한국식물병리학회지 14(6) : 559-562(1998)
Korean J. Plant Pathol. 14(6) : 559-562(1998)

Purification of *Odontoglossum* Ringspot Virus by DEAE-Cellulose Column Chromatography

Cheol-Ho Lee*, Jong-Oo Park[1], Hyo-Won Jung[1] and Yong-Joon La[1]
**Department of Biological Engineering, College of Natural Sciences and Technology, Seokyoung University, Seoul, Korea*
[1]Division of Applied Biology and Chemistry, College of Agriculture and Life Sciences, Seoul National University, Suwon, Korea

DEAE 셀루로오즈 컬럼 크로마토그래피 기법에 의한 *Odontoglossum* 윤문 바이러스의 정제

이철호* · 박종오[1] · 정효원[1] · 나용준[1]
*서경대학교 이공대학 생물공학과, [1]서울대학교 농업생명과학대학 응용생물화학부

ABSTRACT: *Odontoglossum* ringspot virus (ORSV) was finally purified from ORSV-infected orchid plants by diethylaminoethyl (DEAE) cellulose anion exchange column chromatography. The virus was reliably eluted by potassium chloride at the concentration from 0.1 M to 0.13 M. Partial purification was done by solubilization with Triton X-100 (alkylphenoxypolyethoxy ethanol) and precipitation with polyethylene glycol (PEG; MW8,000). The finally purified ORSV represented one distinct homogeneous band and the molecular weight of its capsid protein was about 17,500 Dalton in electrophoretic analysis. Electron microscopy showed not only intact particles ranged from 280 nm to 340 nm in length, but also segmented particles ranged from 140 nm to 220 nm and even disks. Enzyme-linked immunosorbent assay (ELISA) showed that final yield was 12 mg/100 g of the infected leaves. Bioassay demonstrated that the purified ORSV had the normal infectivity to orchid plants and *Nicotiana glutinosa*. Based on these data, anion exchange column chromatography could be efficiently applied to the purification of ORSV and other viruses similar to ORSV.

***Key words*: anion exchange column chromatography, diethylaminoethyl cellulose, Odontoglossum ringspot virus, purification.**

Odontoglossum ringspot virus (ORSV), one of the most prevalent orchid viruses, causes the reduction of plant vigor and flower quality in a wide genera of orchid plants, which affects economic value of cultivated orchid plants (7, 10). The method of reliable and rapid purification of ORSV is very important for the production and certification of virus-free orchid stocks (1, 5, 12). Up to now, although several procedures for purifying ORSV have been reported in many previous works, these have many troubles such as insolubilization, loss of viral infectivity, and contamination with host plant proteins, routinely encountered in purifying plant viruses (2, 4, 9). Furthermore, the usual purification procedure such as differential centrifugation imposes considerable stress on the integrity of virus particles and may lead to disruption of the native structure with subsequent loss of biological activity. Chromatographic methods, mainly used for protein and animal virus purification, have been thought to be one of the alternatives for purifying plant viruses and recently a few applications to plant virus purification have been reported (11). On the other hand, Triton X-100 (alkylphenoxypolyethoxy ethanol) has been used for the purification of many plant viruses (3, 6, 8), because of its high capability of solubilizing virus particles from plant tissues.

In this communication, we reported a new method for the reliable and rapid purification of ORSV from the intact orchid plants. Triton X-100 was applied to the clarification step and final purification was carried out by anion exchange column chromatography. The final yield and viral quantities at each purification step were determined by ELISA and spectrophotometry, respectively and physicochemical and biological properties of the finally purified virus were also examined.

*Corresponding author.

MATERIALS AND METHODS

Plant sources and anti-ORSV antibodies. Tissue-cultured or ORSV-infected *Cymbidium* orchid plants, were kindly given by Dr. Geol-Bo Sim, professor of Yeonam College of Livestocks and Horticulture, Chungcheong Nam-Do, Korea and assayed immediately through agar gel double diffusion test. The assayed plant leaves were segmemted into small parts and stored at -20°C prior to use. Anti-ORSV antibodies were purchased from American Type Culture Collection (ATCC, Rockville, MD).

