Purification and Some Properties of a Virus Associated with Cardamom Mosaic, a New Member of the Potyvirus Group

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ABSTRACT

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The properties of cardamom mosaic virus (CarMV), which causes a severe disease of cardamom (*Elletaria cardamom*) in Guatemala, were investigated. CarMV was purified at Geneva, NY, using leaves collected from a cardamom plantation in Guatemala. Purified preparations contained numerous flexuous rod-shaped particles similar to those observed from leaf-dip extracts of infected leaves. Particles from purified preparations had a normal length of 700–720 nm. Cells of infected plants also contained pinwheel-shaped inclusion bodies that are typically induced by potyvirus infections. Three protein species with molecular weights of about 37,500, 32,200, and 29,000 were detected after purified preparations of CarMV were denatured with sodium dodecyl sulfate (SDS) and analyzed by electrophoresis in polyacrylamide gels. Presumably, the largest protein is the native capsid protein of CarMV and the smaller ones are proteolytic products of it. Antiserum prepared to CarMV reacted specifically with CarMV in SDS-agar immunodiffusion and in both indirect and direct enzyme-linked immunosorbent assay (ELISA). None of 16 potyvirus isolates tested reacted positively to CarMV antiserum in direct ELISA, but four viruses (zucchini yellow mosaic, papaya ringspot types w and p, cowpea aphidborne mosaic virus, and a severe strain [NL-8] of bean common mosaic) consistently gave positive reactions in indirect ELISA. We conclude that CarMV is a new potyvirus. The rapid and specific detection of infected plants by ELISA will help to implement control measures of the disease in Guatemala and elsewhere.

Additional key words: katte disease

Cardamom (*Elettaria cardamom* Maton) is a perennial herb belonging to the ginger family, Zingiberaceae. The dried seeds or entire seed pods are used as a spice to flavor curries, cakes, breads, and coffee (22). Among spices, only saffron and vanilla command a higher price. The major cardamom-producing countries are India, Sri Lanka, and Guatemala.

Although virus diseases have caused substantial losses to cardamom production in Sri Lanka and India for many years, cardamom grown in Guatemala remained free of virus diseases until the 1970s. In 1975, a disease with viruslike symptoms was observed on cardamom growing in the Quezaltenango region of Guatemala. Within 5 yr, the disease had appeared in nearly all cardamom plantations of the country's southern pacific coast region, where 60% of Guatemala's cardamom is produced. The disease is referred to as *el mosaico del cardamomo*, or cardamom mosaic (6). Both lamina and petioles of leaves from infected plants have numerous dark green stripes in a light green background, which gives a distinct mosaic pattern (Fig. 1). Subsequent developing leaves also show these symptoms. Infected plants become less vigorous and the production of fruits, which are smaller, decreases progressively. Plants usually die 4–5 yr after symptoms appear. The virus agent is apparently transmitted by the banana aphid (*Pentalonia nigronervosa*) (3).

The rapid spread of cardamom mosaic virus (CarMV) in Guatemala can be attributed to the use of infected rhizomes to establish new plantations. Many growers have unwittingly spread the virus because rhizomes from recently infected plants do not show symptoms. Thus, a rapid and reliable method to detect CarMV is needed to minimize its spread through planting material and to aid in developing control measures.

In this paper, we report on the purification and serology of CarMV and on the adaptation of enzyme-linked immunosorbent assay (ELISA) for detecting CarMV in plant tissues. We also show that CarMV belongs to the potyvirus group (5,9) on the basis of its particle morphology and the detection of pinwheel-type inclusion bodies in infected tissue.

MATERIALS AND METHODS

Virus source and purification. Young to almost mature leaves with prominent mosaic symptoms were collected from infected cardamom plants growing on a single plantation in the Departamento of San Marcos, Guatemala, and transported the next day to Geneva, NY. The leaves were processed for purification within 7 days of collection; otherwise, they were

Fig. 1. Mosaic pattern on leaves of cardamom infected with cardamom mosaic virus. Healthy leaves (not shown) do not have mosaic patterns.

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frozen until used. Corresponding leaves from healthy plants were also collected.

