

## Factors Affecting Purification of Maize Dwarf Mosaic Virus from Corn

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### ABSTRACT

Maize dwarf mosaic virus (MDMV) was extracted from corn leaves by homogenization in 0.1 M sodium citrate plus 0.5% mercaptoethanol, and was clarified with chloroform and one cycle of differential centrifugation. Pellets were resuspended in 0.005 M sodium citrate, pH 7.0. Purified virus was obtained from sucrose density-gradient columns prepared with the same buffer, and was concentrated by centrifugation. Purified MDMV had an absorption spectrum typical for filamentous viruses containing 5% RNA, showed birefringence, and sedimented at 160-162 S. The concentration of virus after each purification step was measured by analytical

density-gradient centrifugation or by serological and infectivity assays. Typical final yields of 9.6-23.6  $\mu\text{g/g}$  fresh wt represented a total recovery of only 25% of the initial virus concentration in the clarified sap. Variations in the purification procedure failed to improve the recovery of virus nucleoprotein. Systemic infectivity assays on corn, analyzed by maximum likelihood and loglog transformation, demonstrated that less than 0.5% of the infectivity of the crude sap was recovered in the purified virus.

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A number of reports of purification of flexuous rod-shaped viruses, including maize dwarf mosaic virus (MDMV) (1, 10, 11, 12, 13, 14), tobacco etch virus (TEV) (6, 8), and wheat streak mosaic virus (WSMV) (2, 5), have indicated that these viruses are difficult to purify. Lack of success has been attributed to low initial concentrations of virus in host tissue, instability of virus following extraction from the host, and aggregation during the purification process. Purification of MDMV has been evaluated qualitatively by infectivity, by formation of a zone upon density-gradient centrifugation, and by electron microscopy (1, 7, 10, 11, 12, 13, 14). We describe here a re-examination of MDMV purification utilizing three different types of assays to measure quantitatively the amount of virus in clarified sap and at each step of purification. The amount of virus lost at each step gave an indication as to whether the reported low yield of MDMV was due either to a low initial concentration or to aggregation and/or degradation during purification. Variations in the purification procedure were investigated in attempts to increase virus recovery. Certain physical properties were determined using virus purified by density-gradient centrifugation.

**MATERIALS AND METHODS.**—*Cultural conditions.*—The MDMV isolate used was isolated from naturally infected corn plants in Nelson County, Va. Its host range indicated it to be strain A (Johnson grass strain), as reported by Roane & Tolin (9). The virus was maintained in the greenhouse in *Zea mays* L. corn hybrid Hy X C103 grown in 4- or 5-inch pots. Inoculations were made either by rubbing leaves, dusted with 600-mesh Carborundum, with inoculum prepared by grinding infected tissue in an equal volume of neutral 0.01 M sodium phosphate buffer, or by spraying leaves with an artist's airbrush (Thayer & Chandler, Inc., Chicago, Ill., Model C) with

inoculum containing 1% Carborundum. Inoculum for the airbrush was prepared by grinding infected tissue in 0.1 M sodium citrate plus 0.1% mercaptoethanol (R. W. Toler, *personal communication*), straining through cheesecloth, and diluting to a final concentration of 1:10 (w/v). Air pressure was maintained at 120 psi for inoculation with the airbrush in the greenhouse. Corn seedlings in the greenhouse were inoculated in the 1- to 2-leaf stage, 7-10 days after planting, and were harvested 10-14 days after inoculation. Corn grown under field conditions was inoculated in the 2- to 3-leaf stage with an airbrush at 80-100 psi, and was harvested 14-35 days after inoculation.

*Purification and analysis.*—Virus was extracted from systemically infected corn leaves, with the midrib removed, by homogenizing the leaves in a cold Waring Blendor in two-three stages with a volume of cold 0.1 M sodium citrate plus 0.5% mercaptoethanol equal to the weight of tissue. The homogenate was strained through cheesecloth, then centrifuged for 10 min at 10,000 g. The volume of the supernatant fluid was measured, emulsified with 0.5 volume of cold chloroform, and centrifuged for 5 min at 4,000 g to separate the emulsion. The aqueous, upper phase was collected, held at 4 C for 3-4 hr, then centrifuged for 15 min at 12,000 g. The supernatant fluid was centrifuged at 30,000 rpm for 90 min in the Type 30 rotor of the Spinco Model L-2 65B ultracentrifuge. The resulting pellets were overlaid with 0.005 M sodium citrate, pH 7.0, stirred gently with a glass rod, and allowed to stand overnight at 4 C. The resuspended virus pellet was centrifuged at 10,000 g for 10 min; the supernatant fluid was adjusted with 0.005 M sodium citrate to 0.1 the volume of the clarified sap. Two to 4 ml of the virus suspension were layered on sucrose density-gradient columns and centrifuged for 2.5 hr at 27,000 rpm in the SW 27

