Purification and Immunological Analyses of Cylindrical-Inclusion Protein Induced by Papaya Ringspot Virus and Watermelon Mosaic Virus 1

S.-D. Yeh and D. Gonsalves

Graduate research assistant and associate professor, respectively, Department of Plant Pathology, Cornell University, New York State Agricultural Experiment Station, Geneva 14456.

We wish to thank D. K. Hummer and E. D. Williams for technical assistance. This study was supported by the U.S. Department of Agriculture under Agreement 58-9AHZ-1-615.

Accepted for publication 1 May 1984.

ABSTRACT

Yeh, S.-D., and Gonsalves, D. 1984. Purification and immunological analyses of cylindrical-inclusion protein induced by papaya ringspot virus and watermelon mosaic virus 1. Phytopathology 74:1273-1278.

Cytoplasmic cylindrical inclusions induced by papaya ringspot virus (PRV) and watermelon mosaic virus 1 (WMV-1), members of the potyvirus group, were partially purified by differential centrifugation. Subunits of cylindrical inclusions were further purified by preparative polyacrylamide gel electrophoresis. UV absorption spectra of cylindrical inclusion proteins (CIPs) of PRV and WMV-1 were typical of protein. Both CIPs had molecular weights of about 70,000. Results from sodium dodecyl sulfate (SDS)-immunodiffusion tests with highly specific antisera showed that PRV CIP and WMV-1 CIP were serologically indistinguishable, whereas they were serologically unrelated to coat protein. The antisera did not react to CIPs of watermelon mosaic virus 2 (WMV-2) and some other potyviruses tested. An indirect enzyme-linked immunosorbent assay (ELISA) method for detecting purified CIP or CIP in plant extract was described. The method could easily detect as little as 1.6 ng of purified CIP per milliliter or 3.2×10^{-5} dilution of the crude extract, using γ-globulin to CIP at the concentration of 1 μg/ml. Results of indirect ELISA confirmed SDS-immunodiffusion tests that PRV CIP and WMV-1 CIP were serologically identical, but not related to WMV-2 CIP. This indirect ELISA method was considered to be a good alternative serological probe to study potyvirus relationships and for virus diagnosis.

Members of the potyvirus group of plant viruses induce the formation of characteristic inclusion bodies in the cytoplasm of infected cells (4,11–14,26). Cytoplasmic inclusions such as pinwheels, scrolls, bundles, and laminated aggregates are considered to be cross sections of a cylindrical (11,12) or conical (1) structure that can be mathematically described as elliptic hyperboloids (28). The cylindrical inclusions can be isolated and the protein subunits with molecular weights of about 67–70,000 are serologically distinct from both coat protein and host proteins (21,22,27,34). Evidence from in vitro translation indicates the cylindrical inclusion protein (CIP) is encoded by viral RNA and represents about 20% of the genome (9,10). Because of the relatively small amount of viral genetic information reflected in the coat protein (10%), the limitation of serology with antiserum to coat protein of potyviruses has been recognized (17,35). Thus, immunochemical studies of cylindrical inclusions could enhance the potential of serology for classifying and diagnosing viruses in the potyvirus group.

Papaya ringspot virus (PRV), one of the major limiting factors in papaya (*Carica papaya* L.)-growing areas, is nonpersistently transmitted by aphids and has been placed in the potyvirus group (20,30). The host range of PRV is very similar to that of watermelon mosaic virus 1 (WMV-1), which is also of great economic importance wherever cucurbits are grown. However, WMV-1 does not infect Caricaceae (33,38,43). Both PRV and WMV-1 react similarly in some cucurbitaceous species that have genes for resistance to WMV-1 (29,43). With antisera to WMV-1 and to coat protein of PRV, no serological difference could be detected between PRV and WMV-1 (18,33,43). Thus, the similarity in host reaction and the identity in coat-protein serology indicate that PRV and WMV-1 are very closely related.

PRV and WMV-1 induce cytoplasmic cylindrical inclusions which appear either as fibrous bodies when examined by light microscopy (4), or as pinwheels, scrolls, and bundles when examined by electron microscopy (4,13,15,32,36,44). In this study, an attempt was made to further characterize PRV and WMV-1 by investigating the serological relationships of cylindrical inclusions. A purification method has been developed and the results of SDS (sodium dodecyl sulfate)-immunodiffusion tests indicate the cylindrical inclusion proteins (CIPs) of these two viruses are serologically indistinguishable.

The use of the enzyme-linked immunosorbent assay (ELISA) as a sensitive and practical method for detecting and assaying plant viruses is well documented (5,6,40). Here we have successfully utilized an indirect ELISA method (2,7,8,24,39) with antiserum to cylindrical inclusion protein to study the serological relationship of PRV and WMV-1. Qualitative results of simple and sensitive indirect ELISA tests were consistent with SDS-immunodiffusion tests.

MATERIALS AND METHODS

Culture of viruses. An isolate of PRV from Hawaii, PRV HA (18), and an isolate of WMV-1 from Florida, WMV-1 F (kindly provided by R. Provvidenti, New York State Agricultural Experiment Station, Geneva 14456), were cultured in zucchini squash *Cucurbita pepo* L. 'President.' To avoid the possibility of contamination with each other, *Carica papaya* L. 'Kapoho Solo' was periodically used to ascertain the purity of PRV HA since WMV-1 F does not infect papaya.