Virus was usually purified as follows: Leaves from which the midrib had been removed were frozen with liquid nitrogen and pulverized with a mortar and pestle. The macerated tissue was transferred to a warm mortar and mixed with three volumes (1 g/3 ml) of 0.5 M potassium phosphate, pH 7.1, containing 0.01 M EDTA and 0.1% 2-mercaptoethanol. The extract was squeezed through cheesecloth and the debris reextracted with one volume of the same buffer. Triton-X 100 was then added to the supernatants to a final concentration of 1%. The preparation was clarified by centrifugation at 10,400 *g* (maximum) for 10 min, and virus particles in the supernatant were concentrated by centrifugation (78,000 *g* for 2 hr) in a Beckman type 30 rotor (Beckman Instruments, Palo Alto, CA). The resulting pellets were resuspended in 0.025 M potassium phosphate, pH 7.1, buffer (KP) containing 1 M urea. After low-speed centrifugation (10,400 *g* for 10 min), the supernatant was layered over an 8-ml pad of 10% sucrose in KP buffer containing 0.5 M urea and centrifuged at 78,000 *g* for 2.75 hr in a Beckman type 30 rotor. The pellets were resuspended in KP buffer containing 0.5 M urea. Final purification was by centrifugation in a Cs_2SO_4 gradient as follows: Cs_2SO_4 was slowly dissolved in a partially purified virus preparation (0.15 g/ml) and layered over a 3-ml pad of 53% (w/w) Cs_2SO_4 dissolved in KP buffer containing 0.5 M urea and centrifuged in a Beckman SW 40 Ti rotor at 30,000 rpm for 24 hr at 6 C. The viral band, which was generally 56 mm from the bottom of the tube, was collected, diluted 1/3 with KP buffer containing 0.5 M urea, and centrifuged (9,000 *g* for 10 min) to eliminate most of the aggregated host material. The supernatant was dialyzed against 0.125 M potassium phosphate, pH 7.1, then freeze-dried after adding 5% sucrose to the virus preparation. The preparations were kept at −10 C until use for further study.

Serology. A white New Zealand rabbit was sensitized by injection with purified virus mixed 1:1 with Freund's complete adjuvant into both hip muscles. A second injection of virus mixed with Freund's incomplete adjuvant was given 7 days later. Subsequent booster injections were given 8, 14, and 17 wk after the initial injection. Serum was collected weekly starting 3 wk after the first injection.

Sodium dodecyl sulfate (SDS)-agar immunodiffusion (19) and ELISA were used to test the activity of the antiserum. SDS-agar gels contained 0.8% Ionagar, 0.5% SDS, and 1% sodium azide. Antigen preparations were mixed in 0.5% SDS before the wells were charged. Direct ELISA was done as described by Clark and Adams (1), except antibodies to healthy host proteins in the antibody-enzyme conjugate preparations were usually absorbed before use (12). Absorption was accomplished by adding the required amount of stock-conjugated gamma globulin to a volume of healthy leaf extract (1:20, w/v), which was equal to 1/10 the volume of diluted conjugated gamma globulin required for the test. After incubation at room temperature for 15 min, the mixture was diluted with conjugate buffer and the preparation was dispensed into test wells immediately. Indirect ELISA was done using the general method of Lommel et al (13) with the following modifications: 1) Tissues were extracted in direct ELISA coating buffer (1), 2) gamma globulin was purified by ammonium sulfate precipitation and DEAE column chromatography (1), 3) incubation steps were at 6 C for overnight or at 30 C for 4–6 hr, and 4) gamma globulin to CarMV antiserum was absorbed, as described for direct ELISA, with healthy cardamom tissue extracts prepared in phosphate-buffered saline (1), pH 7.4, containing 0.05% Tween 20, 2% polyvinylpyrrolidone-40, and 0.2% ovalbumin. Appropriate buffer, healthy, and known infected test samples were included in all tests. Reactions were measured at 405 nm with a Dynatech MR 580 reader (Dynatech Instruments, Inc., Torrance, CA).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight of CarMV capsid protein was determined by SDS-PAGE using the buffer and gel (5% stacking gel, 12% separating gel) system of Laemmli (11). Virus and molecular weight markers were degraded by heating at 100 C for 2 min in 0.1 M Tris-HCl, pH 6.8, containing 2.5% SDS, 5% 2-ME, and 5% sucrose. Molecular weight markers were bovine serum albumin (66,000), egg albumin (45,000), glyceraldehyde-phosphate dehydrongenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), and trypsin inhibitor (20,100) (Sigma Chemical Co., St. Louis, MO). Gels were stained with Coomassie Blue R250.