rotor. Sucrose gradients were made by layering 6, 9, 9, and 10 ml of solutions containing 100, 200, 300, and 400 g sucrose/liter, respectively, into 1-inch X 3.5-inch cellulose nitrate tubes. The sucrose was dissolved in distilled H<sub>2</sub>O; citrate buffer (0.5 M sodium citrate adjusted to pH 7.0 with 0.5 M citric acid) was added to make a final concentration of 0.005 M citrate. Gradients were allowed to stand overnight at 4 C before centrifugation.

Gradients were scanned photometrically by the method of Brakke (4). The ISCO (Instrumentation Specialties Co., Lincoln, Neb.) ultraviolet analyzer was adjusted to give a full-scale deflection equal to 0-1.0 absorbance units at 254 nm. The virus zone was collected manually, diluted with an equal volume of 0.005 M citrate, pH 7.0, and centrifuged for 50 min at 50,000 rpm in the Type 65 rotor. The virus pellets were resuspended in a volume of 0.005 M citrate, pH 7.0, equal to 0.1 the volume of the clarified sap. Following an additional low-speed centrifugation, the "purified virus" was obtained. The concentration of virus was determined as previously described (8). The ultraviolet absorption spectrum was determined with a Unicam Model Sp 800A spectrophotometer. The sedimentation coefficient for a single concentration of virus was determined in a Spinco Model E ultracentrifuge using Schlieren optics.

**Infectivity assay.**—For systemic infectivity assays, 7-day-old Hy X C103 corn seedlings were used. Each sample to be assayed was first adjusted to 1 ml/g tissue and then diluted to 1:10 (v/v) and 1:20, and by 10-fold steps thereafter. Crude sap and clarified sap were diluted with 0.1 M sodium citrate plus 0.5% mercaptoethanol. Approximately 1% Carborundum (600-mesh) was mixed with each dilution. Purified virus samples were diluted with 0.005 M citrate, pH 7.0. Inoculations were made by spraying 4.5 ml of each dilution on 10 seedlings with an artist's airbrush, which was washed thoroughly with distilled water between inoculations. Within each series, the highest dilution was inoculated first and the most concentrated was last. Infected plants were recorded 7 and 14 days after inoculation. The titer was calculated using a loglog transformation. The results are reported as the logarithm and the standard deviation of the number of infectious particles per infection-initiating volume that infected 63% of the plants (2). The percentage loss of infectivity was calculated from the actual number of particles per infection-initiating volume for each assay.

**RESULTS.—Properties of purified MDMV.**—When purified by chloroform clarification, differential and sucrose density-gradient centrifugation, MDMV was colorless, opalescent, birefringent, and formed a visible band in sucrose density-gradients at concentrations greater than 0.5 mg/ml. Density-gradient scanning patterns at three stages of the purification procedure for MDMV-infected and healthy corn tissue are shown in Fig. 1. Results with field-grown corn are presented here, but similar results were obtained with corn grown under greenhouse conditions. MDMV from clarified sap formed a symmetrical peak indicating uniformity in

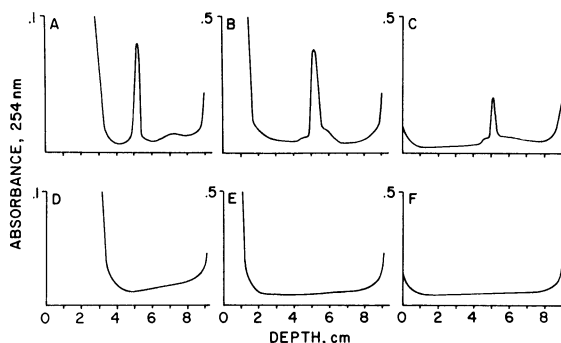


Fig. 1. Density-gradient scanning patterns of field-grown maize dwarf mosaic virus-infected *Zea mays* L. 'Hy X C103' at three stages of the purification procedure. A) Clarified sap from 1.5 g of leaf tissue. B) Resuspended first high-speed pellet from 15 g of tissue. C) Purified virus from 15 g of tissue. D, E, F) Comparable treated healthy corn tissue.

particles (Fig. 1-A). The concentration of virus decreased with purification. Absorbance on the leading and trailing edges of the peak indicates aggregation and degradation of virus from the resuspended first high speed pellet (Fig. 1-B). Aggregation and degradation were also evident in the scanning pattern of purified virus in which nearly all of the slowly sedimenting contaminating material was removed (Fig. 1-C). Samples from the gradient columns from above, in, and below the zone showed short rods, rods ca. 750 nm, and rods fragmented into short segments or aggregated, respectively. Analysis of correspondingly treated healthy corn confirmed that no components sedimenting at the same rate as MDMV were present (Fig. 1-D, E, F).

