Purification and Partial Characterization of a Virus from Pineapple

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ABSTRACT

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Double-stranded RNA and long, flexuous, rod-shaped virus particles (1,200 × 12 nm) were recovered from mealybug wilt-affected pineapple (*Ananas comosus*) plants from plantations in Hawaii. A purification protocol was developed, and the virus was partially characterized. Based upon its morphology, the molecular weight of the coat protein, and analysis of double-stranded RNA, we tentatively assign this virus to the type II closterovirus group.

Additional keyword: toxin.

A wilt disease of pineapple (*Ananas comosus* (L.) Merr.) described in Hawaii more than 75 years ago (16) has remained a serious problem for the pineapple industry worldwide (2,3). The consistent association of mealybugs with this disease earned it the name mealybug wilt of pineapple. It was postulated to be caused by a toxin secreted by mealybugs during their feeding upon pineapple plants (2,4). Singh and Sastry (20) described the characteristic early symptoms as the drying and wilting of mature leaves beginning at the leaf tips and the appearance of a reddish-yellow color. At later stages, the central whorl of affected plants loses turgor as the leaf tips become brown and curl back tightly. If the plants become affected early, no fruit is formed and root development is retarded. The history and characteristics of this interesting disease have been reviewed recently (18).

Much of the available biological data (4–6,12,14,20) may be explained by postulating that mealybug wilt of pineapple is caused by a virus transmitted by mealybugs. This study was initiated to investigate the possibility of a viral etiology for wilt-affected plants, to isolate the virus, and to determine some of its properties.

MATERIALS AND METHODS

Plant material. Wilt-affected pineapple plants were selected from commercial plantations on the Hawaiian islands of Oahu, Maui, and Lanai. Wilt-free plants (subsequently shown to be free of double-stranded RNA and virus) were supplied from the breeding stock collection of Maui Pineapple Company.

Extraction of double-stranded RNA. Double-stranded RNA (dsRNA) was extracted from pineapple plants using the procedure of Dale et al (8) without modification. The plant tissue obtained was from about 1-yr-old infected plants, and care was taken to use only green leaf parts of partially wilted plants. Molecular weights of dsRNA isolated from pineapple were calculated by comparing their electrophoretic mobility to that of dsRNA isolated from *Citrus paradisi* Macfad. 'Marsh' infected with citrus tristeza virus (CTV), *Nicotiana tabacum* L. 'Xanthi' infected with tobacco mosaic virus (TMV), and *Hordeum vulgare* L. infected with brome mosaic virus (BMV). Electrophoresis was performed in a 6% polyacrylamide slab gel (7 cm × 10 cm × 0.75 mm) run at 25 mA for 10 hr. Gels were stained with 25 ng/ml ethidium bromide in electrophoresis buffer (40 mM Tris, 20 mM sodium acetate, and 1 mM ethylenediaminetetraacetic acid, pH 7). After staining, gels were photographed over ultraviolet light (310 nm) using a LP-3 Polaroid land camera and type 665 Polaroid film (Polaroid Corp., Cambridge, MA). Nucleic acid bands observed by polyacrylamide gel elctrophoresis were shown to be dsRNA by incubating the whole gel with ribonuclease A (RNase A) (1 μg/ml) either in 2× standard saline citrate (SSC) for 10 hr or in 0.1× SSC for 4 hr (1× SSC 150 mM NaCl, 15 mM-sodium citrate, pH 7.4). After being incubated with RNase A, gels were restained with ethidium bromide and observed on ultraviolet light and photographed. All nucleic acid samples were incubated with

deoxyribonuclease (10 μg/ml, RNase free) in 50 mM Tris-HCL, pH 8.0, and 10 mM $MgCL_2$ for 1 hr at 37 C for 1 hr, extracted with phenol:chloroform (1:1), incubated on ice for 20 min, and centrifuged. The nucleic acids in the supernatant were precipitated with 1/20 volume of sodium acetate and 2 1/2 volume of ethanol and stored overnight at −20 C. The nucleic acids were pelleted by centrifugation, dissolved in TE (0.01 M Tris-Cl, 0.001 M EDTA, pH 8.0) buffer, and analyzed by polyacrylamide gel electrophoresis as described above.