Electron microscopy. For sectioned material, segments (2 × 1 mm) excised from young unrolled leaves showing mosaic symptoms were fixed in 4% glutaraldehyde buffered with 75 mM potassium phosphate, pH 6.8, for 2 hr. Healthy control leaves were similarly prepared. The samples were then rinsed in buffer and postfixed in 2% OsO_4 (nonbuffered) for 3 hr. After a water rinse, the samples were dehydrated in a graded acetone series, embedded in an Epon-Araldite resin mixture, and polymerized. Sectioned material was stained with aqueous uranyl acetate and lead citrate.

Virus particles from either leaf extracts or purified preparations were stained with 2% aqueous phosphotungstic acid and examined for morphological characteristics.

RESULTS

Electron microscopy. Flexuous rod-shaped particles were consistently observed in extracts of leaves from infected plants showing mosaic symptoms (Fig. 1), although the numbers of particles observed were far lower than those observed in comparable preparations of bean common mosaic virus. Viruslike particles and pinwheel-shaped inclusion bodies, typical of those associated with potyvirus (5,9) infections, were also observed in cells of infected leaves (Fig. 2). In some cells, viruslike particles were lined up side by side and enclosed in membranelike structures.

Virus purification. Initial attempts to use leaves from infected or healthy plants grown in greenhouses at Geneva, NY, were unsuccessful. Both healthy and infected plants grew poorly and did not yield enough tissue for virus purification. Thus we were compelled to use leaves of field-grown plants from Guatemala. The leaves did not show signs of deterioration during the 24 hr it took to hand carry them to Geneva and process for purification.

Virus purification methods commonly used for potyviruses (2,7,9,21) were tried because our electron microscopy evidence indicated that CarMV was a potyvirus. Our attempts to concentrate the virus particles by polyethylene glycol (PEG) precipitation failed because huge quantities of a clear gelatinous matrix, often precipitated by PEG, interfered with further purification. Because the presence of urea (2) in resuspension buffer reduced aggregation considerably, we were able to use differential centrifugation to concentrate the virus without unacceptable loss. The virus, along with host material, formed a matted band in Cs_2SO_4 similar to that observed for papaya ringspot virus (PRV) (7). As with PRV, dilution of the collected viral zone with buffer followed by low-speed centrifugation eliminated visible host contaminants as judged by electron microscopy (Fig. 2) and SDS-PAGE (Fig. 3). Purified preparations appeared to have a number of particles aggregated end to end (Fig. 2). Limited trials indicated that CsCl may be a suitable substitute for Cs_2SO_4. Virus yields were low compared with those of other viruses, such as PRV (7,20), which we have purified in our laboratory. The lack of a continual supply of fresh infected leaves, however, precluded quantitative virus yield measurements.

Properties of CarMV. Three major protein bands with molecular weights of about 37,500, 32,200, and 29,000 were observed in SDS-denatured CarMV preparations that were analyzed by SDS-PAGE (Fig. 3). We assumed that the heaviest protein was the native coat protein, whereas the other two were partially proteolyzed products as have been observed with other potyviruses

(8,21).

A modal particle length of 620–660 nm was obtained for CarMV by measuring 100 particles from leaf-dip extracts. The low number of particles observed in leaf extracts made it difficult to take measurements from a larger population of particles. Thus we also measured particles from purified virus preparations (Fig. 2). A modal particle length of 701–720 nm was obtained from among 1,255 measured particles.