Partial purification by differential centrifugation. Cylindrical inclusions of PRV HA and WMV-1 F were partially purified according to the method of Dougherty and Hiebert (9) with some modifications. Systemically infected tissue of zucchini squash, 25–30 days after inoculation, was homogenized in a Waring blender for 2 min with 2 ml of potassium phosphate buffer (0.5 M, pH 7.5, containing 0.25% sodium sulfite) per gram of tissue, and clarified by adding 0.5 ml of chloroform and 0.5 ml of carbon tetrachloride per gram of tissue. The homogenate was centrifuged in a Sorvall GSA rotor at 1,000 rpm for 5 min and the supernatant was saved. The pellets were reextracted with 1 ml of extraction buffer per gram of starting tissue and recentrifuged at 1,000 rpm for 5 min. The supernatants were combined and centrifuged in a GSA

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

rotor at 2,500 rpm for 5 min. Pellets were discarded and the supernatant was further centrifuged in a GSA rotor at 8,000 rpm for 20 min. The pellets were resuspended in 0.05 M potassium phosphate buffer, pH 8.2, containing 0.1% 2-mercaptoethanol (0.5 ml/g of initial tissue), with a tissuemizer (Tekmar Company) set at 40% of full speed for 30 sec. Triton X-100 was added to a final concentration of 5% and the mixture was stirred at 4 C for 1 hr and then centrifuged in a Beckman 30 rotor at 18,000 rpm for 15 min. The pellets were resuspended in 0.02 M potassium phosphate buffer, pH 8.2, containing 0.1% 2-mercaptoethanol (5 ml/100 g initial tissue) and homogenized with the tissuemizer for 30 sec. Triton X-100 treatment and high-speed centrifugation were repeated once. The pellets were resuspended in small volumes of 0.02 M tris-HCl, pH 8.2 (2 ml/100 g of starting tissue).

To monitor the procedures of partial purification, fractions of supernatants and pellets from different steps of partial purification were electrophoresed in polyacrylamide minislab gels (Idea Scientific Co., Corvallis, OR). Samples were dissociated in 0.1 M tris-HCl, pH 6.8, 2.5% SDS, 5% 2-mercaptoethanol, 5% sucrose, and 0.001% bromophenol blue. Gels and buffers were prepared according to Laemmli (23). Electrophoresis was with constant voltage at 18 V/cm for 1 hr. Protein bands were visualized by staining with Coomassie blue R-250.

Further purification by gel electrophoresis. The proteins of partially purified cylindrical inclusions were further purified by preparative slab-gel electrophoresis. Running buffer and gels (5% stacking gel, 12% separating gel) were prepared as described by Laemmli (23). Partially purified inclusions were degraded by adding 1/3 volume of 4× degradation buffer (0.4 M tris-HCl, pH 6.8, 10% SDS, 20% 2-mercaptoethanol, 20% sucrose, and 0.005% bromophenol blue). The sample was heated in boiling water for 2 min, followed by centrifugation with an International Clinical centrifuge at full speed for 5 min to eliminate insoluble material and then loaded onto the stacking gel (2–3 ml/gel). Electrophoresis was with constant voltage (7 V/cm) for 6–8 hr. The inclusion protein band was visualized by soaking the gel in cold 0.2 M KCl and the protein was eluted from the gel by the method of Hager and Burgess (19). The solution containing the protein was pressed through disk filter paper (diameter 2.3 cm, Whatman 3) by using a 20-ml syringe and an adaptor. After adding 20% (final concentration) trichloroacetic acid the liquid was placed in an ice bucket for 10–15 min to precipitate the protein. The mixture was centrifuged at 10,000 rpm for 10 min in a Sorvall SS 34 rotor. The pellet was rinsed with anhydrous ethyl ether to eliminate residual TCA and resuspended in 6.0 M guanidine-HCl. The purified protein was scanned in a Beckman model 25 spectrophotometer to estimate the yield ($OD_{280\ nm}$ 1.0 as 1 mg/ml) and then dialyzed against 0.12 M guanidine-HCl, 0.02 M tris-HCl, pH 8.2, for at least 18 hr. Purified inclusion protein was stored in a refrigerator (6 C) or a freezer (−20 C).

