Purification and Some Properties of Two Strains of Soybean Dwarf Virus

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ABSTRACT

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A dwarfing (SDV-D) and a yellowing (SDV-Y) strain of soybean dwarf virus from Japan were purified from Wayne soybeans 15–20 days after inoculation. Yields were highest when the virus was extracted by pulverizing stem and leaf tissue in liquid nitrogen, mixing the powder with 0.5 M sodium phosphate, pH 6, (1:2, w/v) and a 2% industrial-grade pectin glycosidase, and stirring the slurry at room temperature for 6 hr. Extracts were clarified with 10% each (v/v) of chloroform and butanol. Concentration and final purification were by two cycles of differential centrifugation and sucrose density gradient centrifugation. Each isolate sedimented as a single band containing 26-nm-diameter icosahedral particles. Yields were 0.1–0.2 mg/kg for the dwarfing strain and 0.15–0.3 mg/kg for the yellowing strain. Each strain had an absorbance maximum at 259 nm and a minimum at 242 nm. $A_{260/280\ nm}$ ratios, corrected for light-scattering, were 1.85 (SDV-D) and 1.83 (SDV-Y). Sedimentation coefficients corrected to 20 C in water were 108 (SDV-D) and 114 (SDV-Y). SDV-D banded isopycnically in CsCl at a density of 1.39 and SDV-Y at 1.41 g/ml. The relative molecular masses of the major proteins were 22,200 (SDV-D) and 22,700 (SDV-Y).

Soybean dwarf was first recognized as a disease of soybeans in 1952 in the southern area of Hokkaido in Japan (26). Since that time the disease has spread slowly throughout the island and in 1971 was reported across the Straits of Tsugaro in northern Honshu (25). In 1969 the disease was determined to be viral in origin and soybean dwarf virus (SDV) was described (27). Although disease incidence varies in different areas and in different years, soybean dwarf is considered one of the most important soybean diseases in northern Japan. Tamada (25) found yield reductions up to 40% when 50% of the plants were infected. No immunity to SDV was found in more than 2,300 germ plasm entries screened, although some varieties exhibited significant levels of tolerance (9).

SDV isolates have been grouped into two strain types, dwarfing (SDV-D) and yellowing (SDV-Y), on the basis of host range and symptoms (24). SDV, like other luteoviruses, is persistently transmitted by aphids, has about 25-nm icosahedral particles, and is localized in the phloem (27). SDV has been purified and some serological comparisons have been made (7,16). Preliminary results indicate a serological relationship between a New Zealand isolate of subterranean clover red leaf virus and SDV (2). Little, however, is known about the physiochemical properties of the virus.

Soybean dwarf disease has not been observed in the United States, but because of the seriousness of the disease in Japan, the importance of the soybean industry in the United States, and the fact that forage legumes are known to host the virus, SDV is being studied in a containment facility at the Plant Disease Research

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Laboratory in Frederick, MD (17). Purification and physiochemical characterization of two strains of SDV are reported here. Preliminary reports have appeared elsewhere (Damsteegt *unpublished*, 13,14).

MATERIALS AND METHODS

All purification procedures were done at room temperature. Except for the initial extraction of virions from infected tissue with 0.5 M sodium phosphate buffer, pH 6, 0.1 M sodium phosphate at the same pH (purification buffer) was used throughout. When proteins, nucleic acids, or partially purified virus preparations were compared with known standards, a standard curve was plotted from which physiochemical properties were determined. All purification experiments were repeated at least twice; the numerical values reported for physiochemical properties are averages of at least three determinations.

Virus isolates and aphid maintenance. Two strains of SDV and the aphid vector, *Aulacorthum* (=*Acyrthosiphon*) *solani* Kaltenbach, were provided by T. Tamada and hand carried from Japan to the containment facility at Frederick under permits from the Animal and Plant Health Inspection Service and the Maryland Department of Agriculture. Colonies of nonviruliferous *A. solani* were established as needed and maintained on curly dock (*Rumex crispus* L.), lettuce (*Lactuca sativa* L. 'Black-seeded Simpson'), or soybean (*Glycine max* (L.) Merr.) in Plexiglas cages in isolation cubicles. After a 48-hr acquisition access period on excised, infected Wayne soybean leaves, 5–10 aphids were transferred to each 1-wk-old soybean seedling. The aphids were confined to the seedlings with small cages, transferred to a Plexiglas isolation cubicle, and allowed a 48-hr inoculation access period. While still in the cubicle, the aphids were killed with malathion. The plants were then moved to benches in the containment glasshouse for 15–20 days for symptom development. Leaves and stems were harvested and held at −20 or −80 C until needed.