Purification of ORSV. The assayed plant leaves were homogenized with sap presser in equal volume of 0.1 M sodium-phosphate buffer (pH 7.4) containing 1% (w/v) sodium sulfite. The homogenate was filtered through several layers of cheesecloth and treated with 2% Triton X-100 with continuous stirring and then incubated for 2 hr at 4°C. This solution was centrifuged at 8,000 rpm and the supernatant was harvested. After the addition of polyethylene glycol (PEG; MW8,000) and sodium chloride to the supernatant with the final concentration of 6% and 2%, respectively, the solution was incubated for 1hr at 4°C with continuous stirring. The pellets saved after centrifugation at 12,000 rpm for 20 min were resuspended with one-hundred volume of the cold same buffer and incubated at 4°C overnight. After the removal of insoluble materials by the centrifugation at 5,000 rpm for 5 min, proper volume of the same buffer was added to the supernatant and reprecipitated with PEG as before. The resultant was applied to DEAE cellulose column (1×15 cm) equilibrated with 100 mM sodium phosphate buffer (pH 7.4) and eluted with potassium chloride in the same buffer. Each eluate was concentrated by ultrafiltration over PD10 filter, scanned by UV spectrophotometer and then active fractions were pooled.

Enzyme-linked immunosorbent assay and immunodiffusion analysis. The anti-ORSV IgG was purified from anti-ORSV antisera by protein-A Sepharose CL-4B (Goding, J.W.) affinity column chromatography according to manufacture's recommendations and used for ELISA. The viral solution and antibodies were appropriately diluted with phosphate buffered saline (PBS)-tween buffer. Immunodiffusion analysis and ELISA were carried out by the method of Ouchterlony (1955) and Hu *et. al.* (5), respectively.

Sodium dodecyl sulfate polyacrylamide electrophoresis and electron microscopy. The viral solution and the molecular weight marker proteins were quantitated by the method of Bradford (1976) with bovine serum albumin (Bio-rad Laboratories Inc., Richmond, CA) as a standard. Protein constituents were analyzed on 12.5% polyacylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli. The molecular weight marker proteins were purchased from Sigma Chemical Co. (St., Louis, Missouri) and the molecular weight of ORSV capsid protein subunit was determined from the logarithmic plots of them. Finally purified virus preparations were negatively stained with 2.0% neutral sodium phosphotungstate and examined by electron microscope (Hitachi H-800, Hitachi Ltd., Co., Japan).

Bioassay. The test plants and orchid plants were cultivated in growth chamber temperature-controlled at 25°C. The finally purified ORSV was used as an inoculum at a concentration of 50 ug/ml. The inoculum was applied on the upper phase of the leaves and the symptoms were examined 7 days after inoculation.

RESULTS AND DISCUSSION

Purification of ORSV. To purify ORSV from ORSV-infected orchid plants, DEAE cellulose column chromatography, combined to solubilization with Triton X-100 and PEG precipitation, was carried out. Table 1 represented the proportionally increased viral quantity, as the purification step proceeded, which was coincident with the results of SDS-PAGE (Fig. 1). The virus was reliably eluted with potassium chloride at the concentration from 0.1 M to 0.13 M. As shown in Fig. 1, the putative band of ORSV was shown in each purification step. Consequently, 12 mg of ORSV was finally purified from the crude extract of 100 g of ORSV-infected leaves over 11.6 fold with 11% yield, as represented in Table 1. The yield was much more less than that of 1 g from 1

Table 1. Purification of *Odontoglossum ringspot* virus from the leaves of *Cymbidium* orchids

Purification step	Specific viral quantity[b] (mg/ml)	Total viral quantity (mg)	Yield (%)
Crude extraction[a]	1.04	110.2	100
PEG precipitation	6.03	62.4	56.6
DEAE cellulose column chromatography	12.1	12.1	11.0

[a]Homogenizing 100 g of ORSV-infected *Cymbidium* orchid leaves in an equal volume of 0.1 M sodium phosphate buffer and solubilized with 2% Triton X-100.
[b]Analyzed by ELISA.

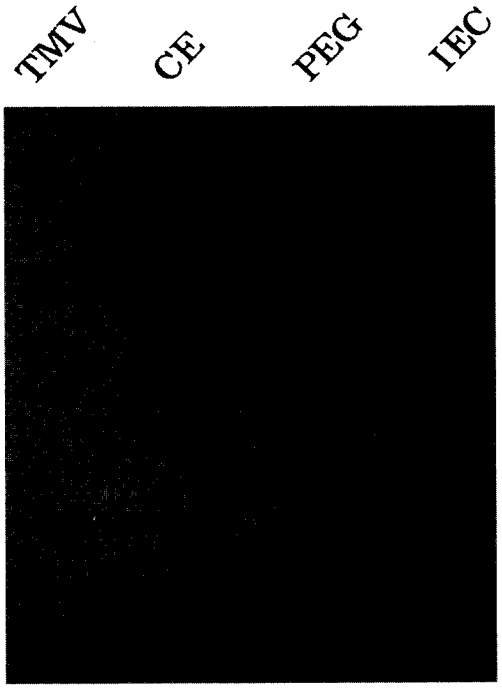

Fig. 1. SDS-PAGE analysis of purification intermediate. TMV, capsid protein of tobacco mosaic virus as molecular weight marker; CE, crude extract of ORSV-infected *Cymbidium* orchid plant; PEG, polyethylene glycol precipitate; IEC, ORSV preparation finally purified by DEAE cellulose column chromatography.

