of soybean mosaic virus (SMV-IL) on detached bean cultivar Top Crop unifoliolate leaves at different steps of purification from soybean cultivar Kanrich trifoliolate leaves

Purification step	Total volume (ml)	Dilution	Lesions per half leaf[a] (no.)	SMV-IL in sample as % of virus in: Starting material	Previous step
Initial extraction	320	0	21.0	100.0	
Water phase of chloroform-butanol	320	0	17.5	83.0	83.0
PEG precipitation	32	0	105.0	50.0	60.0
Differential centrifugation	3.2	1:3	306.0	43.7	87.4
CsCl equilibrium centrifugation	3.4	1:2	171.0	17.3	39.6

[a] Average number of local lesions from 12 half-leaves in 10 different experiments.

Serological relationship between SMV and SMV-coat protein. The antisera to SMV-IL from early bleeding in two rabbits had homologous titers of 1:1,024 and 1:256 in precipitin ring tests. Both antisera were unreactive against SMV-coat protein; however, prolonged immunization did cause development of cross-reactivity. Five weeks after initial injection, antisera to SMV from the two rabbits attained maximum titers of 1:16 and 1:4 against SMV-coat protein with homologous titers of 1:2,048 in both rabbits. Complete removal of cross reactivity to SMV-coat protein from antiserum to SMV left a residual homologous titer of 1:512–1,024. Complete absorption of SMV antiserum with purified SMV removed all the reactivity to SMV-coat protein. The results suggest that the reactivity of SMV antiserum to SMV-coat protein is not due to the presence of SMV-coat-protien-specific antibody.

Antiserum to SMV-coat protein collected to 2 wk after initial immunization had a homologous titer of 1:64 and a heterologous titer of 1:32 to the SMV virion in one rabbit. Prolonged immunization of this rabbit with SMV-coat protein did not cause significant increase of either homologous or cross-reactive antibody titer. The other rabbit respsonded poorly to SMV-coat protein antigen, finally showing a titer of 1:4 13 wk after initial injection. The results suggest that the antigenic specificities in SMV virions and SMV-coat protein are not identical. The SMV-coat protein prepared by LiCl method was serologically identical to the SDS-disrupted SMV-protein, but partially identical to pyridine-treated SMV protein in Ouchterlony double diffusion test using antiserum to SMV-coat protein LiCl (Fig. 3c).

Serological relatedness of SMV-IL and PV-94-ATCC. Antiserum to SMV-IL collected 4 wk after the initial injection had the same homologous and heterologous titers of 1:2,048 when tested against Illinois severe and PV-94-ATCC isolates in precipitin ring tests. Antiserum to PV-94-ATCC collected at the same time as that of the SMV-IL had the same homologous and heterologous titers of 1:256 to PV-94-ATCC and SMV-IL. Absorption with either PV-94 or SMV-IL against the heterologous antisera completely removed the reactivity against the homologous virus. A fused precipitin band was observed when pyridine-degraded SMV-IL and PV-94-ATCC proteins were tested against antisera to either SMV-IL or PV-94-ATCC isolates in a double diffusion test (Fig. 3b). With antiserum to SMV-IL coat protein a similar fused precipitin line between pyridine-degraded SMV-IL and PV-94-ATCC proteins also was observed (Fig. 3a).