Virus purification procedure. The procedure finally adopted was a modification of that used by Zee et al for grapevine leafroll virus (21). Tissue was frozen in liquid nitrogen, ground to a fine powder in a mortar, and thawed in extraction buffer (500 mM Tris-Cl, pH 8.4, 4% Triton-X 100, 0.2% 2-mercaptoethanol) (EB) using 2 ml/g of tissue. The slurry was stirred for 1 hr at 4 C and filtered through cheesecloth, and the filtrate was centrifuged at 5,300 *g* for 20 min in a GSA rotor. The retained supernatant was centrifuged at 200,000 *g* for 35 min in a Ti60 rotor (Beckman Instruments, Inc., Fullerton, CA), and the pellet was dissolved in 100 mM Tris-Cl, pH 8.5, and 10 mM $MgCl_2$ (TM buffer) using 1/8 volume of extraction buffer. The suspension was clarified by centrifugation at 5,000 *g* for 10 min in an SS34 rotor (Dupont Company Medical Products, Sorvall Instruments, Wilmington, DE), and the supernatant was layered over 5 ml of 0.48 molal Cs_2SO_4 in TM buffer and centrifuged at 200,000 *g* for 16 hr in a Ti60 rotor at 8 C. The resulting pellet was dissolved in 200 μl of TM buffer. In a separate experiment, the supernatant from the second low-speed centrifugation was layered over 5 ml of 1.5 M sucrose in TM buffer and centrifuged at 150,000 *g* for 2 hr in a Ti60 rotor at 4 C. The resulting pellet was dissolved in 200 μl of TM buffer.

Electron microscopy. The resuspended pellets from Cs_2SO_4 and sucrose cushions were viewed using either a Hitachi model HS-8-1 (Hitachi America, Ltd., Tarrytown, NY) or a Zeiss 10A (Carl Zeiss, Inc., Thornwood, NY) transmission electron microscope. Samples were prepared by placing 5 μl of a virus suspension on 200-mesh, Formvar and carbon-coated grids (Ted Pella, Inc., Redding, CA). Excess liquid was carefully drained from the grid using filter-paper wicks. Grids then were washed with bacitracin solution in water (300 μg/ml) and stained by floating either for 6 min on a drop of 2% phosphotungstic acid (PTA), pH 6.7, in 250 μg/ml bacitracin solution or for 2 min on a drop of saturated uranyl formate solution in methanol. Photographs, including TMV, were used to calibrate the microscope for length and width measurements of virus obtained from pineapple. Fifty-four particles were measured using an IBAS 1 (Kontron IBAS Interactive Image Analysis System, Federal Republic of Germany) image analyzer.

Viral coat protein. Protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). One hundred grams of diseased tissue was used for virus purification, and the final pellet from a sucrose cushion was dissolved in 200 μl of 1× sample buffer (62 mM Tris-Cl, pH 6.7, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.004% bromophenol blue). Samples of 5, 10, and 15 μl (representing 2.5, 5, and 7.5 g of tissue) were applied to three lanes of a discontinuous slab gel (10 cm × 7 cm × 0.75 mm) system (15). Electrophoresis was performed at room temperature (24 C) at 150 V for 45–60 min. Tissue from healthy plants was processed in parallel with diseased tissue, and aliquots (representing 10 g of tissue) were analyzed by electrophoresis along with virus preparations. TMV coat protein (purified by standard procedures) and molecular-weight markers (Sigma Chemical Co., St. Louis, MO) were