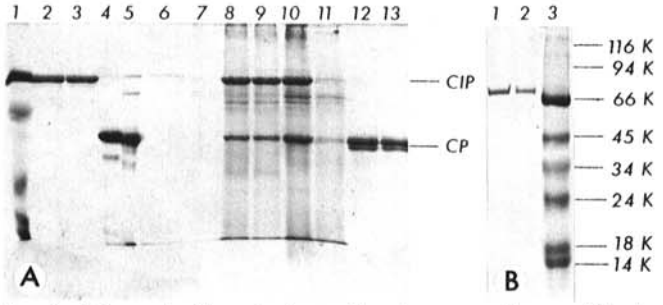

Fig. 1. Polyacrylamide gel electrophoresis to monitor purification procedures of cylindrical-inclusion protein (CIP) and to determine the relative molecular weight of CIPs. **A,** Minislab gel electrophoresis was used to monitor purification steps for the CIP of papaya ringspot virus (PRV). Fractions of different steps were resuspended in 0.02 M tris-HCl, pH 8.2 (8 ml/100 g initial tissue), treated with degradation buffer, and then 15 μl of the aliquot was loaded in each well. Lane 1, protein markers. Lane 2 and 3, gel purified PRV CIP (2 μg). Lane 4 and 5, virus preparations by second and first polyethylene glycol precipitation, respectively, from supernatant after centrifugation at 8,000 rpm for 20 min. Lane 6, healthy tissue processed as 4 and 5. Lane 7, healthy tissue processed the same as CIP partial purification. Lane 8, fraction after the second Triton X-100 treatment. Lane 9, fraction after the first Triton X-100 treatment. Lane 10, fraction from pellet after centrifugation at 8,000 rpm for 20 min. Lane 11, fraction from pellet after centrifugation at 2,500 rpm for 5 min (discarded). Lane 12 and 13, purified coat protein of PRV (2 μg). **B,** Relative molecular weights of CIPs of papaya ringspot virus (PRV) and watermelon mosaic virus 1 (WMV-1) were determined in a 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate and 0.05 M sodium phosphate buffer, pH 7.2. Lane 1, purified PRV CIP. Lane 2, purified WMV-1 CIP. Lane 3, molecular weight markers: lysozyme (14,300), β-lactoglobulin (18,400), trypsinogen (24,000), pepsin (34,700), ovalbumin (45,000), bovine plasma albumin (66,000), phosphorylase b (97,400) and β-galactosidase (116,000); the bands of the last two are not shown.

Molecular weight determination. The molecular weight of cylindrical inclusion protein (CIP) was determined by polyacrylamide gel electrophoresis as described by Weber and Osborn (42) except that the molarity of the electrophoresis buffer was reduced by half (21), and a continuous 7.5% slab gel was used. Lysozyme, β-lactoglobulin, trypsinogen, pepsin, ovalbumin, albumin (bovine plasma), phosphorylase b, and β-glactosidase from Sigma were used as molecular weight markers.

Production of antisera. Purified antigen in 1.0 ml of 0.12 M guanidine-HCl, 0.02 M tris-HCl, pH 8.2, was emulsified with 1.0 ml of Freund's complete adjuvant. Part of the emulsion (about 0.2 ml) was injected into the toe pad of a rabbit, and the rest of the emulsion was injected intramuscularly into the hind legs. The injection procedure was repeated three times at weekly intervals but with Freund's incomplete adjuvant substituted for complete adjuvant. For PRV, 0.2, 0.5, 0.7, and 0.8 mg of purified CIP were injected in order, whereas 1.0 mg of purified WMV-1 CIP was injected each time. Rabbits were bled every 7–10 days after the third injection for a period of 4–7 mo. The titers of antisera were determined in immunodiffusion plates.

SDS-immunodiffusion test. SDS (sodium dodecyl sulfate)-immunodiffusion tests were conducted as described by Purcifull and Batchelor (31). The agar plate consisted of 0.8% Ionagar (No. 2), 1.0% sodium azide, and 0.5% SDS. Purified virus and purified inclusion protein were dissociated in 1% SDS. Crude antigens were prepared from freshly harvested leaves of infected *Cucumis metuliferus* (Naud.) Mey. (Acc. 2459) for PRV or zucchini squash for WMV-1 (43). One gram of tissue was ground in 1 ml of distilled water followed by the addition of 1 ml of 3% SDS. The samples were expressed through cheesecloth and used immediately. Antiserum to PRV CIP collected 70 days after the fourth injection and antiserum to WMV-1 CIP collected 84 days after the fourth injection were used for tests. Undiluted antisera were added to appropriate wells, and the plates were incubated in a moist chamber at room temperature for 24–72 hr.

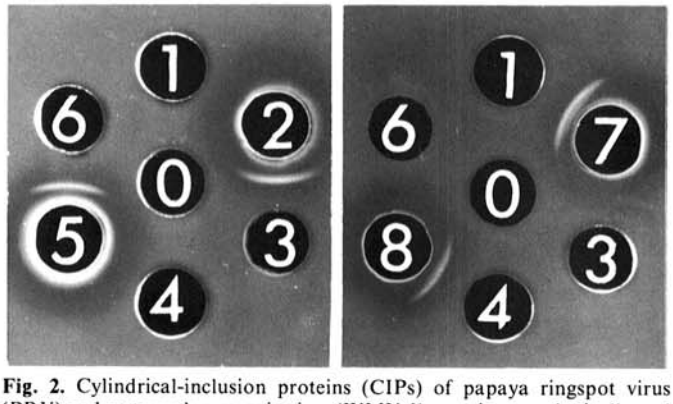

Fig. 2. Cylindrical-inclusion proteins (CIPs) of papaya ringspot virus (PRV) and watermelon mosaic virus (WMV-1) were immunologically and serologically distinct from their coat proteins (CP) as determined by SDS-immunodiffusion tests. Undiluted antisera and 24 μg of PAGE-purified proteins in 60 μl of 1.0% SDS were filled in the wells. Wells contain: 0 = 1.0% SDS, 1 = PRV CP, 2 = antiserum to PRV CIP, 3 = PRV CIP, 4 = WMV-1 CP, 5 = antiserum to WMV-1 CIP, 6 = WMV-1 CIP, 7 = antiserum to PRV CP, and 8 = antiserum to WMV-1.

