

## Use of Triton X-100 and Sephacryl S-500 HR for the Purification of Cymbidium Mosaic Virus from Orchid Plants

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Cymbidium mosaic virus (CyMV) was purified from CyMV infected orchid plant leaves by Sephacryl S-500 HR column chromatography. Partial purification was done by solubilization with Triton X-100 (alkylphenoxypolyethoxy ethanol) and precipitation with polyethylene glycol (PEG 6,000) followed by ultracentrifugation on 30% sucrose cushion. Based on the spectrophotometric analysis, 33 mg of CyMV could be obtained from 100 g of CyMV-infected orchid plant leaves. The purified CyMV represented one distinct homogeneous band by SDS-PAGE, and electron microscopy revealed that it was highly homogeneous and not fragmented. Bioassay demonstrated that the purified CyMV had a normal infectivity to *Chenopodium amaranticolor* and orchid plants. Based on these results, the purification method in this work could be served as an improved method for the purification of CyMV and similar viruses with good yield, high purity and native integrity.

**Keywords :** Cymbidium mosaic virus, Sephacryl S-500 HR column chromatography, Triton X-100.

Cymbidium mosaic virus (CyMV), one of the most prevalent viruses in orchid plants, causes the reduction of plant vigor and flower quality, which affects economic value of the plants. To develop the strategy for health certification program, the method of reliable and rapid purification of CyMV is of primary importance (Frowd and Tremaine, 1977; Hsu et al., 1992; Pearson and Cole, 1986; Wisler et al., 1982). In fact, several procedures for the purification of CyMV from the infected orchids have been reported in many previous works. However, these methods have many troubles such as aggregation, insolubilization, loss of infectivity of the virus, and contamination with host plant proteins, routinely encountered in purifying plant viruses (Corbett, 1960; Lagenberg, 1973). Since Triton X-100

(alkylphenoxypolyethoxy ethanol) was used for the isolation of tobacco mosaic virus from tobacco plants (Nozu and Yamamura, 1971), it has been used for the purification of many plant viruses (Derks et al., 1982; Langenberg, 1973, Noze and Yamamura, 1971). Triton X-100 has been widely accepted to be able to solve insolubilization and proved not to be harmful, while the other organic solvents such as butanol and chloroform were well known to be hazardous and harmful. On the other hand, the usual purification procedures impose considerable stress on the integrity of virus particles and may lead to disruption of the native structure with subsequent loss of biological activity. To solve such problems, chromatographic methods, mainly used for protein and animal virus purification, have been applied to a few plant viruses (Venekamp and Mosch, 1963).

In this paper, we report an improved method for the rapid and reliable purification of CyMV from orchid plants. Triton X-100 was applied to the clarification for solubilizing virus particles effectively and final purification was carried out by Sephacryl S-500 HR column chromatography. The physicochemical and biological properties of purified virus were examined.

### Materials and Methods

**Plant sources and anti-CyMV antibodies.** CyMV-infected orchid plants, *Cattleya* spp. were kindly given by Dr. Geol-Bo Sim, Yeonam College of Livestocks and Horticulture, Korea and infected immediately through agar gel diffusion test. The infected plant leaves were segmented into small parts and stored at -20°C prior to use. Anti-CyMV antibodies were purchased from American Type Culture Collection (ATCC, Rockville, Md).

**Purification of CyMV.** The CyMV-infected orchid plant leaves were macerated with sap pressor in equal volume of 0.1 M sodium phosphate buffer (pH 7.4) containing 1% (w/v) sodium sulfite and filtered through several layers of cheesecloth. And then 2% Triton X-100 (alkylphenoxypolyoxy ethanol, Rhom and Hass, Philadelphia, Pa) was applied to the homogenate with continuous stirring and incubated for 30 min at 4°C. This solution

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was centrifuged at 10,000g and the supernatant was harvested. After the addition of polyethyleneglycol (PEG; MW 6,000) and sodium chloride to the supernatant at the final concentrations of 8% and 1%, respectively, the solution was incubated for 30 min at room temperature with continuous stirring. Pellets saved after centrifugation at 10,000 rpm for 20 min were resuspended with one hundred volume of the same buffer and incubated at 4°C overnight. After the removal of insoluble materials by the centrifugation for 5 min at 5,000g, proper volume of the same buffer was added to the supernatant and this solution was reprecipitated by PEG as before. The resultant was applied onto 30% sucrose cushion and centrifuged for 90 min at 35,000 rpm. The pellets were dissolved into the same buffer and spinned down for 10 min at 9,000 rpm. The resultant was applied to Sephacryl S-500 HR (Pharmacia) column (1×15 cm) equilibrated with 100 mM sodium phosphate buffer (pH 7.4), washed with 10 volumes of the same buffer and stabilized at 4°C for 24 hr and then eluted with the same buffer with a velocity of 40 ml/hr. Virus fractions were collected with a fraction collector (Model 2110, Bio-rad) equipped with a UV detector (Model 112 UV/visible detector, Gilson) and recorder. Each eluate was concentrated by ultrafiltration, scanned by a UV spectrophotometer and then active fractions were pooled.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electron microscopy.** The viral solution was quantitated by the method of Bradford with bovine serum albumin (Bio-rad Laboratories Inc., Richmond, CA) as a standard. Protein constituents were analyzed on 12.5% SDS-PAGE. Finally purified virus preparations were negatively stained with 2.0% neutral sodium phosphotungstate (PTA) and examined by an electron microscope (Hitachi H-800, Hitachi Ltd., Co., Japan).