Purified preparations, as represented in Fig. 1-C, showed an ultraviolet absorption spectrum typical (Fig. 2) for rod-shaped viruses having 5% RNA and 95% protein. The A<sub>260/280</sub> of a typical purified

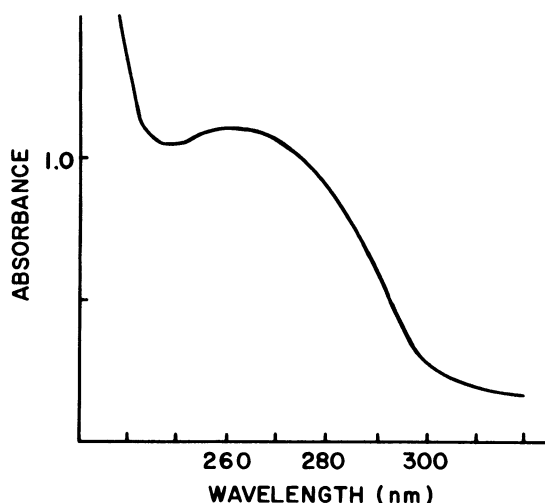


Fig. 2. Ultraviolet absorption spectrum of purified maize dwarf mosaic virus in 0.005 M citrate buffer, pH 7.0.

TABLE 1. Yield of maize dwarf mosaic virus from field-grown *Zea mays* L. 'Hy X C103'

Virus preparation	Density gradient		Titer <sup>c</sup>	Infectivity		Dilution end point
	Yield <sup>a</sup>	% Loss <sup>b</sup>		% Loss		
Crude sap						
Exp. 1			3.43 ± 0.11			10 <sup>-5</sup>
Exp. 2			3.44 ± 0.09			10 <sup>-5</sup>
Clarified sap						
Exp. 1	45.0		2.59 ± 0.11	85.5 <sup>d</sup>		10 <sup>-4</sup>
Exp. 2	41.2		3.13 ± 0.12	51.0		2 × 10 <sup>-4</sup>
First high-speed						
Exp. 1	18.1	58	1.56 ± 0.23	98.6	91.7 <sup>b</sup>	2 × 10 <sup>-3</sup>
Exp. 2	17.8	57	1.35 ± 0.11	99.2	98.4	10 <sup>-3</sup>
Purified virus						
Exp. 1	9.6	79	1.10 ± 0.12	99.53	96.8	10 <sup>-2</sup>
Exp. 2	11.2	73	0.98 ± 0.13	99.65	99.3	10 <sup>-2</sup>

<sup>a</sup>  $\mu$ g of virus per gram fresh weight.

<sup>b</sup> % Loss from clarified sap.

<sup>c</sup> Logarithm and standard deviation of the number of infectious particles per infection-initiating volume.

<sup>d</sup> % Loss from crude sap.

preparation was 1.18. The spectrum also showed a slight shoulder at 290 nm similar to that for TMV, but not as prominent, indicating the presence of tryptophan in the coat protein.

Purified virus at 0.42 mg/ml suspended in distilled H<sub>2</sub>O or in 0.01 M NaCl had a sedimentation coefficient of 160 or 162 S, respectively, in the Spinco Model E analytical ultracentrifuge. Only one component was detected in the purified virus.

*Variation in purification procedure.*—All variations in the purification procedure were conducted using greenhouse-grown Hy X C103 or Golden Cross Bantam. The effect of a variation in one step of the procedure was determined while all other steps remained constant. Sucrose density-gradient analysis was used to evaluate the effects of each variation.

For homogenization and clarification, the tissue was ground in five molarities of sodium citrate: 0.5, 0.1, 0.05, 0.01, or 0.005. The highest yield of virus and the sharpest peak in sucrose density-gradients were obtained from tissue ground in 0.1 M sodium citrate. Two to 4-fold less virus was obtained using 0.5 M sodium citrate and the virus peak was usually broad, whereas little or no virus was recovered using 0.05 M, 0.01 M, or 0.005 M.