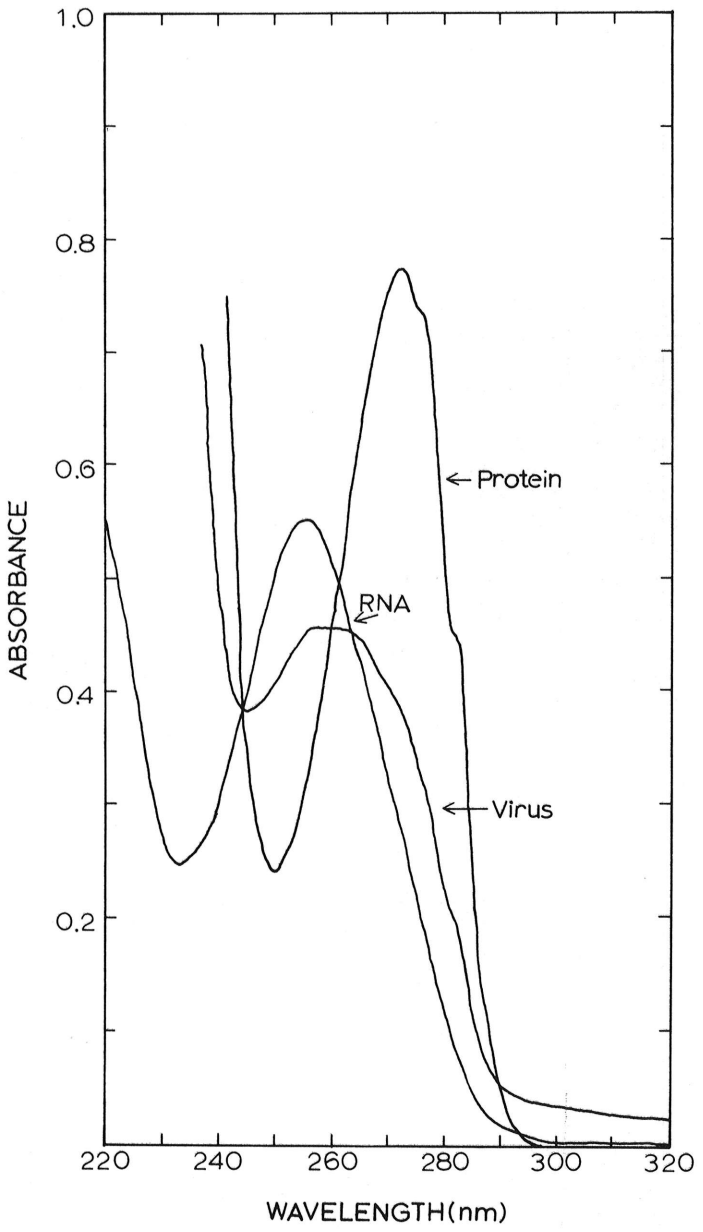

Fig. 1. Ultraviolet absorption spectra of purified SMV and SMV-nucleic acid and protein. The virus was in 50 mM sodium phosphate buffer, pH 7.0, containing 500 mM urea. The protein was in 50 mM borate buffer, pH 8.2, and the nucleic acid was in 10 mM NN-*bis*-(2-hydroxyethyl) glycine, pH 6.8.

DISCUSSION

Soybean mosaic virus (SMV-IL) isolated from cultivar Amsoy soybean in Illinois was difficult to purify and required modifications of established procedures. The modifications included polyethylene glycol precipitation of the virus. Precipitation of SMV with 4% PEG in the presence of 0.3 M NaCl, then pelleting the virus through 30% sucrose solution effectively removed most of the host protein contaminants. Damirdagh and Shepherd (5) reported that 0.5–1.0 M urea was useful in preventing lateral aggregation of viruses in the potato virus Y group. The presence of 0.5 M urea and Na_2SO_3 in the resuspending buffer markedly reduced the aggregation of SMV-IL.

The ultraviolet absorption spectrum and the RNA content of purified SMV-IL are similar to those reported by Ross (12). Han

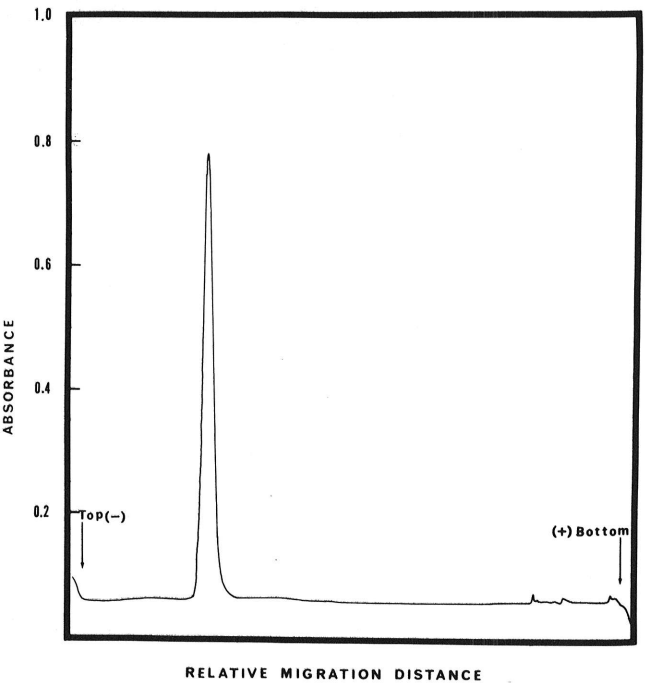

Fig. 2. SDS-polyacrylamide gel electrophoretic profiles of SMV-IL protein. Electrophoresis was done at 6 mA/gel for 4 hr in 7.5% gels using 100 mM phosphate buffer containing 1% SDS and 1% mercaptoethanol. The migration is from top (cathode) to bottom (anode).