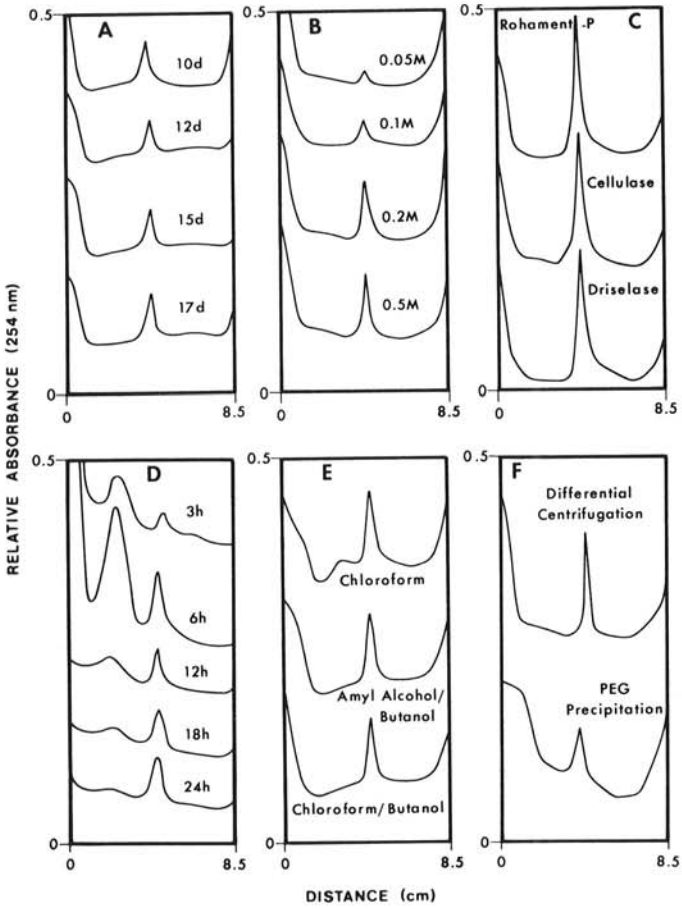

Fig. 1. Ultraviolet absorption profiles of sucrose density gradients to determine the effect of time of harvest and purification procedures on yield and purity of soybean dwarf virus (SDV) yellowing (Y) and dwarfing (D) strains. **A**, Yield of SDV-Y harvested 10, 12, 15, and 17 days after inoculation. **B**, Yield of SDV-D after extraction in 0.05, 0.1, 0.2, and 0.5 M sodium phosphate buffer, pH 6. **C**, Yield of SDV-D after extraction with 2% (w/v) Rohament-P, cellulase, and Driselase. **D**, Yield of SDV-Y after shaking ground infected tissue with 0.5 M sodium phosphate buffer and 2% Rohament-P for 3, 6, 12, 18, and 24 hr. **E**, Effect of clarification with 20% (v/v) chloroform, 10% amyl alcohol, and 10% butanol, and 10% chloroform and 10% butanol. **F**, Purity and yield of SDV-Y after concentration by differential centrifugation and precipitation with 0.25 M NaCl and 10% polyethylene glycol. SDV-D and SDV-Y were analyzed in linear 10–40% sucrose gradients in 0.1 M sodium phosphate buffer, pH 6, after centrifugation at 40,000 rpm (201,800 *g*) for 2.5 hr at 4 C. The 0-cm mark represents the top of the gradient.

Virus purification. To ensure that tissue used for comparisons of extraction, clarification, and concentration methods was equivalent in both quality and quantity, the following process was used: Frozen tissue was pulverized in liquid nitrogen, transferred while still frozen into an aluminum blender jar, ground in buffer until the tissue was in small pieces and thoroughly mixed, then divided by weight into equal quantities.