Table 1. Serological reaction of potyviruses to antiserum prepared against cardamom mosaic virus in enzyme-linked immunosorbent assay (ELISA)[a]

Virus[b]	Experiment no. 1	2	3	4	Tissue type	Virus source (reference)
CarMV	0.59[c]	1.28	0.24	0.88	Cardamom	Gonsalves
ZYMV-Fla	0.94	0.26	>1.50	NT[d]	Zucchini	Provvidenti (15)
ZYMV-Conn	0.93	0.41	>1.50	NT	Zucchini	Provvidenti (15)
PRV-w (Fla)	0.37	0.35	0.70	NT	Zucchini	Purcifull (20)
PRV-w (NY)	0.74	0.96	0.85	NT	Zucchini	Provvidenti (16)
PRV-p (HA)	0.25	0.35	0.60	NT	Papaya	Gonsalves (7)
BCMV-NL8	0.21	0.70	0.14	0.88	Beans	Provvidenti (17)
CAMV-Mor	0.22	0.00	1.13	0.66	Cowpea	Gonsalves (27)
CYVV	0.60	0.00	0.21	0.01	Beans	Provvidenti (18)
BICMV-Fla	0.22	0.00	0.05	0.16	Cowpea	Purcifull (27)
BYMV, BCMV	0.00	0.00	0.00	NT	Beans	Provvidenti (17,18)
WMV-2 (NY, China)	0.00	0.00	0.00	NT	Zucchini	Provvidenti (16)
PVY, TEV	0.00	0.00	0.00	NT	Tobacco, tomato	Provvidenti
BYMV-A	0.00	0.00	0.00	NT	*Alpinia*	Zettler (14)

[a] ELISA conditions = tissue extract at 1/20, coating gamma globulin at 5 μg/ml, and antirabbit gamma globulin at 1/1,000 dilution.

[b] CarMV = cardamom mosaic virus, ZYMB = zucchini yellow mosaic virus, PRV = papaya ringspot virus, BCMV = bean common mosaic virus, CYVV = clover yellow vein virus, CAMV = Cowpea aphidborne mosaic virus, BICMV = blackeye cowpea mosaic virus, BYMV = bean yellow mosaic virus, WMV = watermelon mosaic virus, and PVY = potato virus Y.

[c] Optical density at 405 nm, readings of corresponding healthy reactions were subtracted from those of infected reactions. For example, the absorbance obtained with healthy zucchini leaves was subtracted from infected reactions of ZYMV, PRV, and WMV-2. The highest average healthy reading was cardamom (0.14); the rest averaged 0.06.

[d] NT = not tested.

Serology. Antiserum prepared to CarMV reacted specifically to purified CarMV and extracts of infected tissues to form specific precipitin bands in SDS-immunodiffusion tests (reaction not shown). The dilution end point of the serum was 1/2 using infected cardamom tissue. Slight healthy reactions were observed, especially with antiserum taken from later bleedings.

Healthy and infected tissues were easily differentiated by direct and indirect ELISA (Fig. 4). Positive reactions were obtained with infected tissues diluted up to 10^3–10^6 and 10^5–10^6 times in direct and indirect ELISA, respectively (Fig. 4). With indirect ELISA, it was necessary to absorb the gamma globulin with healthy leaf extracts to remove background reaction. Although it helped improve the test, it was not essential to absorb the enzyme gamma globulin conjugate for direct ELISA. The antiserum was successfully used in both types of ELISA to detect CarMV in field-grown plants in Guatemala (28; Julio Tejada, Universidad del Valle, Guatemala, *personal communication*).