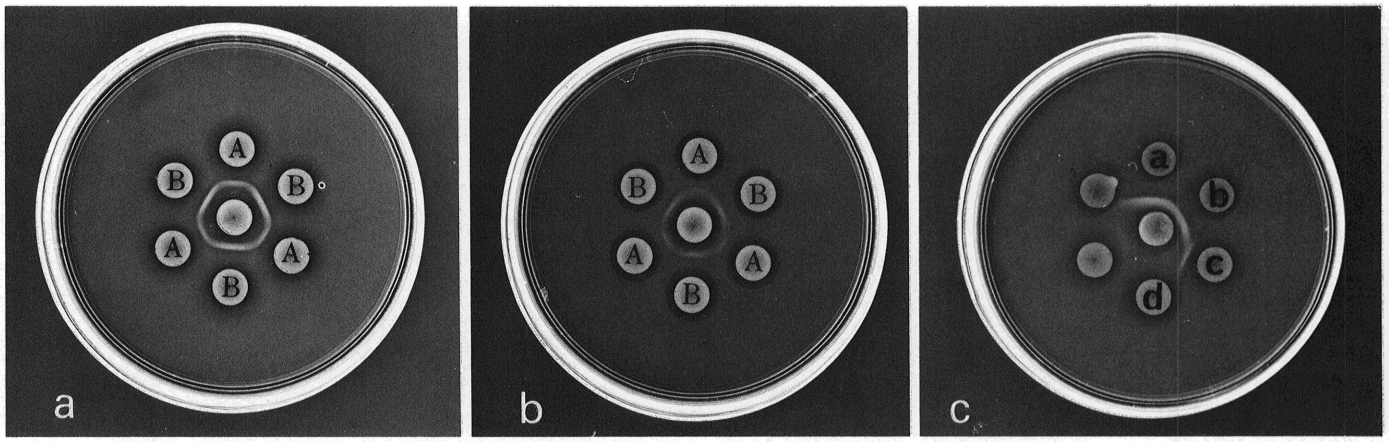

Fig. 3. a, Double immunodiffusion plate with antiserum against SMV-IL protein in the central well and pyridine-degraded SMV-IL (A) and PV-94-ATCC (B) **a,** isolates in the peripheral wells. **b,** The same as that of **a** except antiserum against SMV-IL was placed in the central well. **c,** Double immunodiffusion plate with antiserum against SMV-IL protein in the central well and SDS-dissociated SMV-IL protein (a), LiCl degraded SMV-IL protein (b), pyridine-degraded SMV-IL protein (c), and 50 mM borate buffer, pH 8.2 (d) in the peripheral wells.

and Murayama (8) reported an RNA content of 8.76 to 15%. SMV was sensitive to RNase inactivation. RNase sensitivity has been reported on maize dwarf mosaic virus (MDMV), a member of the PVY group, and it was hypothesized that incomplete protection of the viral nucleic acid by the coat protein permitted the inactivation (13).

The failure of SMV antiserum collected 2 wk after initial immunization to react with SMV coat protein suggests that intact SMV and SMV coat protein have different antigenic specificities. Cross-reactivity between SMV antiserum and SMV coat protein did appear when the immunization was prolonged. The cross-reactivity was not due to the presence of SMV coat protein-specific antibody; complete absorption of SMV antiserum with purified SMV also removed the cross-reactivity. Atabekov et al (1) indicated that the cross-reactivity between barley stripe mosaic virus antiserum and the degraded protein was largely accounted for by the presence of structural unit aggregates in the degraded protein. The cross-reactivities in the SMV and SMV-protein antisera also may possible be due to the presence of small amounts of protein subunit aggregates in the purified protein, which have an antigenic specificity similar to intact SMV. The SMV-coat protein antiserum had a low homologous titer, similar to the corresponding antisera to potato virus X coat protein and tobacco etch virus coat protein (16,17). Unlike PVX and TEV coat protein antisera, which display very little initial cross-reactivity toward the respective intact viruses (16,17), the SMV coat protein antiserum has an initial cross-reactive antibody as high as the homologous titer. The high cross-reactive antibody titer indicates that intact SMV and SMV coat protein share common antigenic specificities.

Symptom severity is the principle difference between SMV-IL and PV-94-ATCC isolates. The consistently lower yield of purified PV-94-ATCC may be attributed to the relatively lower virus concentration in PV-94-ATCC-infected soybeans. Based on serological reactions, both isolates possess similar antigenic specificity. Ouchterlony double diffusion tests of antiserum against SMV-IL coat protein suggest the identity of the pyridine-degraded proteins of these two isolates. Due to the difficulties in obtaining sufficient quantities of purified PV-94-ATCC coat protein, a reciprocal test of antiserum against PV-94-ATCC coat protein was not conducted. No differences were detected in the ultraviolet absorption spectra of both isolates and the molecular weights of their proteins estimated by SDS gel electrophoresis are similar. Further studies on the tryptic peptide maps, the amino acid compositions of the coat proteins, and the base ratios of viral RNAs might detect differences between these two isolates.

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