The following purification procedure, modified from one developed for beet western yellows virus (5) was adopted for routine virus purification. Soybean leaves and stems, harvested and frozen 15–20 days after inoculation, were mixed with liquid nitrogen (1:5, w/v), and ground to a fine powder. The powder was mixed with 0.5 M sodium phosphate buffer, pH 6 (2:1, w/v) and a 2% industrial-grade pectin glycosidase (Rohament-P, Fermco Biochemics, Inc., Elk Grove Village, IL) used in the food processing industry. After the slurry was stirred or shaken in a 30 C waterbath for 6 hr, a 1:1 mixture of butanol and chloroform was added at the proportion of 1 ml of solvents to 5 ml of slurry and stirred for 15 min. The emulsion was broken by centrifugation in a Sorval GSA rotor at 7,500 rpm (9,150 *g*) for 20 min. The aqueous phase was recovered and allowed to stand at 4 C overnight. After a second, low-speed centrifugation, the virus was concentrated by high-speed centrifugation in a Beckman Type 60 rotor at 28,000 rpm (55,220 *g*) for 3.5 hr. The pellets were covered with purification buffer and allowed to stand at 4 C overnight and then subjected to a second cycle of differential centrifugation. Sucrose gradients were prepared by a method modified from Davis and Pearson (6). Centrifuge tubes were filled with 25% sucrose in purification buffer and stored at −20 C. As needed, the tubes were removed from the freezer and allowed to stand at 4 C, during which time the gradient was formed. After differential centrifugation, preparations of both strains were layered onto the gradients and centrifuged in an SW 40 rotor at 40,000 rpm (201,800 *g*) for 2.5 hr. After centrifugation, the gradients were scanned with an ISCO UA-5 absorbance monitor at 254 nm. Absorbing zones were collected and concentrated by centrifugation in a type 75 Ti rotor at 50,000 rpm (132,561 *g*) for 1.25 hr. The pelleted particles were allowed to resuspend overnight at 4 C in purification buffer and frozen at −20 or −80 C until needed.

Ultraviolet absorbance profiles and spectra. Ultraviolet absorption (220–320 nm) scans of 0.1–0.5 mg/ml of each particle type were made in a Beckman DU-7 spectrophotometer. After correction for light scattering (8), absorption ratios were calculated and the average of 10 preparations was determined.

Sedimentation coefficients. Sedimentation coefficients were estimated by linear log sucrose density gradient centrifugation (3). Approximately 10 μg of each of the following viruses were used as markers: brome mosaic virus, 87 S; cowpea mosaic virus, 95 S and 115 S; potato virus Y, 145 S; and tobacco mosaic virus, 194 S. Preformed gradients were layered with virus and centrifuged for 2.75 hr at 6 C in a SW 40 rotor at 40,000 rpm (201,800 *g*).

Buoyant densities in CsCl. Buoyant densities were determined by equilibrium centrifugation in a SW 60 Ti rotor. Cesium chloride was dissolved in purification buffer containing approximately 10 μg of SDV-D or SDV-Y at an initial density of 1.4 g/ml. The preparations were centrifuged to equilibrium at 40,000 rpm (164,000 *g*) for 18 hr at 4 C. Immediately thereafter the gradients were scanned at 254 nm and fractionated. Refractive indices of

representative 0.2-ml fractions were measured with an American Optical Abbe Mark II refractometer at 25 C. After the refractive indices of the fractions containing SDV-D and SDV-Y were determined, they were converted to buoyant densities (15).

Protein subunits. The molecular mass (M_r) of the protein subunits was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein standards (Biorad Laboratories, Rockville Center, NY) and intact virus particles were boiled for 3 min in dissociation buffer (0.1 M Tris, pH 8, 1% 2-mercaptoethanol, 1% sodium dodecyl sulfate, 0.2% bromphenol blue, and 20% glycerol) and applied separately and together to a 1.5 × 13 × 14 mm three-layer discontinuous slab gel system (4). Electrophoresis was at room temperature at 11 mA for 18 hr. After electrophoresis the gels were fixed and stained overnight in 25% methanol, 8% acetic acid, and 0.2% Coomassie brilliant blue R. The gels were destained in three changes of a solution of 10% methanol and 8% acetic acid and scanned at 565 nm.