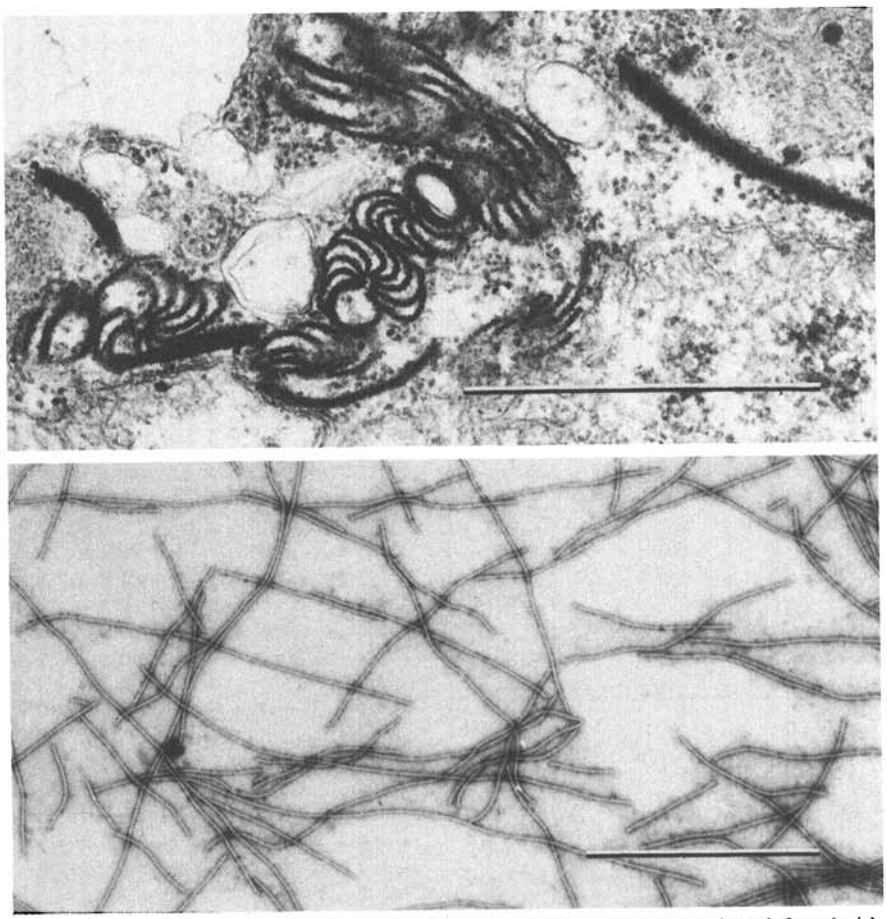

Fig. 2. Electron micrographs of **(top)** cylindrical inclusion bodies in cardamom tissue infected with cardamom mosaic virus (CarMV) and **(bottom)** purified preparation of CarMV. Scale bar = 1 μm.

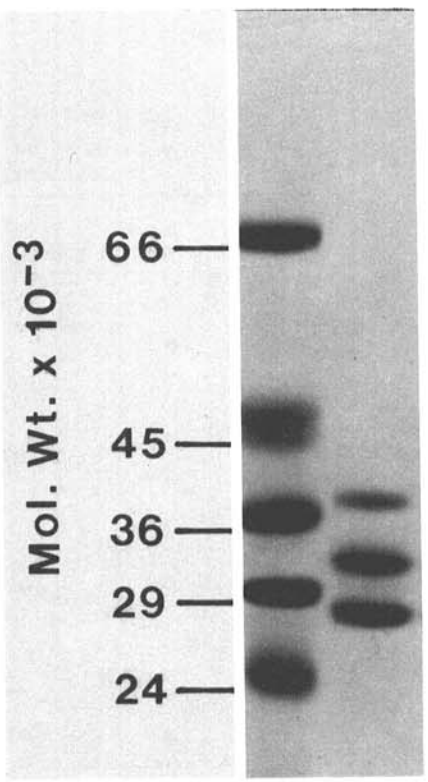

Fig. 3. Analysis of sodium dodecyl sulfate (SDS) denatured cardamom mosaic virus (CarMV) capsid protein by electrophoresis in 12% polyacrylamide gels permeated with SDS. Molecular weight markers are in the left lane and CarMV is in the right lane. Note the three distinct CarMV protein bands of 37,500, 32,200, and 29,000 mol wt. The latter two bands are presumably partially proteolyzed products of the native (37,500) protein.

Relation to potyviruses. Indirect and direct ELISA were used to determine the reactivity of CarMV antiserum to 16 isolates of potyviruses (Table 1). No reactions were obtained with any of the 16 virus isolates in direct ELISA. However, zucchini yellow mosaic virus (ZYMV), PRV type w and type p, and the NL 8 isolate of bean common mosaic virus (BCMV) gave positive reactions in all indirect tests. Cowpea aphidborne mosaic virus (CAMV), clover yellow vein virus (CYVV), and blackeye cowpea mosaic virus (BlCMV) were positive in one or more of the first three experiments (Table 1, experiments 1–3). In a fourth test, which involved BCMV-NL 8, CAMV, CYVV, and BCMV, CYVV was negative and BlCMV had very weak reactions in ELISA, whereas CAMV and BCMV-NL 8 gave strong positive reactions.