liter of sap of *Nicotiana tabacum* cv. Samsun inoculated with the virus by Paul *et. al.* (9). Such difference in yield was thought to be due to that of total amount of the virus in starting material, considering that the viral preparation, finally purified in this work, contained not only intact particles, but also segmented particles and even disks (Fig. 2).

Physical characteristics of purified ORSV. To examine physical properties of the finally purified ORSV, SDS-PAGE and electron microscopy were carried out. The ORSV preparation obtained from the final step, DEAE cellulose column chromatography, represented one distinct homogeneous band and the molecular weight of its capsid protein was estimated to be about 17,500 Dalton in electrophoretic analysis (Fig. 1), which was almost equal to that of CMI or the other previous reports. Electron microscopy revealed that the virus particle was rod-shaped and 300×18 nm in average size (Fig. 2). Especially, the finally purified ORSV preparation, compared to that of 30% sucrose cushion (Fig. 2B), contained not only intact particles ranged from 280 nm to 340 nm in length, but also segmented particles ranged from 140 nm to 220 nm and even disks (Fig. 2A), which means that DEAE cellulose column chromatography, the final purification step used in this work, is able to purify even the virus being assembled.

Fig. 2. Electron microscopy of ORSV preparation. A) eluate from DEAE celulose column, B) suspended pellet after 30% sucrose cushion centrifugation. V designates virus particle and the bar represents 100 nm.

Biological and immunological activity of purified ORSV. To examine the biological and immunological activity of the finally purified ORSV, immunodiffusion and infectivity assay were performed. Immunodiffusion analysis demonstrated that anti-ORSV antibody remarkably cross-reacted with crude extract of *Nicotiana glutinosa* and other test plants inoculated with the finally purified ORSV (Table 2). Furthermore, bioassay showed typical symptoms on both the test plant, *Nicotiana glutinosa* and *Cymbidium* orchid plants (Fig. 3). These facts mean that the finally purified ORSV has normal integrity.

It has been generally accepted that effective control of viral infection in orchid plants depends on the selection and propagation of virus-free plants or eradication of diseased specimens and therefore an accurate diagnosis is the first requisite for disease management. ELISA

Table 2. Biological and serological activity of the finally purified Odontoglossum ringspot virus

Plant tested	Symptom[a]	Serological reaction[b]
Cassia occidentalis	–	–
Chenopodium amaranticolor	LN	+
C. quinoa	LN	+
Datura stramonium	–	–
Gomphrena globosa	LN	+
Nicotiana glutinosa	LN	+
N. tabacum cv. Barley 21	–	–
N. tabacum cv. nc-82	–	–
N. tabacum cv. Xanthi-nc	LN	+
Phaseolus vulgaris	–	–
Tetragonia expansa	LN	+
Vigna sinensis	–	–

[a]Observed within one or two weeks after inoculation. LN, localized necrosis; –, uninfected.

[b]Each crude extract reacted with anti-ORSV antibody at the concentration of 50 ug/ml. +, positively reacted; –, negatively reacted.

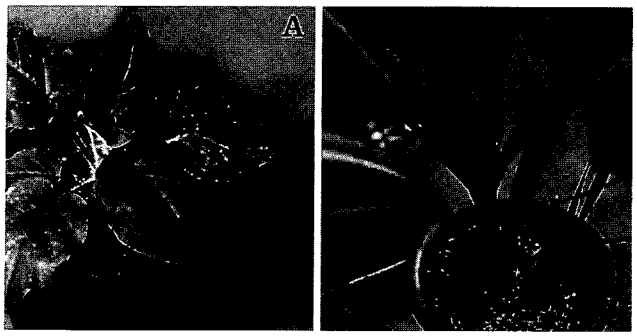

Fig. 3. Typical local lesions on *Nicotiana glutinosa* (A) and necrotic spots on *Cymbidium* orchid plants (B), inoculated with finally purified ORSV.

has been widely accepted as one of the most reliable techniques for such purpose. There is no exception to ORSV. In this point of view, the rapid and reliable purification of ORSV was essential for the production of remarkable and high-titer antibodies. Especially, considering that DEAE cellulose column chromatography, the final step used in this work could purify the virus being assembled, the purification method used in this work has been thought to be very effective for the purification of ORSV and other viruses similar to ORSV.