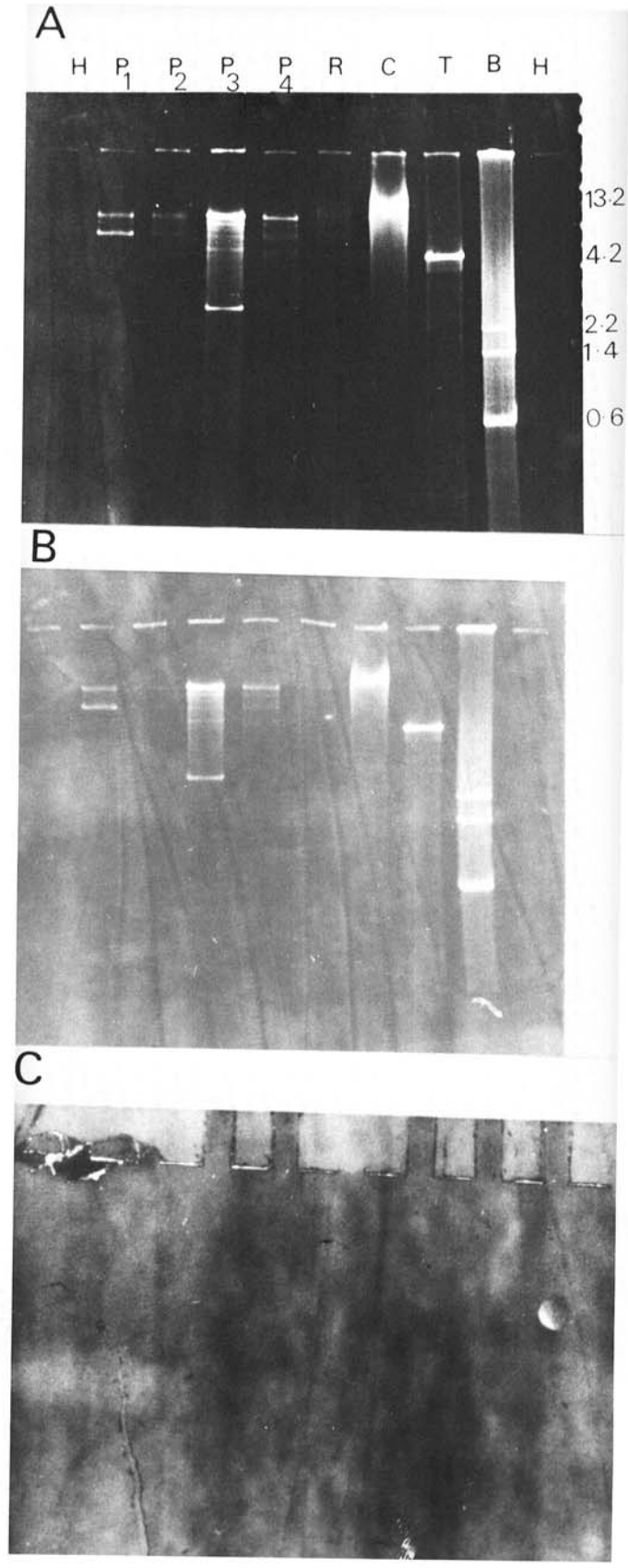

Fig. 1. Polyacrylamide gel electrophoresis of double-stranded RNA (dsRNA). **A,** Polyacrylamide gel electrophoresis of dsRNA isolated from pineapple. Lane H: preparation from healthy pineapple. Lanes P1–P4: dsRNA isolated from four different plants. Lane R: dsRNA isolated from diseased roots. Double-stranded RNA representing 20 g of plant tissue were loaded in these lanes. Lane C: dsRNA isolated from citrus infected with citrus tristeza virus (CTV). Lane T: dsRNA isolated from tobacco infected with tobacco mosaic virus (TMV). Lane B: dsRNA from barley infected with brome mosaic virus (BMV). The numbers on the right side of the figure are molecular weights of dsRNA of CTV, TMV, and BMV in millions of daltons. **B,** The gel in A after incubation with ribonuclease A (RNase A) in 2× standard saline citrate (SCC) for 10 hr at 25 C. **C,** The same gel as B after incubation with RNase A in 0.1× SSC for 4 hr. After each incubation, the gel was restained with ethidium bromide before visualizing on ultraviolet light.