Nine isolates of PRV (43) (PRV Su-sm, PRV Su-smn, PRV Su-mm, PRV T-Chen, and PRV T-Wang from Taiwan; PRV HA and PRV HB from Hawaii; PRV F-340 from Florida; and PRV ED from Ecuador) and three isolates of WMV-1 (WMV-1 F from Florida, WMV-1 NY from New York, and WMV-1 VG from Virginia) were used in studies of the relationship between PRV and WMV-1. Three isolates of WMV-2 (WMV-2 F from Florida, WMV-2 NY from New York, and WMV-2 C from China) were also used for comparison. Crude antigens from plant tissue infected by other potyviruses (potato virus Y [PVY], tobacco etch virus [TEV], bean yellow mosaic virus [BYMV], bean common mosaic virus [BCMV], cowpea aphidborne mosaic virus [CAMV], blackeye cowpea mosaic virus [BlCMV], lettuce mosaic virus [LMV], and turnip mosaic virus [TuMV]) were used to study their relationships with PRV and WMV-1. All isolates of viruses except PRV were kindly provided by R. Provvidenti (New York State Agricultural Experiment Station, Cornell University, Geneva). Epidermal cells from the infected tissues used in tests were processed by the orange-green (O-G) staining procedure (4) (calcomine orange 2 RS and brilliant green BL were kindly provided by T. A. Zitter, Cornell University, Ithaca, NY 14853) and examined under light microscopy for the presence of cylindrical inclusions.

Indirect ELISA. The double-antibody sandwich (DAS) method of ELISA described by Clark and Adams (6) was initially used to detect CIP, but the DAS test was not evaluated further because of a nonspecific background. However, we were able to eliminate this problem by using the indirect method of ELISA (7,8,24). Purified antigens were resuspended in 0.125 M tris-HCl, pH 6.8, containing 0.5% SDS at 1 mg/ml and stored as a stock. Wells of polystyrene microtitration plates (Immulon™ MICROELISA®, Dynatech Lab., Inc.) were coated with different concentrations of purified antigen diluted in coating buffer (0.05 M sodium carbonate, pH 9.6, and 0.01% sodium azide) and incubated at 6 C for 15–18 hr (or at 30 C for 4–6 hr). Plates were then rinsed three times with phosphate-buffered saline, pH 7.4, containing 0.05% Tween-20 (PBS-T). Gamma-globulin to PRV CIP was prepared from antiserum, which was collected 28 days after the fourth injection, by ammonium sulfate precipitation and DEAE column chromatography (6). Two hundred microliters was added to the wells at 1 μg/ml diluted in enzyme buffer (PBS-T/2% PVP-40/0.2% ovalbumin) and incubated at 30 C for 4 hr. After being rinsed three times with PBS-T, 200 μl of goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma, A-8025) was used at 1/1,000 dilution in enzyme buffer and incubated at 30 C for 4 hr. After further rinsing, the bound conjugate was detected by adding 220 μl of the substrate ρ-nitrophenyl phosphate at 1 mg/ml in 0.1 M diethanolamine buffer, pH 9.6, and incubating the plate at room temperature. Reactions were recorded by a MICROELISA® auto reader (MR 580, Dynatech) 5–60 min after the addition of substrate. To stop the reactions, 50 μl of 3.0 M NaOH was added to each well.

Crude antigens from PRV HA-infected, WMV-1 F-infected, and WMV-2 NY-infected *C. metuliferus* were also used for study. The presence of cylindrical inclusions was verified by O-G staining as described above. The tissues were ground and diluted in coating buffer, and then processed by using the same procedures as those used to process the purified antigens.

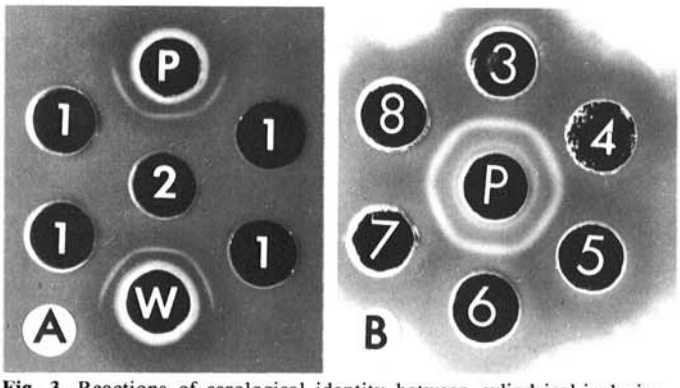

Fig. 3. Reactions of serological identity between cylindrical-inclusion protein (CIP) of papaya ringspot virus (PRV) and watermelon mosaic virus 1 (WMV-1) in SDS-immunodiffusion tests. **A,** PAGE-purified PRV CIP and WMV-1 CIP were tested against their antisera. **B,** Crude antigens from tissue of *Cucumis metuliferus* infected with PRV or WMV-1 were tested against PRV CIP antiserum (similar results obtained with WMV-1 CIP antiserum). Wells contained: P = undiluted PRV CIP antiserum, W = undiluted WMV-1 CIP antiserum, 1 = purified PRV CIP (24 μg in 60 μl of 1% SDS), 2 = purified WMV-1 CIP (24 μg in 60 μl of 1% SDS), 3 = WMV-1 NY, 4 = PRV HB, 5 = WMV-1 F, 6 = PRV F-340, 7 = WMV-1 VG, and 8 = PRV HA.