**Bioassay.** The test plant, *Chenopodium amaranticolor*, and orchid plants were cultivated in the temperature-controlled growth chamber. The purified virus was used as an inoculum at concentration of 50 µg/ml. The inoculum was applied on the upper surface of the test plant leaves or orchid plants and the symptom development was examined 7 days after inoculation.

## Results and Discussion

### Effect of Triton X-100 on the purification of CyMV.

To examine the efficacy for the purification of CyMV, Triton X-100 was compared to other organic solvents. As

**Table 1.** Effect of different organic solvents on solubilization of Cymbidium mosaic virus

Organic solvent	Absorbance at			Virus conc. (mg/ml) <sup>a</sup>
	260 nm	280 nm	280 nm/260 nm	
Triton X-100 <sup>b</sup>	0.321	0.377	1.174	0.107
Chloroform & butanol	0.069	0.083	1.200	0.023
Chloroform	0.050	0.062	1.240	0.016

<sup>a</sup>Twentieth-fold dilution of the original crude sap was analyzed by spectrophotometry.

<sup>b</sup>Concentration of Triton X-100 was 4%.

shown in Table 1, Triton X-100 was the best among the organic solvents tested. For the practical application, optimum concentration and treating time of Triton X-100 were also investigated. Table 2 shows that the best yield could be obtained when Triton X-100 was treated after macerating the orchid plant leaves at the concentration of 2%.

Based on these results, the effect of hydrogen ion concentration of maceration buffer on the yield of CyMV was examined when the concentration of Triton X-100 was fixed at 2%. As shown in Table 3, the best yield was obtained at pH 8.0.

**Final purification of CyMV by Sephacryl S-500 HR column chromatography.** To purify CyMV, Sephacryl S-500 HR column chromatography was carried out to obtain 33 mg of CyMV from 100 g of CyMV-infected *Cat-*

**Table 2.** Effect of Triton X-100 concentrations on solubilization of Cymbidium mosaic virus

Conc. of Triton X-100 (%)	Absorbance at			Virus conc. (mg/ml) <sup>a</sup>
	260 nm	280 nm	280 nm/260 nm	
2.0	0.667	0.627	0.940	0.222
4.0	0.653	0.583	0.893	0.218
8.0	0.637	0.611	0.959	0.212

<sup>a</sup>Ten-fold dilution of the original crude sap was analyzed by spectrophotometry.

**Table 3.** Effect of hydrogen ion concentrations of the maceration buffer on solubilization of Cymbidium mosaic virus

Hydrogen ion conc. (-log[H] <sup>+</sup> )	Absorbance at			Virus conc. (mg/ml) <sup>a</sup>
	260 nm	280 nm	280 nm/260 nm	
6.5	0.215	0.306	1.423	0.071
7.0	0.217	0.291	1.341	0.072
7.5	0.272	0.308	1.132	0.090
8.0	1.002	1.576	1.572	0.334

<sup>a</sup>Ten-fold dilution of original crude sap was analyzed by spectrophotometry.

**Table 4.** Purification of Cymbidium mosaic virus from *Cattleya* orchid leaves

Purification step	Specific viral quantity <sup>a</sup> (mg/ml)	Total viral quantity (mg)	Yield (%)
Crude extraction <sup>b</sup>	1.1	107.0	100
PEG precipitation	6.5	67.2	62.8
Sucrose cushion centrifugation	19.8	48.5	45.3
Sephacryl S-500 HR column chromatography	33.0	33.0	30.8

<sup>a</sup>Analyzed by spectrophotometry.

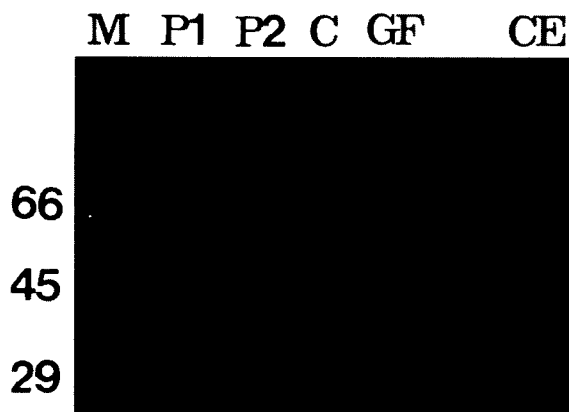
<sup>b</sup>Homogenizing 100 g of CyMV-infected *Cattleya* orchid leaves in an equal volume of 0.1 M sodium phosphate buffer and solubilized with 2% Triton X-100.