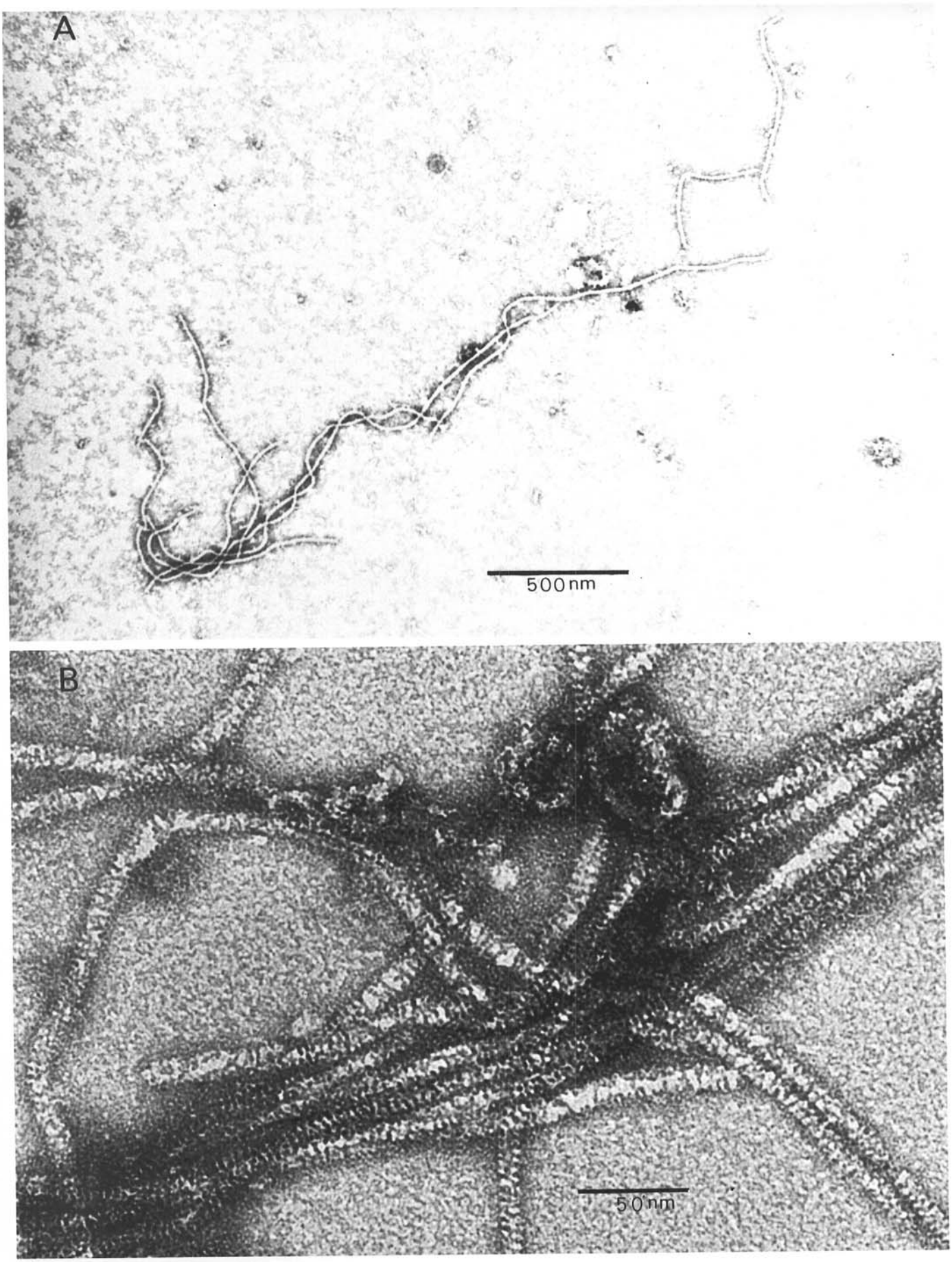

Fig. 2. A, Electron micrograph of virus particles stained with 2% phosphotungstic acid in 200 μg/ml of bacitracin in water. **B,** Electron micrograph of virus particles stained with saturated uranyl formate in methanol. The open structure characteristic of closteroviruses can be seen easily. The bending of the filaments suggests that this virus isolated from pineapple is flexible.

included, and their electrophoretic mobilities were used to estimate the molecular weight of the coat protein of the virus isolated from pineapple. After electrophoresis, proteins were visualized by staining with Coomassie blue (Bio-Rad Laboratories, Richmond, CA).

RESULTS

Analysis of dsRNA. Using green leaf material from wilting plants, we consistently recovered dsRNA from more than 50 plants tested throughout this study and we never observed dsRNA in healthy plant material. The observation that the molecules are resistant to RNase treatment under high-salt conditions but are degraded by RNase in low-salt buffer indicates that they are dsRNA. SDS-PAGE resolved the dsRNA into several components, the slowest moving of which was the most consistent. Bands of higher mobility were observed but their intensity was variable (Fig. 1, lanes P1–P3). The relationship between log molecular weight and distance migrated was determined for the standards using least squares linear regression, and the resulting equation was used to calculate a molecular weight of 8.35 million daltons for the slowest moving dsRNA band from infected pineapple samples.

Virus purification and electron microscopy. Purification using differential centrifugation followed by pelleting through a Cs_4SO_4 cushion produced greater yields of virus particles than other extraction methods including polyethylene glycol (PEG) precipitation, ammonium sulfate precipitation, and chloroform extraction followed by PEG precipitation. The superiority of the method was determined by transmission electron microscopy. PTA (2%), pH 6.7, in 250 μg/ml of bacitracin gave the best staining of virus particles (Fig. 2A). Uranyl acetate (2%) in 250 μg/ml bacitracin solution or a saturated solution of uranyl formate in methanol produced higher backgrounds and resulted in aggregation of virus particles. However, saturated uranyl formate in methanol allowed visualization of structural details of the virus particles at higher magnification. As shown in Figure 2B, the open structure and flexuous nature characteristic of closteroviruses (1,7) are very distinct at higher magnification. Based on the measurements of 54 virus particles, about 50% measured from 900 to 1,500 nm with an average length of 1,200 nm. The width of the particles was about 12 nm.