RESULTS

Factors affecting cytoplasmic inclusion protein (CIP) purification. Cylindrical inclusion purification was assessed by monitoring fractions from different steps of differential

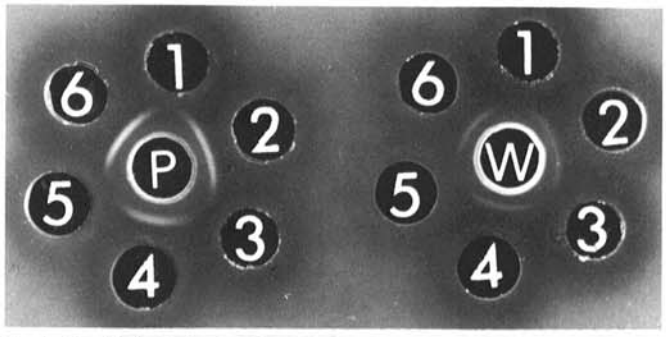

Fig. 4. Serological unrelatedness of papaya ringspot virus (PRV) and watermelon mosaic virus 1 (WMV-1) to watermelon mosaic virus 2 (WMV-2) in SDS-immunodiffusion tests using antisera to cylindrical-inclusion proteins (CIPs). Crude antigens of cylindrical inclusions were extracted from infected tissue of *Cucumis metuliferus* and tested against undiluted PRV CIP antiserum and WMV-1 CIP antiserum. Wells contain: 1 = WMV-2 NY, 2 = PRV HA, 3 = WMV-2 F, 4 = PRV HB, 5 = WMV-2 C, 6 = WMV-1 F, P = undiluted PRV CIP antiserum, and W = undiluted WMV-1 CIP antiserum.

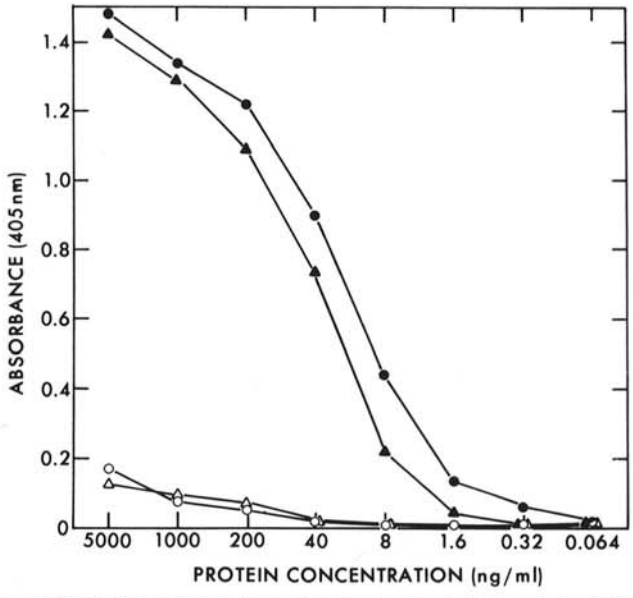

Fig. 5. Serological relationships of cylindrical-inclusion proteins (CIPs) and coat proteins (CPs) of papaya ringspot virus (PRV) and watermelon mosaic virus 1 (WMV-1) as detected by indirect ELISA. PAGE-purified proteins were diluted in coating buffer at the initial concentration of 5 μg/ml, γ-globulin to PRV CIP was used at 1 μg/ml, and goat anti-rabbit IgG alkaline phosphatase conjugate at 1/1,000 dilution. Readings were recorded 12 min after adding substrate at 1 mg/ml. •——•, PRV CIP; ▲——▲, WMV-1 CIP; o——o, PRV CP; and Δ——Δ, WMV-1 CP.

centrifugation in minislab gel electrophoresis. The concentration of CIP increased as the differential centrifugation proceeded (Fig. 1A). Coat protein of the virus, which was identified by its molecular weight and serological reaction, was found in every fraction indicating that virus aggregates or virus particles associated with cylindrical inclusions may sediment with low-speed centrifugation. When the healthy tissue was processed in the same way, no CIP or coat protein could be detected.

In initial trials, the purification procedure developed by Dougherty and Hiebert (9) for tobacco etch virus and pepper mottle virus was followed. However, inclusions did not form a discrete zone above the 80% sucrose layer after sucrose step gradient centrifugation. Instead, diffuse opalescent zones were present in the 80 and 60% sucrose layers, and most of the inclusions sedimented out in the pellet as judged by minislab gel analysis. In an effort to minimize the loss of the inclusions and to simplify the purification procedure, sucrose step gradient centrifugation was omitted and further purification was achieved by preparative gel electrophoresis after differential centrifugation.

A low-speed centrifugation to eliminate insoluble material was essential before gel electrophoresis; otherwise, granular aggregates interfered with the penetration of the sample into the stacking gel. The CIP band could be easily seen 5–10 min after staining in cold 2.0 M KCl solution. If the background was too strong, the gel could be destained in cold distilled water. We have found this detection procedure easier than using Coomassie blue or dansyl chloride (37). Because of polyacrylamide residues, it was not possible to determine the UV absorption spectrum of CIP which diffused directly in the elution buffer. Filtration through filter paper and precipitation by TCA removed polyacrylamide residues and made UV scanning possible.

Ultraviolet absorption and molecular weight. The purified viral CIP resuspended in distilled water or 0.5% SDS was too turbid for UV scanning. This turbidity could be reduced by resuspending the sample in 6.0 M guanidine-HCl. The ultraviolet absorption spectra of PRV CIP and WMV-1 CIP were similar. Both showed maxima at 277–278 nm and minima at 250–251 nm, with a $A_{280\ nm}/A_{260\ nm}$ ratio of 1.32 (uncorrected for light scattering). Purified PRV and WMV-1 CIP had a single protein species with a molecular weight of 70,000 as determined by electrophoresis in 7.5% polyacrylamide gel (Fig. 1B).

