A Simplified Procedure for the Purification of Curly Top Virus and the Isolation of Its Monomer and Dimer Particles

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ABSTRACT

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Curly top virus was purified from shepherd's purse plants (*Capsella bursa-pastoris*) 4–5 wk after inoculation with viruliferous beet leafhoppers (*Circulifer tenellus*). The virus-containing sap was clarified with chloroform, the virus precipitated and concentrated by polyethylene glycol, and subjected to two cycles of high-speed ultracentrifugation. Monomer and dimer particles were isolated from partially purified preparations by sucrose density gradients. Infectivity assays indicated that the dimer particle is required for infection. Virus yields obtained averaged 500 μg/kg of plant material. The $A_{260\text{-}280\ nm}$ ratio for dimers was ~1.4.

Additional key words: electron microscopy, geminivirus, sugar beet, trimers.

Since the first report of beet curly top virus (CTV) in 1888, several attempts have been made to characterize and purify this virus, usually with limited success. Previous methods have frequently involved complicated procedures which produced impure virus in low concentration or of unspecified yields. Although workers have reported the occurrence of monomer and dimer particles associated with CTV (6,11–13) and other geminiviruses, little is known about the relationship of these particle types to infectivity.

The objectives of this study were to develop a simple high-yielding method of virus purification to attempt to isolate both monomer and dimer particles of CTV, and to examine some of their biological properties.

MATERIALS AND METHODS

Colonies of beet leafhoppers, *Circulifer tenellus* Baker, were maintained in muslin covered cages on sugar beets, *Beta vulgaris* L. 'US 75' infected with Logan, a severe isolate of curly top virus. Nonviruliferous beet leafhoppers used for infectivity assays were reared on healthy sugar beets. Insects were maintained in an insectary greenhouse at a temperature range of 26–32 C. Three-week-old shepherd's purse seedlings, *Capsella bursa-pastoris* (L.) Medic., were inoculated using two to three viruliferous leafhoppers per plant, and given a 72-hr inoculation access period. Plants were maintained under greenhouse conditions at 26–32 C until symptoms appeared. Infected plants exhibited severe twisting and distortion of leaves and flower stalks accompanied by frequent midvein necrosis and an infectious phloem exudate (2).

Virus purification. All centrifugation steps during virus purification were carried out at 4 C unless otherwise specified. Rotor gravity units were calculated using R_{max}. Infected shepherd's purse plants (200–300 *g*) were harvested 4–5 wk after inoculation and washed in tap water. Plants were ground in a meat grinder using 2:1 (v/w) 0.1 M phosphate buffer (Na_2HPO_4-KH_2PO_4) pH 7.2 containing 0.0025 M EDTA and 0.01 M Na_2SO_3 (SPE buffer). The material was subsequently homogenized in a VirTis-60 homogenizer at 50,000 rpm for 3–4 min, and sap expressed through one layer of cotton muslin. Triton X-100 was added to a final concentration of 1% and the slurry stirred overnight at 4 C. Sap was clarified by adding one-tenth volume of cold chloroform and the mixture stirred for 15 min at room temperature. The clear gold-colored supernatant was decanted after low speed centrifugation for 10 min at 10,400 *g*. The virus was precipitated from the aqueous phase with polyethylene glycol (M.W. 7,000–9,000) at the rate of 12 g/100 ml of extract in 0.2 M NaCl by stirring for 1.5 hr at 4 C and pelleting the precipitate for 15 min at 10,400 *g*. The virus-containing pellets were resuspended in one-tenth of the original volume with SPE buffer, Triton X-100 added to a final concentration of 0.5%, and the suspension stirred for 2 hr or overnight at 4 C, followed by centrifugation at 12,100 *g* for 10 min. The supernatant was centrifuged for 3 hr at 95,000 *g* or 1 hr at 325,000 *g*. Pellets were resuspended in 10 ml SPE buffer with the aid of a glass tissue homogenizer, stirred for 1 hr at 4 C and the suspension centrifuged for 10 min at 10,400 *g*. The supernatant was centrifuged for 2 hr at 125,000 *g*. The final pellet was resuspended in 2 ml SPE buffer and frozen overnight to coagulate and precipitate host plant constituents. After thawing, the preparation was centrifuged for 5 min at 4,300 *g*.