Additional treatments evaluated for their efficacy in clarification of MDMV were the freezing of leaf tissue or crude homogenate, the heating of crude sap at 40 C for 15 min, addition of charcoal, and the lowering of the pH of the crude sap to pH 5.5 with citric acid. None was as effective as chloroform emulsification when evaluated both as clarified sap and after high-speed centrifugation. We recovered no measurable virus after freezing either the leaves or the ground homogenate. Charcoal treatment did not give adequate clarification. Heat and acid treatment caused excessive aggregation after high-speed centrifugation. Even though chloroform treatment resulted in a loss of infectivity (Table 1), it gave good clarification without excessive aggregation.

Several ratios of chloroform to homogenate (v/v) were evaluated. A 1:2 ratio gave adequate clarification and did not increase the total volume as much as the 1:1 ratio used earlier (1, 13), and the sap was a clear, light yellow after separation of the emulsion. Allowing the clarified sap to stand at 4 C for 3-4 hr between the centrifugation to break the chloroform emulsion and the final low-speed centrifugation resulted in the formation of a heavy precipitate. This precipitate, which contained no virus, was removed by centrifugation at 12,000 g for 15 min, leaving a clear, light-yellow supernatant fluid. The high-speed pellets were smaller and contained less nonviral material when the clarified sap was held for 3-4 hr before the final low-speed centrifugation than when it was centrifuged immediately.

Citrate buffer concentrations from 0.5 to 0.001 M, pH 7.0, were compared to determine the optimum molarity of citrate for resuspending high speed pellets; 0.005 M citrate buffer was the most satisfactory. The purified virus was stable in this buffer for at least 1 week, the longest time tested, whereas the virus in clarified sap was destroyed after 2 days. High-speed pellets resuspended in a volume of 0.005 M citrate buffer equal to 0.1 volume of the clarified sap showed birefringence.

The effects of several different additives and buffers on the recovery of virus after the high-speed centrifugation were evaluated. The detergent Igepon at 0.1, 0.01, 0.005, and 0.001% (3); 5% sucrose (15); and 0.1% mercaptoethanol and urea (6) were tested as additives to 0.005 M sodium citrate buffer. Other buffers evaluated were 0.005 M sodium borate, pH 8.0; 0.01 M phosphate, pH 7.0; 0.005 M Tris [tris (hydroxymethyl) amino methane]-HCl, pH 7.0; and distilled H<sub>2</sub>O. None of the additives consistently increased the yield of MDMV after high-speed centrifugation, nor appeared to decrease the degree of aggregation.

*Loss of MDMV during purification.*—The loss of MDMV at each step of the purification procedure was

determined by sucrose density-gradient analyses, by systemic infectivity assays, and by microprecipitin tests. The results of two typical experiments are shown in Table 1.

The yield determined by sucrose density-gradients in a typical experiment was 45.0  $\mu\text{g}$  of virus/g fresh weight of tissue in the clarified sap, 18.1  $\mu\text{g}/\text{g}$  in the pellet resuspended after the first high-speed centrifugation, and 9.6  $\mu\text{g}/\text{g}$  in the purified virus. The results in general showed that 50% of the virus in the clarified sap was lost after the first high-speed centrifugation, and an additional 50% loss occurred during the final purification step. Total ultraviolet absorbance equivalent to the amount in the collected virus zones could be recovered after centrifugation to concentrate the purified virus and remove sucrose, but only half of the absorbance layered on the second density-gradient was recovered in the zone. The microprecipitin test for the two experiments gave virus dilution end points of 1/128-1/256, 1/64, and 1/32 for clarified sap, resuspended first high-speed pellets, and purified virus, respectively. Thus, the over-all yield of purified virus was only 25% that in the clarified sap, based on sucrose density-gradient and serological dilution end point analyses.

Systemic infectivity assays of crude sap and the three purification steps showed that a tremendous loss of infectious virus occurred (Table 1). An average of about 75% of the infectivity was lost during the chloroform clarification step. Virus loss during clarification could not be measured by density-gradient or serological analysis. Another 20-25% of the infectivity was lost by high-speed centrifugation, but little further loss occurred in the purified virus stage. The total recovery of infectious purified MDMV was less than 0.5% of the infectivity in crude sap. Loss could also be expressed in terms of the infectivity dilution end point, which decreased 10-fold at each step.