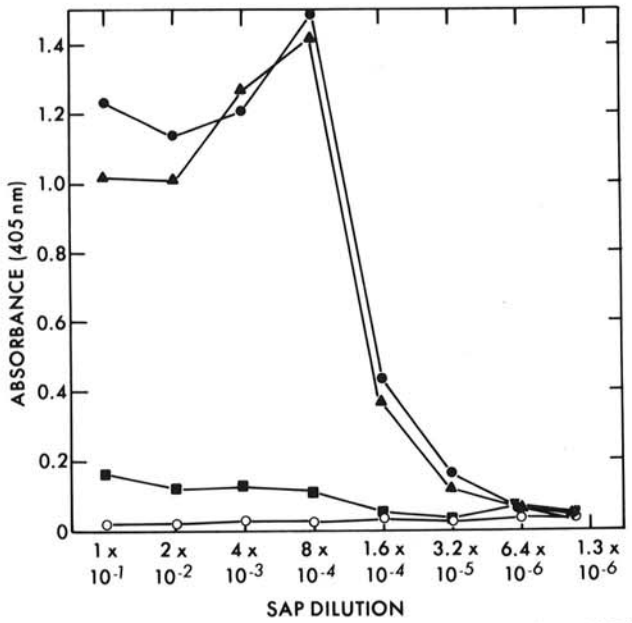

Fig. 6. Serological relationships of papaya ringspot virus (PRV), watermelon mosaic virus 1 (WMV-1), and watermelon mosaic virus 2 (WMV-2) as detected by indirect ELISA with antiserum to cylindrical-inclusion protein of PRV. Crude sap from infected tissue of *Cucumis metuliferus* was diluted in coating buffer by fivefold series, γ-globulin to PRV CIP used at 1 μg/ml, and goat anti-rabbit IgG alkaline phosphatase conjugate at 1/1,000 dilution. Readings were recorded 30 min after adding substrate at 1 mg/ml. •——•, PRV HA; ▲——▲, WMV-1 F; ■——■, WMV-2 NY; o——o, healthy *C. metuliferus*.

SDS-immunodiffusion test. The titers of antisera to PRV CIP and to WMV-1 CIP, as determined in SDS-immunodiffusion plates by using crude antigens from infected tissue, ranged from 1/4 to 1/8 for bleedings taken 2–5 mo after the first injection. However, when 1.0 OD unit per milliliter of purified CIPs were used as antigen, titers up to 1/8 and 1/16 were obtained.

Precipitin lines between CIPs and CIP antisera usually started to appear within 10–12 hr, about 4–6 hr later than the reactions between coat protein and coat protein antisera, and were complete within 24–48 hr. The inclusion antigens in plant extracts gave prominent reactions when tested against CIP antisera. None of the antisera reacted with coat protein of corresponding viruses or with sap from healthy plants. Purified CIPs reacted with CIP antisera but did not react with antisera to intact virus particles or coat protein. The results clearly indicated that purified preparations of CIP were immunogenically and serologically distinct from coat protein (Fig. 2).

No serological difference could be detected when purified PRV CIP and WMV-1 CIP were tested against either PRV CIP antiserum or WMV-1 CIP antiserum (Fig. 3A). Moreover, when crude antigens from infected tissue were tested against both CIP antisera, all nine isolates of PRV and three isolates of WMV-1 were serologically indistinguishable (Fig. 3B).

PRV CIP antiserum and WMV-1 CIP antiserum did not react with sap from plant tissue infected with WMV-2 NY, WMV-2 F, and WMV-2 C, respectively (Fig. 4). Also, no precipitin lines were observed when crude antigens from plant tissues infected with PVY, TEV, BYMV, BCMV, CAMV, BICMV, LMV, and TuMV were tested against both antisera. Numerous linear bodies, bundles, and plates, which are typical types of cylindrical inclusions (4), were observed in stained epidermal strips taken from the infected tissues which were used in the serological tests. This indicated that negative reactions in immunodiffusion tests were not due to low amounts of CIP in plant tissues.

Indirect ELISA. When 1 μg/ml of γ-globulin to PRV CIP was used to detect different concentrations of purified CIP of PRV and WMV-1, the indirect ELISA showed extremely high sensitivity and could detect the antigens down to 1.6 ng/ml. At the 1.6 ng/ml antigen concentration, the color reaction had an $OD_{405\ nm}$ reading of 0.471 and 0.189 for PRV CIP and WMV CIP, respectively, 30 min after the substrate was added to the test wells. These reactions were easily detected by visual inspection. PRV CIP γ-globulin reacted identically to purified PRV CIP and purified WMV-1 CIP but not to purified coat protein of PRV or WMV-1 (Fig. 5). The results clearly showed that PRV CIP and WMV-1 CIP were serologically indistinguishable, whereas the CIP and coat protein of both viruses were immunologically unrelated.

Crude CIP antigens from PRV- or WMV-1-infected *C. metuliferus* reacted similarly to PRV CIP γ-globulin, but CIP antigen from WMV-2-infected tissue failed to react (Fig. 6). Once again, by using purified PRV CIP γ-globulin and indirect ELISA we confirmed the SDS-immunodiffusion test which indicated that PRV CIP and WMV-1 CIP were serologically indistinguishable, but not related to WMV-2 CIP. The reaction to healthy extract in indirect ELISA was very low (Fig. 6) and no cross absorption was needed for eliminating the healthy background. The method was capable of detecting CIP in crude sap diluted to 3.2×10^{-5}. However, in the lower dilution (1/10–1/250) the intensity of reaction did not increase proportionally (Fig. 6). This might be due to some host factors that interfered at higher concentrations with the reaction of CIP to CIP γ-globulin. Another possibility is that the buffering capacity of coating buffer was not sufficient to coat CIP on the plate when host constituents were present in higher concentration.