Density gradient centrifugation. Linear sucrose gradients (10–35% in 0.1 M phosphate buffer, pH 7.2) were prepared with an ISCO model 570 automatic gradient former. The 2 ml of virus suspension was layered onto the density gradient and centrifuged for 3.5 hr at 125,000 *g* in a Beckman SW 27 rotor. The gradient was examined for light scattering bands prior to analysis with an ISCO model 640 automatic fractionator equipped with model UA-5 ultraviolet optics at 254 nm.

Infectivity. Sucrose density gradient fractions (1.2 ml per fraction) were each adjusted to 15% with SPE buffer or SPE-buffered sucrose, and non-viruliferous beet leafhoppers were allowed to feed through membranes as described by Duffus and Gold (5). Two leafhoppers per plant were then fed for 72 hr on healthy sugarbeet seedlings *Beta vulgaris* L. 'US 75'. Assay plants were observed for symptoms and the results of infectivity plotted against their respective fraction number and absorbance profile according to the method utilized by Mumford (13).

Electron microscopy. Purified virus was applied to 200-mesh carbon-formvar grids and allowed to stand for 3–5 min, excess liquid removed with filter paper, and the grids rinsed with a few drops of distilled water. Grids were negatively stained in 2% uranyl acetate containing 2% ETOH and 1% acetic acid and examined and photographed in a Siemens Elmiskop model 101.

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Antiserum production and serology. Antisera against purified CTV were prepared in rabbits by six intramuscular injections at weekly intervals. Preparations containing 75–100 μg/ml of virus were mixed with equal volumes of Freund's complete adjuvant (Difco) just prior to injections. Rabbits were bled from the ear weekly for six consecutive weeks beginning 7 days after the final injection.

Antiserum titer was measured against its homologous CTV antigen by the method described by Ball and Brakke (1). CTV antigen (10 μg) in 0.5 ml 0.1 M phosphate buffer was mixed with 0.5 ml of antiserum for each serial dilution up to 1:128,000, and incubated for 30 min at 37 C. Each dilution was then layered on a linear 10–35% sucrose density gradient and centrifuged for 2.5 hr at 125,000 *g* in a SW 27 rotor. Plotted gradients were scanned and peaks compared against a control using a planimeter.

RESULTS

Virus purification. Light-scattering bands, not present in preparations made from healthy, uninoculated shepherd's purse plants, were observed in sucrose density gradients at 2.1 and 2.8 cm below the meniscus. Ultraviolet absorbance profiles showed two peaks corresponding to the light-scattering bands (Fig. 1A). The lower zone (II) was usually 2–3 times larger than the upper zone (I).

Zone fractions representing the peaks were collected and sedimented by centrifugation for 4–5 hr at 125,000 *g*. Pellets were resuspended in 2 ml 0.1 M phosphate buffer pH 7.2, and again centrifuged through density gradients for 3 hr at 125,000 *g* (Fig. 2). Gradients were fractionated and the virus collected and pelleted as before. Pellets were resuspended in 1 ml 0.1 M phosphate buffer pH 7.2 and analyzed in a Beckman DB spectrophotometer for concentration of nucleoprotein. Purified virus yields were typically about 500 μg/kg of plant material calculated using the formula $E^{0.1\%}_{1\ cm} \cdot A_{260\ nm} = 7.7$ derived for bean golden mosaic virus (7). On several occasions, virus yields reached about 2.8 mg/kg.

Ultraviolet absorption. Purified CTV from both peak fractions had a UV absorbance spectrum typical of nucleoprotein (Fig. 3). The $A_{260-280\ nm}$ ratio for the first and second virus zone was 1.35 and 1.38–1.4, respectively, uncorrected for light scattering. The $A_{240-260\ nm}$ ratio for the second peak was 0.85.

Electron microscopy. Virus particles stained readily in uranyl acetate and maintained their particle integrity without fixation.

Examination of material from both zones I and II showed a high concentration of viruslike particles. Particles from the upper zone were isometric and pentagonal or roughly circular in shape and were 21 nm in diameter. A few particles were pentagonal in shape, and were not isometric (Fig. 4A), due to one side of the pentagonal particles being noticeably longer than the others.