Electron microscopy. Purified virus preparations were negatively stained with 2% solution of uranyl acetate, pH 6.0, and viewed on a JOEL JEM-100C electron microscope at 80 kV. For each isolate, 25 particles were measured between faces on each of three micrographs.

Infectivity assay. Sucrose preparations (20%) of SDV-D and SDV-Y in purification buffer were placed between two stretched Parafilm membranes. Nonviruliferous *A. solani* were allowed access to the virus for 24 or 48 hr, after which 5–10 aphids were transferred to each of 10 1-wk-old Wayne soybean seedlings. Two days later the aphids were killed and the plants were observed for 3–5 wk until symptoms developed.

RESULTS

Purification. Attempts to purify SDV-D from tissue harvested 30 or more days after infestation failed. When tissue was harvested earlier, at 10, 12, 15, or 17 days, virus yields peaked at or before 10 days and remained at approximately the same level for at least 7 days (Fig. 1A). Density gradient analysis indicated that more virus was recovered when the virus was extracted with 0.5 or 0.2 M sodium phosphate than with 0.1 or 0.05 M (Fig. 1B). Three enzymes, Type I Practical Grade Cellulase (Sigma Chemical Co., St. Louis, MO), Driselase (Sigma), and Rohament-P gave equal yields of virus (Fig. 1C). Combinations of Rohament-P and cellulase (data not shown) yielded no more virus than any of the enzymes used individually. Rohament-P was selected for routine use because it is an effective, inexpensive product.

More virus was recovered when tissue was incubated with enzymes for 6 hr than for 3, 12, 18, or 24 hr (Fig. 1D). Unfortunately, more low molecular weight contaminants were released at 6 hr than at the other times tested. Increasing the time that the tissue was exposed to the enzymes reduced the amount of low molecular weight contaminants, but the amount of virus recovered also was reduced. Addition of butanol, whether combined with chloroform or amyl alcohol, removed the unwanted material in the 6-hr preparations (Fig. 1E). Chloroform-butanol clarification was made part of the routine procedure. Still more contaminants were removed when the clarified extracts were allowed to stand at 4 C overnight and centrifuged at low speed before concentration. Acid rather than organic solvent clarification was tested, but no virus was recovered when the pH of enzyme-treated plant extracts was lowered to 4, 4.5, or 5 with the addition of thioglycollic acid or 0.1 N HCl. Triton X-100 (1%) was tested as a clarification agent in combination with the various organic solvents tested. In all cases yields were reduced slightly and no improvement in purity was observed.

Because yields of luteoviruses are low and a limited volume of

TABLE 1. Infectivity of soybean dwarf virus

Soybean dwarf virus strain	Virus source[a]	Acquisition access 1 day	Acquisition access 2 days
Dwarfing	10 μg/ml (CB)	0/4[b]	1/38
Dwarfing	50 μg/ml (CB)	0/4	2/14
Dwarfing	Wayne soybean leaves	3/4	8/8
Yellowing	10 μg/ml (CB)	0/4	13/32
Yellowing	50 μg/ml (CB)	3/4	9/14
Yellowing	Wayne soybean leaves	0/4	3/6
Dwarfing	10 μg/ml (CB)	1/20	
Dwarfing	50 μg/ml (CB)	2/18	
Dwarfing	10 μg/ml (C)	5/19	
Dwarfing	50 μg/ml (C)	8/17	

[a] Aphids were allowed access to two different concentrations of purified preparations clarified with chloroform only (C) or with chloroform/butanol (1:1 v/v) (CB) and to excised midcanopy leaves from Wayne soybean plants infected with SDV for 21 days.
[b] Number of plants with symptoms/number of test plants.