DISCUSSION

Various isolates of PRV and WMV-1 have previously been shown to be closely related by their similar reactions to resistant

genes in three cucurbitaceous species and by their indistinguishable reactions with antisera to dissociated coat protein or virus (43). We successfully purified cylindrical inclusion subunits of PRV and WMV-1 and produced highly specific antisera. Results from SDS-immunodiffusion tests and indirect ELISA tests clearly show that PRV CIP and WMV-1 CIP are serologically indistinguishable and are immunologically unrelated to both coat proteins. The serological identities respectively in their coat proteins and CIPs strongly indicate that PRV and WMV-1 are very closely related. Thus, PRV and WMV-1 should be considered as strains of the same virus instead of two different viruses.

WMV-2 is not related to PRV or WMV-1 according to serological studies with antisera to coat protein or virus (18,33,41). Also, Baum and Purcifull (3) reported that the cylindrical inclusions of WMV-1 and WMV-2 did not cross react when tested with antisera to their cylindrical inclusions. In this study, antisera to PRV CIP or WMV-1 CIP did not react to three different strains of WMV-2 in SDS-immunodiffusion tests or in indirect ELISA tests. The detection of numerous cylindrical inclusions in WMV-2-infected leaf tissue by O-G staining strongly indicates that the lack of serological relationship between WMV-1 and WMV-2 was not due to low concentration of WMV-2 CIP. Thus, serological data coupled with very different host ranges (33,38,41) suggest that WMV-2 and WMV-1 are two different potyviruses and not strains of the same virus. However, serological analyses conducted with antisera to WMV-2 CIP are needed to confirm our conclusion.

Falk and Tsai (16) reported that the indirect ELISA, but not the DAS ELISA, was able to detect the noncapsid viral protein of maize stripe virus in infected tissue extracts. Although SDS-immunodiffusion studies on the serological relationships of the cytoplasmic inclusions of some potyviruses have been well described (22,27,34), we do not know of any reports in which ELISA techniques were used to study these nonstructural viral proteins. In this report, we have shown that indirect ELISA is a highly sensitive method for detecting purified CIP or CIP in plant extracts and that no cross absorption was needed to eliminate the healthy background. It may be easier to purify CIP of a potyvirus than the virus itself since the concentration of CIP is often high in plant tissue and the purification procedure is relatively simple. Thus, the indirect ELISA method with CIP antisera can be a simple, alternative serological probe for studying potyviral relationships and making diagnoses. In our preliminary trials, nonspecific reactions occurred when we used PRV CIP antiserum in DAS ELISA. Further studies are needed to determine whether CIP antisera of PRV and other potyviruses can be used in DAS ELISA tests.

Although morphology and features of cylindrical inclusions of PRV and WMV-1 have been extensively studied (4,13,15,25,36,44) and the subunit proteins have been purified, the function of the nonstructural viral protein still remains unknown.