The lower zone yielded doublet particles, or dimers, 20 nm by 38 nm. The individual components of these geminate pairs were

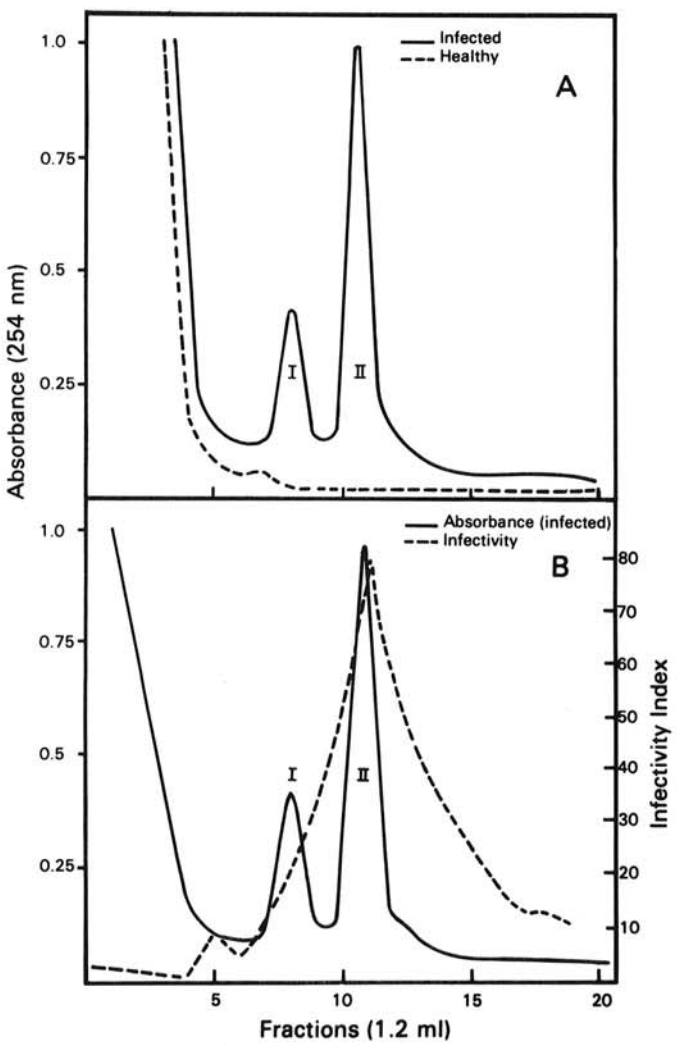

Fig. 1. Beet curly top virus monomers and dimers. **A,** Absorbance profiles of CTV-infected and healthy shepherd's purse plants from the first sucrose density gradient. Centrifugation zones I and II show sedimentation position of monomers and dimers, respectively. **B,** Plot of relative infectivity against viral absorbance zones. Note that the highest infectivity index is associated with the second zone.