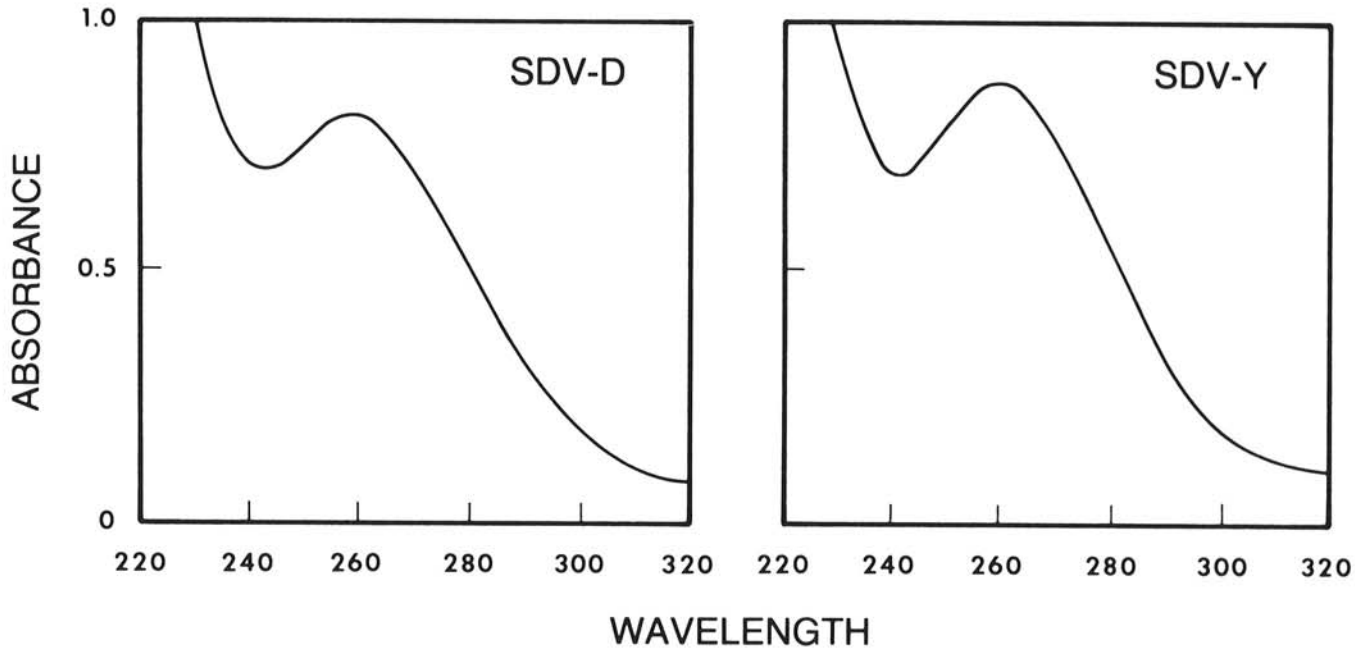

Fig. 2. Ultraviolet absorption spectra of purified soybean dwarf virus (SDV) dwarfing (D) and yellowing (Y) strains. After final purification, pelleted particles were allowed to resuspend in 0.1 M sodium phosphate buffer, pH 6, overnight at 4 C before spectrophotometry.

clarified extract can be centrifuged at one time, attempts were made to precipitate the virus with polyethylene glycol (PEG) and NaCl. Concentration of virus by ultracentrifugation yielded 2–3 times more virus than precipitation with any combination of 4–10% PEG and 0.01–0.2 M NaCl (Fig. 1G). Sometimes the PEG preparations were as free of contaminants as those concentrated by ultracentrifugation. Usually, however, the virus peak was located on a shoulder of unidentified absorbing material.

Isolation, electron microscopy, and infectivity of the particles from sucrose gradients. In sucrose gradients, SDV-D and SDV-Y sedimented as single components after rate-zonal centrifugation (Fig. 1A–F). When examined in the electron microscope, fractions at a depth of 4 cm, corresponding to the peaks obtained by ultraviolet scanning of the gradients, were free of visible contamination and contained icosahedral particles. The mean diameters of SDV-D and SDV-Y, 26.1 nm (SD = 1.3 nm) and 26.2 nm (SD = 1.4 nm), respectively, were not different. The same fractions were fed to aphids through membranes. When aphids were allowed access to excised leaves, SDV-Y was always more efficiently transmitted than SDV-D. In contrast, purified preparations of SDV-Y were more infectious than SDV-D (Table 1). If a mixture of chloroform and butanol rather than chloroform alone was used in the purification procedure, SDV-D infectivity was reduced. Test plants developed typical symptoms of SDV-D in 7–10 days and symptoms of SDV-Y in 10–14 days.