요 약

면역 확산법에 의해 *Odotoglossum* 윤문 바이러스(ORSV)에 감염된 것으로 확인된 국내 배양난의 葉조직을 인산 완충액으로 마쇄, Triton X-100와 PEG 8000을 이용하여 부분 순화한 후, DEAE celllulose 컬럼 크로마토그래피 기법을 이용하여 최종 분리하였다. ORSV의 분리 수율은, 면역 효소 항체법으로 확인한 결과, 감염 조직 100 g당 12 mg으로 확인되었으며 분리한 바이러스의 물리, 화학적 특성을 조사하기 위하여 전기 영동 및 전자현미경 관찰, 생물 활성 검정 등을 수행하였다. 전기 영동 결과, 바이러스 단백질의 뚜렷한 단일 밴드를 확인할 수 있었고 전자 현미경下에서 입자가 손상되지 아니한 균일한 형태의 바이러스 입자뿐만 아니라 조립 과정중에 있는 바이러스 입자까지도 관찰할 수 있었다. 한편, 최종 분리한 ORSV는 배양된 무감염 *Cymbidium* 蘭과 지표 식물인 *Nicotiana glutinosa*에서 정상적인 생물 활성을 지니는 것으로 확인되었다. 이상의 결과로부터 본실험에서 사용한 분리 기법은 ORSV 뿐만 아니라, 이와 유사한 특성을 지니는 tobamovirus群의 분리에 효율적으로 사용될 수 있을 것으로 생각된다.

ACKNOWLEDGEMENTS

We thank Geol-Bo Sim, professor of Yeonam College of Livestocks and Horticulture, Chungcheong Nam-Do, for providing ORSV-infected or tissue-cultured orchid plants.

REFERENCES

1. Allison, A. V., Adams, E. B. and Steinagel, L. 1981. Enzyme-linked immunosorbent assay (ELISA) as a rapid method for screening for the presence of *Cymbidium* mosaic virus and *Odontoglossum* ringspot virus. *Phytopathology* 71 : 103.
2. Chang, M. U., Chun, H. H., Baek, D. H. and Chung, J. D. 1991. Studies on the viruses in orchids in Korea. 2. *Dendrobium* mosaic virus, *Odontoglossum* ringspot virus, orchid fleck virus and unidentified potyvirus. *Kor. J. Plant Pathol.* 7(2) : 118-129.
3. Derks, A. F. L. M., Vink-van den abeele, J. L. and Van schadewijk, A. R. 1982. Purification of tulip breaking virus and production of antisera for use in ELISA. *Neth. J. plant Pathol.* 88 : 87-98.
4. Francki, R. I. B. 1966. Isolation, purification and some properties of two viruses from cultivated *Cymbidium* orchids. *Aust. J. Biol. Sci.* **19** : 555-564.
5. Hu, J. S., Ferreira, S., Wang, M. and Xu, M. Q. 1993. Detection of *Cymbidium* mosaic virus, *Odontoglossom* ringspot virus, tomato spotted wilt virus, and potyviruses infecting orchids in Hawaii. *Plant Dis.* 77 : 464-468.
6. Izaguirre-Mayoral, M. L., Carballo, O. and Gil, F. 1990. Purification and partial characterization of isometric virus-like particles in *Kalanchoe* species. *J. Phytopathol.* 130 : 303-311.
7. Lawson, R. H. and Brannigan, M. 1986. Virus diseases of orchids. In: Handbook on orchid pests and diseases. *Amer. orchid Soc.*, West palm beach, FL. pp.2-49.
8. Nozu, Y. and Yamamura, R. 1971. Use of Triton X-100 for isolation of tobacco mosaic virus from some plants other than tobacco. *Virology* 43 : 514-515.
9. Paul, H. L. 1965. Odontoglossum Ringspot Virus. C.M.I./A.A.B. Descriptions of Plant Viruses No. 155.
10. Pearson, M. N and Cole, J. S. 1986. The effects of *Cymbidium* mosaic and *Odontoglossom* ringspot virus on the growth of *Cymbidium* orchids. *J. Phytopathology* 117 : 193-197.
11. Venekamp, J. H. and Mosch, W. H. M. 1963. Chromatographic studies on plant viruses I. The isolation of potato virus X by means of various systems of adsorption chromatography. *Virology* 19 : 316-321.
12. Wisler, G. C., Zettler, F. W. and Purcifull, D. E. 1982. A serodiagnostic technique for detecting *Cymbidium* mosaic and *Odontoglossom* ringspot viruses. *Phytopathology* 72 : 835-837.

(Received September 22, 1998)