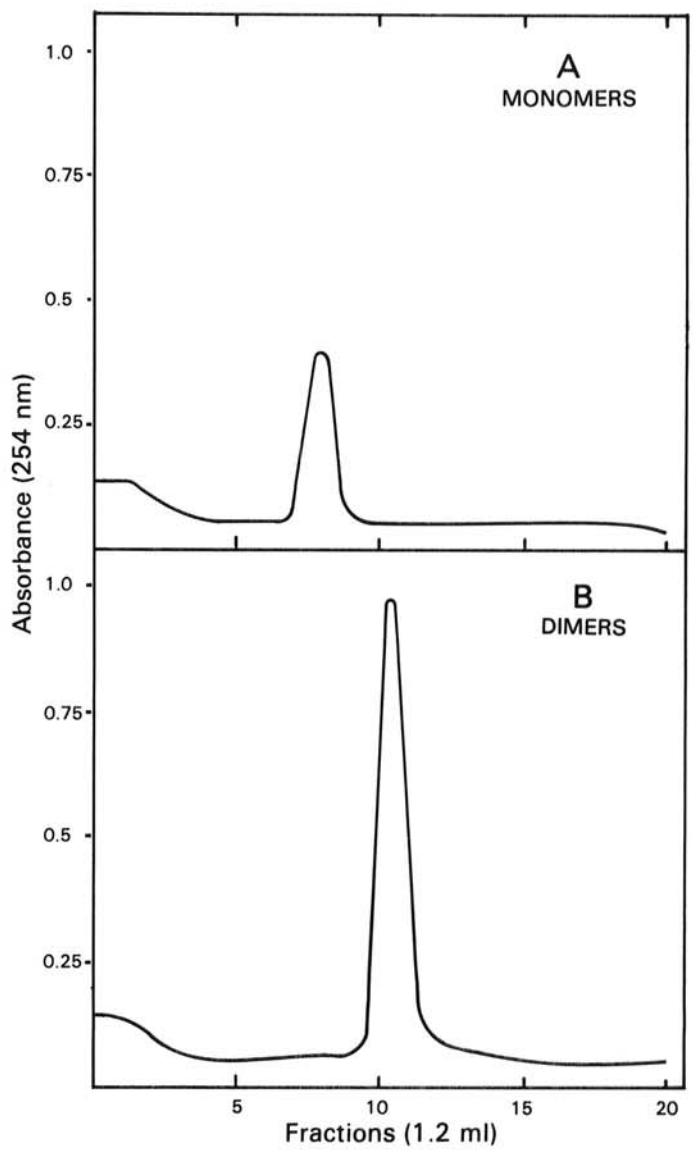

Fig. 2. Absorbance profiles of purified beet curly top virus from the second sucrose density gradient. **A,** Monomers. **B,** Dimers.

always morphologically pentagonal in shape, and in size are similar to other geminiviruses (Fig. 4B). On several occasions triplet particles 52–54 nm in length were observed, but these were found in relatively low concentrations only. They contained a rectangular-shaped middle member bound on both sides by a pentagonal member (Fig. 4C).

Infectivity. The distribution of infectivity indicates a broad zone, with the most concentrated area of infectivity corresponding to peak II (dimers). There is no distinct zone of infectivity associated with peak I (monomers) (Fig. 1B). Infectivity assays using insects are much more sensitive than ultraviolet optics so there is naturally an overlapping of infectivity in peak I and in other areas of the gradient with very little absorbance. To further test the hypothesis that monomers were not infective and that infectivity was associated only with dimers, further infectivity tests were conducted with pelleted monomers and dimers derived from the second density gradient centrifugation (Fig. 2). Equal concentrations of monomer and dimer virus particles as measured at $A_{260\ nm}$ were used for these membrane-fed infectivity tests. In all tests, results suggested that most virus infectivity was obtained from zones with primarily dimers and very little infectivity (three plants infected of 103 inoculated) from zones with primarily monomers (Table 1). The three plants infected by leafhoppers fed on monomers showed typical BCTV symptoms and purified preparations produced typical virus peaks (Fig. 1A). Purified preparations derived from the symptomless monomer-inoculated plants exhibited an absorbance profile similar to that derived from healthy plants.

Antiserum production and serology. Antiserum prepared against the second absorbance zone (dimers:optical density $A_{260\ nm}$ = 0.57–0.65) had a titer of 1:128,000 against both monomer and dimer particles in tests using the density-gradient method of Ball and Brakke (1), and a titer of 1:1,024 in agar diffusion tests (Fig. 5A).

Antiserum was conjugated and tested for host plant contaminations and the relationship of monomers and dimers by the direct double antibody sandwich method described by Clark and Adams (4). The coating globulin was used at 1 μg/ml and the enzyme-conjugated globulin was 1:400. A strong positive reaction was demonstrated against CTV-infected plant material, but no reaction was observed against healthy shepherd's purse. No serological difference was found between monomer and dimer particles with the ELISA technique (Fig. 6) (H.-Y. Liu, *personal communication*).

Additional serological tests comparing purified monomer and dimer particles against the homologous antiserum were performed utilizing agar diffusion. Both particle types reacted with CTV antiserum without producing spurs showing that the monomers and dimers are serologically identical (Fig. 5B).