LITERATURE CITED

1. Andrews, J. H., and Shalla, T. A. 1974. The origin, development, and conformation of amorphous inclusion body components in tobacco etch virus-infected cells. Phytopathology 64:1234-1243.
2. Bar-Joseph, M., and Malkinson, M. 1980. Hen egg yolk as a source of antiviral antibodies in the enzyme-linked immunosorbent assay (ELISA): a comparison of two plant viruses. J. Virol. Meth. 1:179-183.
3. Baum, R. H., and Purcifull, D. E. 1981. Serology of the cylindrical inclusions of several watermelon mosaic virus (WMV) isolates. (Abstr.) Phytopathology 71:202.
4. Christie, R. G., and Edwardson, J. R. 1977. Light and electron microscopy of plant virus inclusions. Fla. Agric. Exp. Stn. Monogr. 9. 150 pp.
5. Clark, M. F. 1981. Immunosorbent assays in plant pathology. Annu. Rev. Phytopathol. 19:83-106.
6. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
7. Crook, N. E., and Payne, C. C. 1980. Comparison of three methods of ELISA for baculoviruses. J. Gen. Virol. 46:29-37.
8. Crowther, J. R., and Abu-El Zein, E. M. E. 1979. Detection and quantification of foot and mouth disease virus by enzyme-labelled immunosorbent assay technique. J. Gen. Virol. 42:597-602.
9. Dougherty, W. G., and Hiebert, E. 1980. Translation of potyvirus RNA in a rabbit reticulocyte lysate: Identification of nuclear inclusion proteins as products of tobacco etch virus RNA translation, and cylindrical inclusion protein as a product of the potyvirus genome. Virology 104:174-182.
10. Dougherty, W. G., and Hiebert, E. 1980. Translation of potyvirus RNA in a rabbit reticulocyte lysate: Cell-free translation strategy and a genetic map of the potyviral genome. Virology 104:183-194.
11. Edwardson, J. R. 1966. Electron microscopy of cytoplasmic inclusions in cells infected with rod-shaped viruses. Am. J. Bot. 53:359-363.
12. Edwardson, J. R. 1966. Cylindrical inclusions in the cytoplasm of leaf cells infected with tobacco etch virus. Science 153:883-884.
13. Edwardson, J. R. 1974. Some properties of the potato virus Y group. Fla. Agric. Exp. Stn. Monogr. 4. 398 pp.
14. Edwardson, J. R., and Christie, R. G. 1978. Use of virus-induced inclusions in classification and diagnosis. Annu. Rev. Phytopathol. 16:31-55.
15. Edwardson, J. R., Purcifull, D. E., and Christie, R. G. 1968. Structure of cytoplasmic inclusions in plants infected with rod-shaped viruses. Virology 34:250-263.
16. Falk, B. W., and Tsai, J. H. 1983. Assay for maize stripe virus-infected plants by using antiserum produced to a purified noncapsid protein. Phytopathology 73:1259-1262.
17. Gibbs, A. 1969. Plant virus classification. Adv. Virus Res. 14:263-328.
18. Gonsalves, D., and Ishii, M. 1980. Purification and serology of papaya ringspot virus. Phytopathology 70:1028-1032.
19. Hager, D. A., and Burgess, R. R. 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: Results with sigma subunit of *E. coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal. Biochem. 109:76-86.
20. Harrison, B. D., Finch, J. T., Gibbs, A. J., Hollings, M., Shepherd, R. J., Valenta, V., and Wetter, C. 1971. Sixteen groups of plant viruses. Virology 45:356-363.
21. Hiebert, E., and McDonald, J. G. 1973. Characterization of some proteins associated with viruses in the potato Y group. Virology 56:349-361.
22. Hiebert, E., Purcifull, D. E., Christie, R. G., and Christie, S. R. 1971. Partial purification of inclusions induced by tobacco etch virus and potato virus Y. Virology 43:638-646.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227:680-685.
24. Lommel, S. A., McCain, A. H., and Morris, T. J. 1982. Evaluation of indirect enzyme-linked immunosorbent assay for the detection of plant viruses. Phytopathology 72:1018-1022.
25. Martelli, G. P., and Russo, M. 1976. Unusual cytoplasmic inclusions induced by watermelon mosaic virus. Virology 72:352-362.
26. Martelli, G. P., and Russo, M. 1977. Plant virus inclusion bodies. Adv. Virus Res. 21:175-266.
27. McDonald, J. G., and Hiebert, E. 1975. Characterization of the capsid and cylindrical inclusion proteins of three strains of turnip mosaic virus. Virology 63:295-303.
28. Mernaugh, R. L., Gardner, W. S., and Yocum, K. L. 1980. Three-dimensional structure of pinwheel inclusions as determined by analytic geometry. Virology 106:273-281.
29. Provvidenti, R., and Gonsalves, D. 1982. Resistance to papaya ringspot virus in *Cucumis metuliferus* and its relationship to resistance to watermelon mosaic virus 1. J. Hered. 73:239-240.
30. Purcifull, D. E. 1972. Papaya ringspot virus. No. 84 in: Description of Plant Viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England.
31. Purcifull, D. E., and Batchelor, D. L. 1977. Immunodiffusion tests with sodium dodecyl sulfate (SDS)-treated plant viruses and plant viral inclusions. Fla. Agric. Exp. Stn. Bull. 788 (Tech.). 39 pp.
32. Purcifull, D. E., and Edwardson, J. R. 1967. Watermelon mosaic virus: Tubular inclusions in pumpkin leaves and aggregates in leaf extracts. Virology 32:393-401.
33. Purcifull, D. E., and Hiebert, E. 1979. Serological distinction of watermelon mosaic virus isolates. Phytopathology 69:112-116.
34. Purcifull, D. E., Hiebert, E., and McDonald, J. G. 1973. Immunochemical specificity of cytoplasmic inclusions induced by viruses in the potato Y group. Virology 55:275-279.
35. Shepard, J. F., Secor, G. A., and Purcifull, D. E. 1974. Immunochemical cross-reactivity between the dissociated capsid protein of PVY group plant viruses. Virology 58:464-475.

36. Story, G. E., and Halliwell, R. S. 1969. Identification of distortion ringspot virus disease of papaya in the Dominican Republic. Plant Dis. Rep. 53:757-760.
37. Talbot, D. N., and Yphantis, D. A. 1971. Fluorescent monitoring of SDS gel electrophoresis. Anal. Biochem. 44:246-253.
38. Van Regenmortel, M. H. V. 1971. Watermelon mosaic virus. No. 13 in: Description of Plant Viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England.
39. Van Regenmortel, M. H. V., and Burckard, J. 1980. Detection of a wide spectrum of tobacco mosaic virus strains by indirect enzyme-linked immunosorbent assays (ELISA). Virology 106:327-334.
40. Voller, A., Bartlett, A., Bidwell, D. E., Clark, M. F., and Adams, A. N. 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). J. Gen. Virol. 33:165-167.
41. Webb, R. E., and Scott, H. A. 1965. Isolation and identification of watermelon mosaic viruses 1 and 2. Phytopathology 55:895-900.
42. Weber, K., and Osborn, M. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.
43. Yeh, S.-D., Gonsalves, D., and Provvidenti, R. 1984. Comparative studies on host range and serology of papaya ringspot virus and watermelon mosaic virus 1. Phytopathology 74:1081-1085.
44. Zettler, F. W., Edwardson, J. R., and Purcifull, D. E. 1968. Ultramicroscopic differences in inclusions of papaya mosaic virus and papaya ringspot virus correlated with differential aphid transmission. Phytopathology 58:332-335.