DISCUSSION

We conclude that the virus disease infecting cardamom in Guatemala is caused by a potyvirus (5,9). Our conclusion is based on four primary lines of evidence:

1. Inclusion bodies observed in infected tissues (Fig. 2) are similar to those induced by other potyviruses. It is generally agreed that the presence of such inclusion bodies in infected tissues is a unique characteristic of potyvirus infections (5).

2. Particle morphology and modal length are similar to those of potyviruses (9). We have no explanation for the discrepancy between the modal length obtained from leaf-dip extracts (about 660 nm) and from purified preparations (700–720 nm). It should be noted, however, that the latter was from 1,255 particles, whereas the former was from only 100.

3. It is transmitted by aphids (3).

4. It has serological affinities to other potyviruses (Table 1). Also, we have recently produced antiserum to the inclusion bodies isolated from leaves infected with CarMV infection (D. Gonsalves, *unpublished*). The cylindrical inclusion body protein has a molecular weight (70,000) similar to those of other potyviruses (9,20). In ELISA, plants that gave positive reactions to the inclusion body antiserum also reacted positively with antiserum to CarMV, confirming the constant association of virus particles and inclusions in infected plants. Recently, Dimitman et al (4) reported in an abstract that cardamom from Guatemala that are infected with CarMV have flexuous rod-shaped particles and pinwheel inclusions.

Serological affinity of CarMV with some potyviruses was demonstrated with indirect (Table 1) but not direct ELISA. Others have shown that indirect ELISA detects a wider range of serologically related viruses than direct ELISA (10,29). In fact, serological cross-reactivity among distinct potyviruses is not uncommon (26). We feel that CarMV should be regarded as a distinct new member of the potyvirus group. Our data further point out the abundance and economic importance of this group (5,9).

The disease caused by the virus we describe appears similar to a virus disease of cardamom commonly found in India that is also referred to as cardamom mosaic or "katte" disease (31). Plants infected with the katte disease have symptoms very similar to those caused by CarMV in Guatemala, and the causal agent is transmitted nonpersistently to semipersistently by the same aphid vector, *P. nigronervosa* (23–25,30,31). However, some have reported that katte virus can be mechanically transmitted to cowpea (*Vigna unguiculata* subsp. *unguiculata*) on which it produces local lesions (24). They speculated that perhaps the katte disease was caused by more than one component, one of which was mechanically transmitted. In limited trials at Geneva, we were unable to mechanically transmit Guatemalan CarMV to cardamom and cowpea or other herbaceous hosts (*unpublished*). As far as we know, the virus particles that cause the katte disease have not been seen. With the availability of antiserum to