DISCUSSION

The purification procedure described here is an efficient method for the extraction and purification of CTV free of cellular plant components. Previous purification attempts of CTV have been reported (11–13), although yields from those procedures were not indicated. With our purification method, average virus yields were about 500 μg/kg of plant material, and on several occasions yields as high as 2.8 mg/kg have been obtained. The reason for these large variations in yields is not yet understood, but possibly is due to the age of the plants at time of purification, seasonal variation or other environmental factors. A time-course study on virus titer similar to the work of Shock and Goodman (14) would most likely provide a better understanding of the variations in CTV virus concentrations in infected plants. The high yields we have achieved, together with concentration of the virus into visible density gradient light scattering zones, present strong evidence against the earlier hypothesis that CTV occurs in low concentrations in plants (11).

Although others (3,6,8,9,11,12) have cited the presence of both single and paired geminivirus particles, this is the first report of isolation of monomers and dimers from freshly prepared plant tissue. The occurrence of these two particle types along with occasional triplet particles indicates the need for more information

TABLE 1. Transmission of curly top virus from purified virus preparations

Test	Virus conc. (μg/ml)	Purified preparation[a]	
		Monomers	Dimers
1	100	2/20[b]	18/19
2	100	0/10	10/10
3	75	1/15	9/15
4	40	0/18	4/17
5	40	0/20	5/20
6	40	0/20	4/20

[a] Purified virus from second sucrose density gradient.
[b] Ratio is the number of plants infected (numerator) over the number of plants tested (denominator).

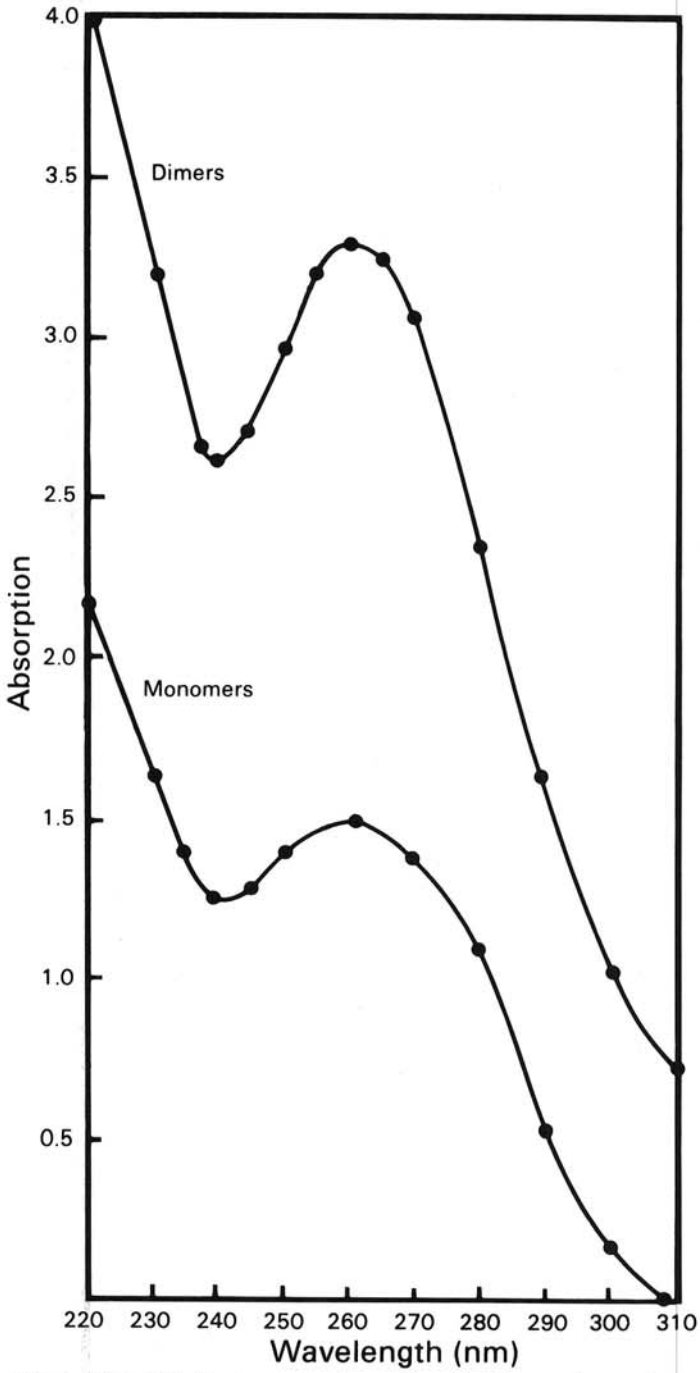

Fig. 3. Ultraviolet absorbance spectrum of purified beet curly top virus monomers and dimers.