Yield of purified MDMV varied from one run to another, but was probably influenced more by the concentration of virus in the tissue than by differences in the efficiency of the technique. For three different experiments in which the yield of purified virus was 9.6, 15.0, and 23.6  $\mu\text{g}/\text{g}$ , the percentage loss from clarified sap to purified virus was 82, 79, and 73, respectively.

**DISCUSSION.**—Our purification procedure differs from others (1, 10, 11, 12, 13) in the use of citrate instead of borate or phosphate buffers, and in the use of sucrose density-gradient centrifugation, but is similar to that used by Snazelle et al. (14). There appeared to be an optimum molarity of citrate for tissue extraction and for virus suspension after high-speed centrifugation. Although clarification by chloroform emulsification caused considerable loss of virus, it was the most satisfactory and consistent treatment of any of those tested. The fact that several additives failed to increase the efficiency of resuspension indicates that MDMV differs from WSMV (5) and TEV (6) in chemical properties controlling aggregation. These data support the results of Snazelle et al. (14). Purified by this

method, MDMV appears to be stable. Fairly high yields of purified virus were obtained consistently. The sedimentation coefficient of 160-162 S is between those previously reported for MDMV (1, 13) and is similar to values reported for TEV (8) and WSMV (2). The slight shoulder at 290 nm in the ultraviolet absorption spectrum varies from Sehgal & Jean's results (12), but may indicate a high degree of purity of the virus. The  $A_{260/280}$  agrees with the value of 1.18 obtained by Sehgal & Jean (12).

It is difficult to compare the infectivity of purified virus reported here to that reported by others because insufficient data were presented. Shepherd reported a dilution end point of  $10^{-3}$  for purified virus concentrated 50-fold (13). Sehgal (10) reported a dilution end point of  $2.5 \times 10^{-2}$  for purified MDMV, but the initial concentration of the purified virus was not given. The  $10^{-2}$  dilution end point reported here for purified virus apparently is higher than either of the previously reported values, since the inoculum was adjusted to 1 ml/g of tissue prior to dilution. These results indicate a higher percentage recovery of purified virus, a higher virus concentration in the starting tissue, or both. The dilution end point of  $10^{-5}$  for crude sap of field-grown tissue is higher than that reported previously (10, 13).

Analysis of clarified sap and purified virus by density-gradient centrifugation and photometric scanning was a rapid, consistent method for the measurement of MDMV concentration. The results were comparable to infectivity or serological assays of the same preparations. The yield of purified MDMV from field-grown Hy  $\times$  C103 of 9.6-23.6  $\mu\text{g}/\text{g}$  fresh weight of leaf tissue is greater than the 4  $\mu\text{g}/\text{g}$  obtained for WSMV by the same technique (5). Yields of MDMV reported here are less than the 40-60  $\mu\text{g}/\text{g}$  obtained by Damirdagh & Shepherd (6) with TEV from tobacco, but their values were apparently not based on purified virus recovered in zones.

The yield of purified virus represents only about 25% of that present in clarified sap. Of this, 50% was lost by failure to recover virus after the first high-speed centrifugation. Approximately 50% of the absorbance layered on the gradient column was recovered in the virus zone in the subsequent density-gradient centrifugation. This loss of virus was probably due to aggregation. Working with WSMV, Brakke & Ball reported a 70-90% recovery of virus after the first density-gradient centrifugation, and a total recovery from clarified sap to purified virus of 50% (5). Infectivity assays of MDMV indicated a yield of less than 0.5% from crude sap to purified virus, compared with 1% for WSMV (5). Purified MDMV, like WSMV, apparently has a low specific infectivity.

Electron microscopy showed that fragments of virus particles occurred in samples from both above and below the virus zone. The short pieces below the virus zone indicate that degradation occurred in the gradient, since initially degraded particles would have had a lower sedimentation rate and would have

remained above the virus zone. Aggregated virus particles observed below the virus zone and in the pellet also account for part of the losses incurred.

Our results confirm earlier reports on purification of MDMV, suggesting that aggregation (10) and degradation (1, 7, 13) occur during purification. Using our purification procedure, consistent relatively high yields of purified MDMV were obtained provided the starting tissue had a high concentration of virus (8). Additional work is still necessary, however, for improvement of the purification of MDMV. Preventing aggregation and degradation should be the major objective of these studies. Comparative studies should be done with MDMV and TEV to determine why these flexuous rods differ so greatly in reaction to buffers and additives during purification.

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