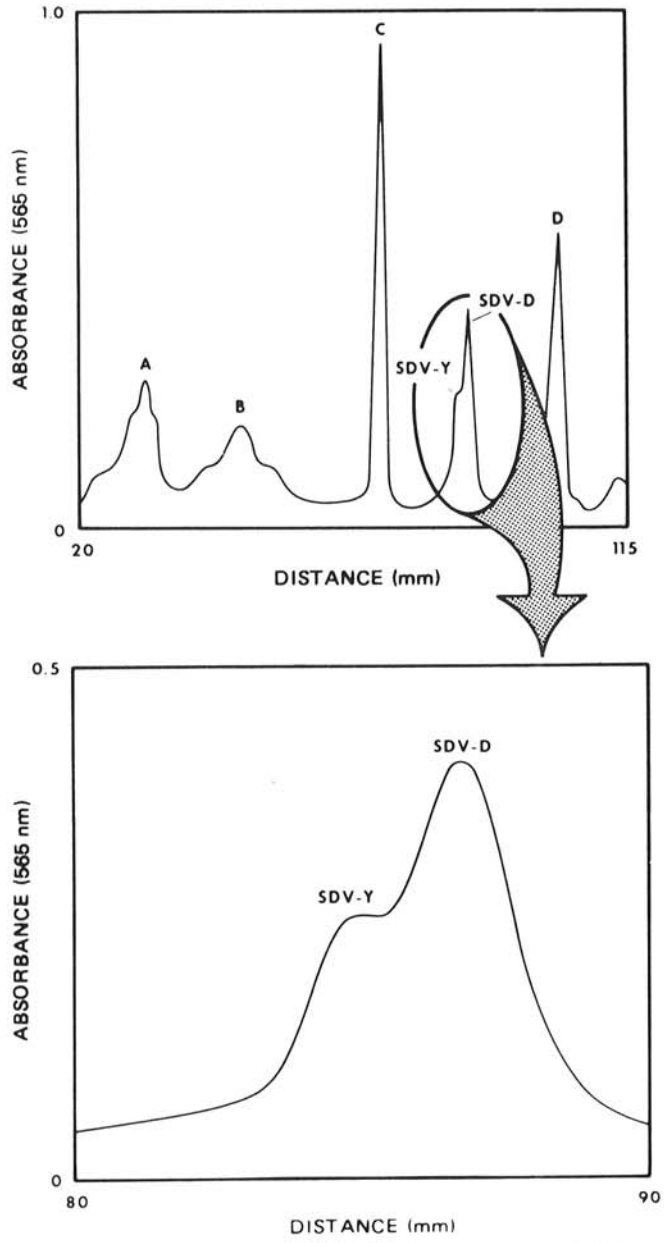

Fig. 3. (Top) Relative molecular mass (M_r) of SDV-D and SDV-Y coat protein subunits of soybean dwarf virus (SDV) dwarfing (D) and yellowing (Y) strains were estimated by electrophoresis with known internal standards in vertical discontinuous polyacrylamide gels. Standards and their M_r were bovine serum albumin, 66,200 (A); ovalbumin, 45,000 (B); carbonic anhydrase, 31,000 (C); and soybean trypsin inhibitor, 21,500 (D). Phosphorylase B, 92,500 and lysozyme, 14,400 were also used but are not shown. Calculated protein subunit M_r were 22,200 (SDV-D) and 22,700 (SDV-Y). (Bottom) Expansion of the scan above from 80–90 mm to further illustrate differential migration of SDV-D and SDV-Y coat protein subunits.

Ultraviolet absorbance spectra. Absorbance spectra of purified preparations of both isolates were typical of nucleoprotein preparations (Fig. 2). Both isolates had an absorbance maximum at 259 nm and a minimum at 242 nm. Calculated $A_{260/280\,nm}$ absorbance ratios of partially purified virus were 1.85 for SDV-D and 1.83 for SDV-Y.