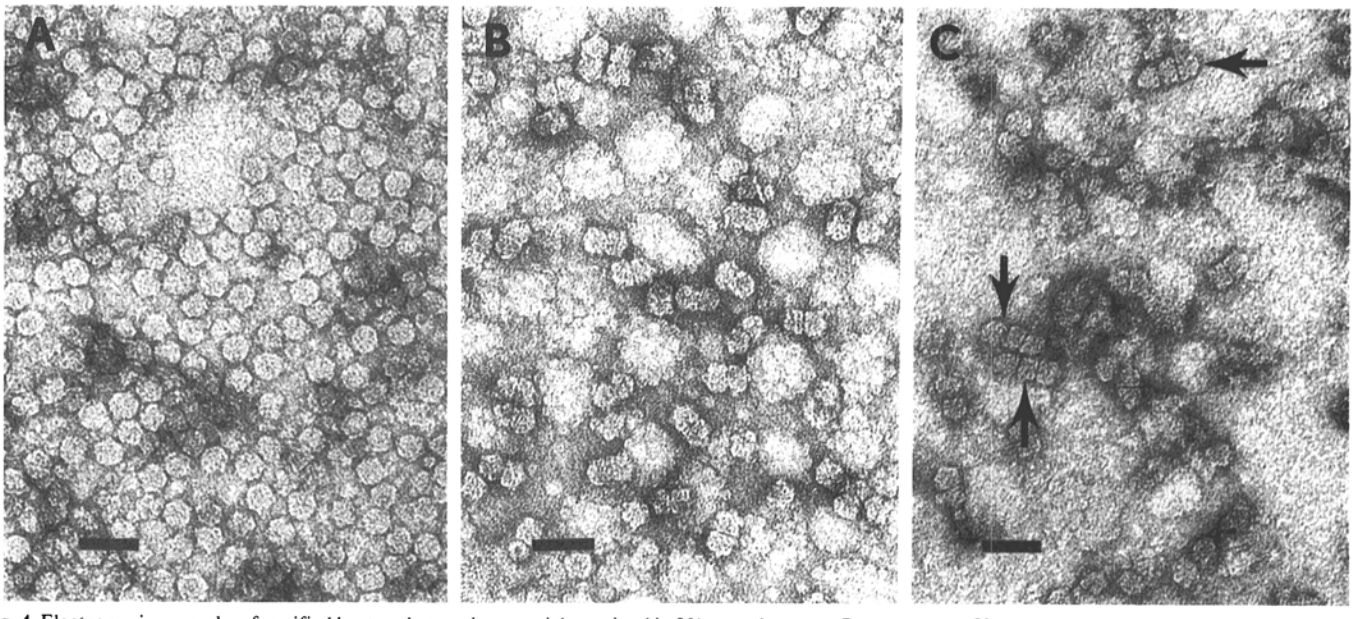

Fig. 4. Electron micrographs of purified beet curly top virus particles stained in 2% uranyl acetate. Bar represents 50 nm. **A,** Monomers. **B,** Dilute preparation of dimers. **C,** Trimers (arrows).