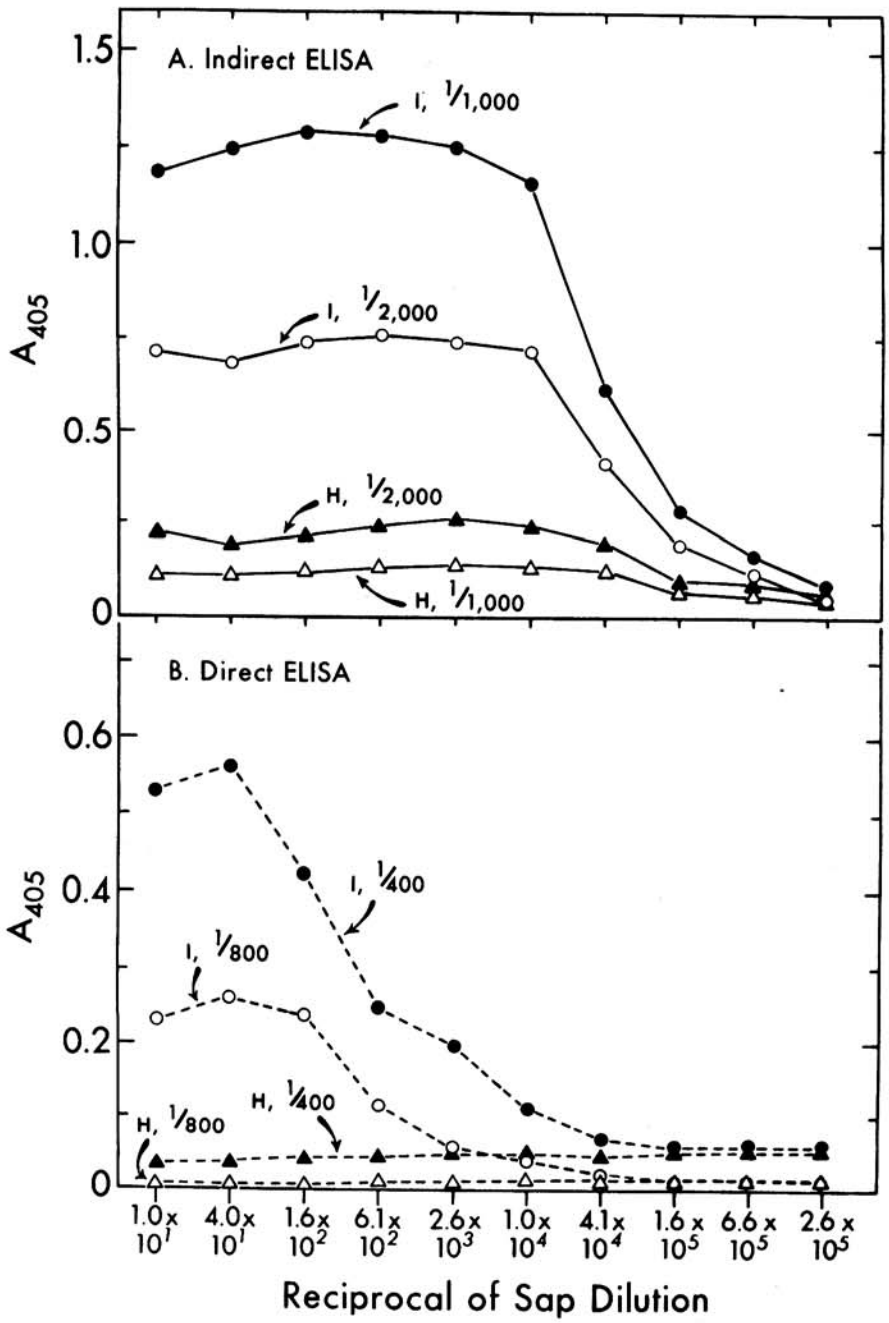

Fig. 4. Detection of cardamom mosaic virus (CarMV) in leaf extracts by enzyme-linked immunosorbent assay (ELISA). **(A)** Indirect ELISA, 5 μg/ml trapping CarMV gamma globulin, 1/1,000 and 1/2,000 diluted alkaline phosphatase conjugated antirabbit gamma globulin. **(B)** Direct ELISA, 5 μg/ml coating CarMV gamma globulin, 1/400 and 1/800 diluted alkaline phosphatase conjugated CarMV gamma globulin. I = infected and H = healthy.

CarMV, the serological relationship between CarMV of Guatemala and of India can now be determined. Until the relationship between katte disease and the virus we worked on is determined, we should refer to the latter as CarMV of Guatemala.

Ideally, it would have been best to use an isolate of CarMV that had been purified biologically through single lesion transfers and to have used tissue grown in greenhouses for virus purification. However, it was impossible for us to satisfy these conditions, considering the nature of the virus and our difficulty in growing infected plants under greenhouse conditions in New York. Nevertheless, our purified preparations appeared homogeneous, as judged by electron microscopy (Fig. 2) and analysis of the viral capsid protein (Fig. 3). Furthermore, we have had perfect correlation between results of ELISA, using antiserum produced to CarMV, and visual diagnosis of the disease in cardamom plants taken from all cardamom-growing regions of Guatemala (*unpublished*). Although we have not fulfilled Koch's postulates, it is likely that further work will show that CarMV is indeed the causal agent of the disease. Efforts are now under way in Guatemala to do this (Julio Tejada, *personal communication*).

Control of CarMV is highly dependent on the use of clean rhizomes or seedlings for establishing plantations. The successful production of antiserum to CarMV and its use to quickly and specifically detect infected plants by ELISA will help growers and nursery workers to select clean planting material. In fact, ELISA is now being used extensively in the cardamom mosaic virus control program of Guatemala (Julio Tejada, *personal communication*).

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