*tleya* leaves over 30 folds with 30.8% yield (Table 4).

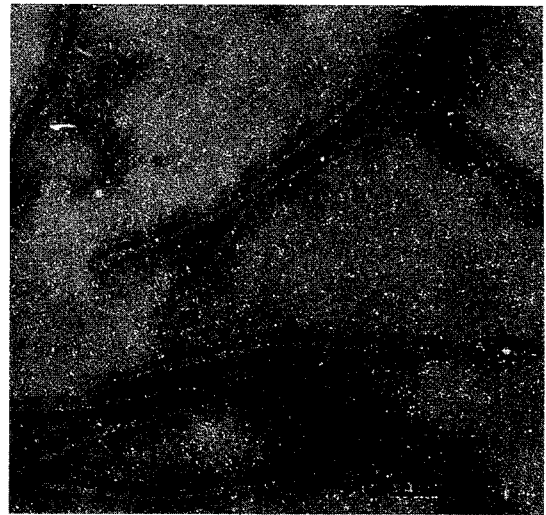
The yield was comparable to that of CMI description, 360 mg/kg of CyMV-infected leaf tissue. In fact, because of mixed infection of several viruses in orchid plants, the separation of CyMV by repeated selection on test plant, *C. amaranticolor* or *Datura stramonium* was generally accepted to be a prerequisite for the purification. However, the separation step has many troubles such as time-consuming and relatively low efficiency. In this point of view, a new purification method reported here may be very effective for the virus separation.

**Physicochemical and biological properties of purified CyMV.** To examine the purity, the size and shape of the finally purified CyMV, SDS-PAGE and electron microscopy were carried out. A putative CyMV band was shown in each purification step, and the final preparation of CyMV obtained from Sephacryl S-500 HR column chromatography represented one distinct homogeneous band (Fig. 1). As shown in Fig. 1, the molecular weight of its capsid protein was estimated to be about 27,000 dalton. This molecular weight was comparable to 27,640 dalton of Frowd and Tremaine (1977), who purified CyMV from *D. stramonium* leaves with local lesions by density gradient centrifugation. Meanwhile, electron microscopy revealed that virus particles were filamentous in shape and  $475 \times 13$  nm in average size (Fig. 2). As shown in Fig. 2, purified CyMV was highly homogeneous and the virus particles were neither aggregated nor fragmented.

To examine the biological activity of the finally purified CyMV preparation, infectivity assay was carried out. The finally purified CyMV has the normal infectivity to the test plant, *C. amaranticolor* and orchid plants, as shown in Fig. 3.



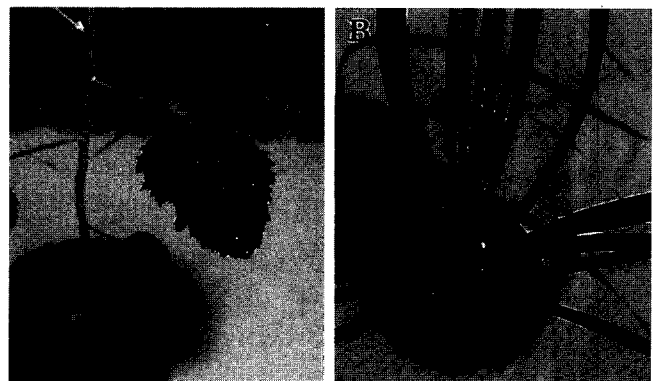
**Fig. 1.** SDS-PAGE analysis of purification intermediates. CE, crude extract of CyMV-infected *Cattleya* leaves; GF, eluate from Sephacryl S-500 HR; SC, pellets after ultracentrifugation on 30% sucrose cushion; P1, pellets after the first precipitation with PEG; P2, pellets after the second precipitation with PEG; M, molecular weight marker (bovine serum albumin, 66,000; egg albumin, 45,000; carbonic anhydrase, 29,000 dalton).



**Fig. 2.** Electron micrograph of purified Cymbidium mosaic virus. The bar represents 100 nm.

Based on these results, it has been thought that the finally purified CyMV has normal biological activity.

On the other hand, Nozu and Yamaura (1971) reported the use of Triton X-100 for the isolation of TMV from some other plants, not tobacco. They demonstrated that the purified TMV was not significantly affected by the treatment of Triton X-100 in electron microscopy. Derks et al. (1982) also reported that Triton X-100 dissolved cell membrane and did not have negative effect on the virus to be purified, and higher yield of the intact virus particle could be obtained. In this work, electron microscopy revealed that the finally purified CyMV was homogeneous in shape and size, and neither fragmented nor aggregated. Based on these results, the purification method in this work, especially with the use of Triton X-100 for solubilization of virus particles from plant cell components and Sephacryl S-500 HR column chromatography, may be used for the puri-



**Fig. 3.** Necrotic local lesions on *Chenopodium amaranticolor* (A) and mild mosaic on Cymbidium plant (B) infected with finally purified Cymbidium mosaic virus.

fication of CyMV and similar viruses with reasonable yield and high purity.

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