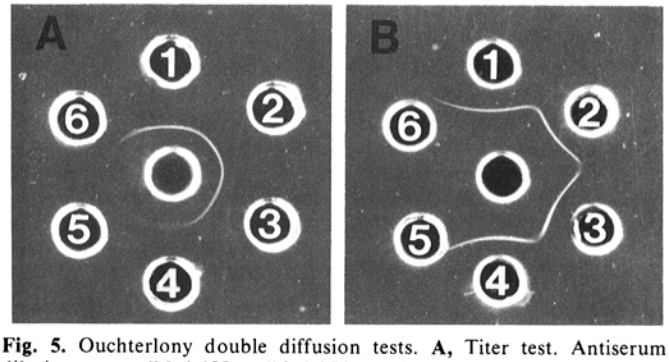

Fig. 5. Ouchterlony double diffusion tests. **A,** Titer test. Antiserum dilutions are: well 1, 1:128; well 2, 1:256; well 3, 1:512; well 4, 1:1,024; well 5, 1:2,048; and well 6, 1:4,096. The center well contained 3 μg/ml of purified CTV dimers. **B,** Relationship of CTV monomers and dimers. Wells 1 and 3 contained 2 μg/ml and 3 μg/ml of monomers, respectively. Wells 2 and 4 contained 2 μg/ml and 3 μg/ml of dimers, respectively. Well 5 contained healthy shepherd's purse sap, and well 6 was a phosphate buffer check. The center well contained a 1:16 dilution of CTV antiserum.

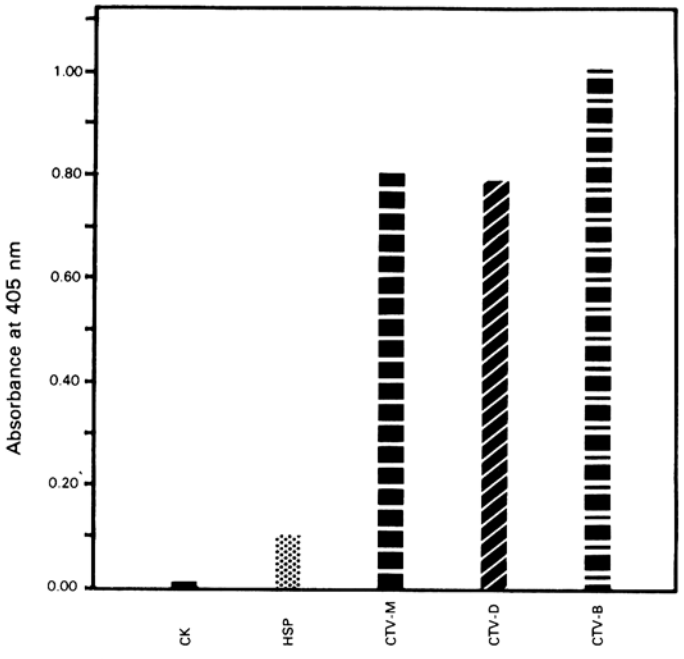

Fig. 6. ELISA test with beet curly top virus and controls with beet curly top virus antiserum. CK = buffer check; HSP = healthy shepherd's purse; CTV-M = purified beet curly top monomers; CTV-D = purified beet curly top dimers; CTV-B = sugar beet (*Beta vulgaris*) infected with the Logan isolate of beet curly top virus. Results are mean $A_{405\ nm}$ values of two experiments 30 min after adding substrate.

regarding the genesis and biological action of CTV as well as other geminiviruses. Whether or not the large numbers of monomers associated with CTV are the result of the purification technique reported here or are merely disrupted dimers is not known. Egbert et al (6) reported that both dimers and monomers were infective. Although our data indicate that both particle types contain nucleic acid, our tests demonstrating that only dimer particles are infective do not support their findings. Only in three instances did monomer-fed leafhoppers transmit virus to test plants (Table 1), and this was probably due to contamination with dimer particles.

Hooker et al (10) have reported the occurrence of isometric triplet particles resembling geminiviruses associated with potato leaf curl virus. Since these particles are apparently the infectious entity, the possibility exists that the triplets associated with CTV are also infective. Only low concentrations of these particles were found with CTV, so their origin and role in infectivity are not yet known. Hatta and Francki (9) describe chloris striate mosaic virus monomers as icosahedra, each containing 24 spherical capsomeres. The dimer particles are reportedly comprised of two icosahedra, each missing one capsomere at their adjoining surfaces. The rectangular-shaped middle segments of the trimers may be a similar icosahedron, but with a capsomere missing from each side. The two adjoining icosahedra may thus each be missing only one capsomere, as in the dimers. Further examinations of the biochemical and biophysical properties of these respective particle types are currently under investigation.

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