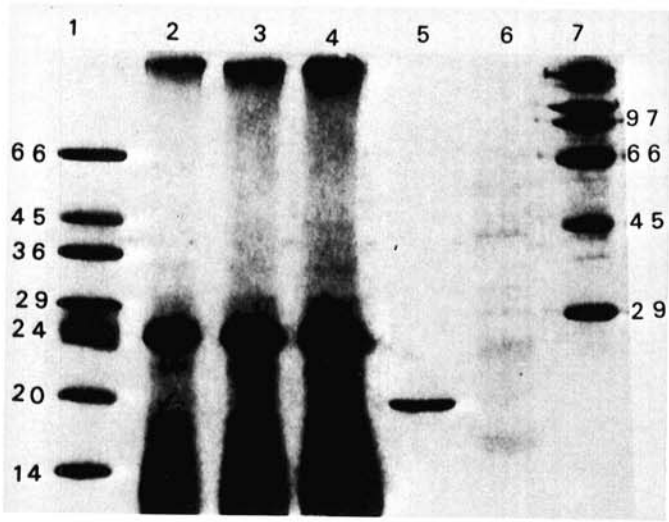

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1: molecular-weight markers (numbers on left side indicate the molecular weight in thousands of daltons). Lanes 2–4: virus isolated from diseased pineapple by the sucrose cushion method. Aliquots representing 2.5, 5, and 7.5 g of tissue were loaded in these respective lanes. Lane 5: coat protein from tobacco mosaic virus. Lane 6: aliqout representing 7.5 g of healthy tissue prepared along with diseased tissue. Lane 7: high-molecular-weight markers (numbers on the side represent the molecular weight in thousands of daltons).

Viral coat protein analysis. SDS-PAGE of disrupted, partially purified virus preparations revealed the presence of one major protein and some minor lower molecular-weight protein components. Electrophoretic analysis of equivalent fractions from a purification starting with uninfected material showed a minor amount of proteins of various molecular weights but no predominant band. The major band shown in Figure 3, lanes 2–4, is absent from the healthy material and is co-purified with virus, suggesting that it is the viral coat protein. Using the molecular-weight standards shown in the figure, a regression analysis of the log molecular weight versus mobility was used to obtain an equation to calculate a molecular weight of 23,800 Da for the major protein component co-purifying with the pineapple virus. A protein of this size is consistent with its being the coat protein of a closterovirus particle.

DISCUSSION

The etiology of mealybug wilt of pineapple has been controversial for many years. The theory that it is caused by a toxin secreted by mealybugs during feeding on pineapple was proposed by Carter (2–4). Later, the concept of a viral etiology began to emerge (5,14,20). In the work presented here, we have shown that dsRNA is present in mealybug wilt-affected pineapple plants and that its presence correlates with virus particles in affected tissues of those plants.

The dsRNA reproducibly recovered from diseased plants, which ran as an intensely staining, high-molecular-weight band on polyacrylamide gels, may be the replicative form of a viral RNA. The dsRNA staining pattern is similar to those shown by Dodds and Bar-Joseph (9) for closteroviruses. The virus particles are longer and narrower (1,200–1,500 nm × 12 nm) than potato virus X or potato virus Y (17) and generally resemble the dimensions of closteroviruses. Closteroviruses are not easily measured because they tend to aggregate, become entangled, and break during purification and may assume different dimensions in various negative stains (10). However, using PTA as a negative stain, we were able to measure enough particles to estimate the length and width of the virus. The open structure and the flexuous nature of the virus particles, as observed with uranyl formate staining, are very similar to the published characteristics of known closteroviruses (1,7,11). SDS-PAGE of purified virus resolved a protein with a molecular weight of 23,800 Da.

The closteroviruses have been divided into three subgroups based upon their biophysical properties (10). The virus from diseased pineapple plants that we have described best fits the closterovirus group for the following reasons: the dsRNA pattern in polyacrylamide gels, the particle dimensions and structure, the molecular weight of the coat protein, and the difficulties in purification. No other approved group can accommodate a virus with these properties. Interestingly, grapevine virus A, also a closterovirus, is one of the few plant viruses known to be transmitted by mealybugs (19).

To our knowledge, this report represents only the second example of the isolation of virus particles from pineapple. Tomato spotted wilt virus has been reported from pineapple (13). This is the first report of the isolation of a flexuous, rod-shaped virus from diseased pineapple and also of the association of this virus with pineapple mealybug wilt.

The presence of virus particles in diseased plants does not by itself prove the pathogenicity of the virus to the host. However, its consistent association with diseased plants leads us to postulate that it may be the causal agent of pineapple wilt disease. Experiments to validate this hypothesis by completing Koch's postulate are in progress.

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