Sedimentation coefficients. Sedimentation coefficients for SDV-D and SDV-Y were 108 and 114, respectively.

Buoyant densities, percent RNA, extinction coefficients, and yields of purified virus. Buoyant densities of purified virions were 1.39 (SDV-D) and 1.41 g/ml (SDV-Y). Using buoyant density in CsCl, the percent RNA of the infectious particles was estimated as 28% for SDV-D and 31% for SDV-Y (22). Calculated extinction coefficients for SDV-D and SDV-Y were 7.3 and 7.9 $(mg/ml)^{-1}cm^{-1}$ at 260 nm. (10).

Relative M_r of coat protein subunits. Single major protein bands, which migrated to a position between those reached by trypsin inhibitor and carbonic anhydrase, were observed when dissociated SDV-D and SDV-Y were electrophoresed in polyacrylamide gels (Fig. 3). The M_r of these proteins were 22,300 for SDV-D and 22,600 for SDV-Y. When SDV-D and SDV-Y were mixed and electrophoresed, two bands were occasionally visualized, but usually they appeared to comigrate in the gel. Two overlapping peaks were distinguished after densitometric analysis (Fig. 3), indicating that the two proteins differ slightly in M_r. When coat protein subunits from each of the two viruses were electrophoresed in separate lanes and scanned, a single peak was observed.

DISCUSSION

Although the factors that influence yield of purified luteoviruses vary with the host and the strain, efficient extraction of particles from the fibrous phloem tissue is the most important step in successful purification of any luteovirus. Grinding in liquid nitrogen (5,20), a fruit press (21), or meat grinder (16), lyophylizing the tissue and grinding in a Wiley Mill (18), prolonged grinding in a blender (11), and grinding first in a blender and then in a Virtis homogenizer (1) have all been used with some success to extract luteoviruses. Some investigators have found, however, that mechanical means of extraction are less effective than enzyme treatment of infected root or shoot tissue (2,5,11,28,29). The use of enzymes is not entirely free of problems. Paliwal (18) used cellulase to extract barley yellow dwarf virus (BYDV) from oat tissue but abandoned its use because the partially purified virus contained too many contaminants. Purified preparations of a vector nonspecific strain of BYDV were cleaner if macerating enzymes were not used (11). Takanami and Kubo (23) increased yields of potato leafroll and tobacco necrotic dwarf viruses with Driselase, a mixture of five enzymes. Driselase, a Japanese product, has become available in North America, but it too has disadvantages in that it is expensive and it has been reported that the virus infectivity is sometimes low (28,29). Others have found that the use of less expensive cellulases (2,11), an industrial-grade pectin glycosidase (5), and an industrial-grade cellulase (29) have made enzyme extraction of luteoviruses economical without the loss of infectivity or a decrease in the purity of the final product.

The physicochemical characteristics of the SDV isolates investigated here are typical of other luteoviruses. Buoyant densities, number and M_r of the coat protein subunits, the size and

shape of the particles, and the spectrophotometric characteristics are all within the reported ranges of these properties for members of the luteovirus group. The sedimentation coefficient for SDV-Y is also typical (114 S), but that of SDV-D (108 S) is low. Low sedimentation coefficients have also been reported for two other luteoviruses, carrot red leaf virus (104 S) (29) and a vector nonspecific strain of barley yellow dwarf virus (106 S) (12). Potato leafroll virus is reported to have a sedimentation coefficient of 127 S (21). These results suggest that the very narrow group range (115–118 S) (19) may have to be broadened to include viruses with sedimentation coefficients both higher and lower than the current range.

Differences in other physiochemical properties measured are less striking, but the two strains investigated here differ in the size of the coat protein subunits, genomic RNAs and dsRNA profiles (14), and bouyant densities. The two strains have many common hosts but can be differentiated by symptomatology on different hosts and on different soybean cultivars (Damsteegt, *unpublished,* 24). In contrast, Kojima and Tamada (16) found that no serological differences between the two strains were detected in ring precipitin, gel double diffusion, or infectivity neutralization tests. Homologous antisera to the SDV isolates studied have been produced and serological studies are in progress.

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