Purification of Cucumber Pale Fruit Viroid*

Ichiro Uyeda**, Teruo Sano** and Eishiro Shikata**

Cucumber pale fruit viroid was purified from infected cucumber leaves and stems. Nucleic acids were extracted from the frozen tissue by phenol-CHCl₃-SDS, and precipitated with ethanol. Polysaccharides were removed by ethylene glycol monomethyl ether treatment and phenolic substances by cetyltrimethyl ammonium bromide. After 2M LiCl soluble fraction was treated with DNase, low molecular weight RNAs were obtained. The viroid was further purified by CF-11 cellulose chromatography and 15% polyacrylamide gel electrophoresis. Yield of the purified viroid was about 3-6 μg/200g of tissue. Five percent polyacrylamide gel electrophoresis of the purified viroid in the presence of urea revealed two bands both associated with infectivity. (Received December 16, 1983)

Key Words: cucumber pale fruit viroid, gel electrophoresis, purification.

Introduction

Sasaki and Shikata have shown that hop stunt disease is caused by a viroid¹⁵–¹⁹ and Takahashi confirmed its viroidal etiology²¹. They¹⁵,¹⁷ also reported that hop stunt viroid (HSV) is mechanically transmissible to several herbaceous plants including cucumber and tomato. Descriptions of symptoms on cucumber and host range of cucumber pale fruit viroid (CPFV) found in Europe²² were very similar to HSV. Sano et al.¹⁴ conducted simultaneous comparison of HSV and CPFV, and showed that they were indistinguishable by host range and symptomatology.

For further comparative studies of the two viroids by their physicochemical properties, it is essential to extract and purify them in a reasonably large quantity. In our preliminary attempts, purification of CPFV and HSV by extraction methods of Morris and Smith⁶ was unsuitable when a large amount of the infected tissue was handled, because remained impurities prevented a sample to dissolve in a small volume of a buffer before 5% polyacrylamide gel electrophoresis (PAGE). Furthermore, 5% PAGE did not separate the two viroids from 7S RNA of the host origin.

Although Sänger at al.¹⁹ and Mühlbach and Sänger¹⁹ reported studies using purified CPFV, detailed procedures have not yet been described. In this paper, we describe

---

* This research was supported in part by Research Grant No. 56480037 and No. 56360004 from the Ministry of Education, Science and Culture of Japan.
* Plant quarantine permission number 54-Yokoshoku-1259 and 55-Yokoshoku-1035.
** Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan
successful purification of CPFV by modification of the methods of Raymer and Diener\textsuperscript{11),} and Singh and Sanger\textsuperscript{20),} and by the use of 15\% PAGE.

\textbf{Materials and Methods}

\textit{Viroids.} Cucumber pale fruit viroid was maintained and propagated on cucumber plants (\textit{Cucumis sativus} L. cv. Suyo) as previously described\textsuperscript{14).}

Test sample were mechanically inoculated to 4 cucumber plants and infectivity was quantitatively assayed according to Raymer and Diener\textsuperscript{11).}

\textit{Purification of the low molecular weight RNAs.} The methods of Raymer and Diener\textsuperscript{11),} and Singh and Sanger\textsuperscript{20) were combined and modified as follows.

Two hundreds g of frozen tissues were blended in 300 ml of 1 M \textit{K}_2\textit{HPO}_4, 4 g of sodium dodesyl sulfate (SDS), 0.4 g of bentonite and 40 ml of water–saturated phenol for 5 min at room temperature. After centrifugation at 6,500 rpm for 20 min, aqueous supernatant was collected. Water–saturated phenol (200 ml) and 200 ml of CHCl\textsubscript{3} were added and stirred with a magnetic stirrer for 30 min at room temperature. Aqueous supernatant after centrifugation at 6,500 rpm for 20 min was collected and 2.5 volume of 95\% ethanol containing 0.2 M sodium acetate were added. After storage at -30 C overnight, nucleic acids were recovered as a skin at the interface between the lower phosphate rich aqueous phase and upper ethanol phase after the low speed centrifugation. Nucleic acids were dissolved in 100 ml of distilled water, precipitated again by 2.5 volumes of 95\% ethanol, and dissolved in 50 ml TES buffer (0.1 M Tris, 0.001 M ethylenediaminetetraacetate disodium salt (EDTA), 0.1 M NaCl, pH 7.5). Fifty ml each of ethylene glycol monomethyl ether and 2.5 M \textit{K}_2\textit{HPO}_4, pH 8.3 was then added, blended at 15,000 rpm for 3 min, and centrifuged at 3,000 rpm for 20 min. The upper aqueous phase is carefully recovered without disturbing the interphase and diluted up to 625 ml with distilled water to make 0.2 M \textit{K}_2\textit{HPO}_4. While stirring with a magnetic stirrer, 10 ml of 2\% cetyltrimethyl ammonium bromide (CTAB) was slowly added. And CTA–nucleic acids comlexes were precipitated by a low speed centrifugation after incubation at 6 C for 30 min. The precipitate was washed twice with 95\% ethanol to remove CTA cations and stored in 70\% ethanol at -30 C overnight. After a low speed centrifugation, the precipitate was dissolved in TES buffer and an equal volume of 4 M LiCl solution was added. The solution was stood at 0 C for 4 hr and centrifuged at 3,000 rpm for 30 min. To the supernatant, which contained 2 M LiCl soluble low molecular weight RNAs and DNA, added 2.5 volumes of 95\% ethanol and the nucleic acid was precipitated by the low speed centrifugation. The precipitate was dissolved in 10 ml of DNase I (Worthington Biochemical) solution (25 μg/ml)\textsuperscript{13),} incubated at 37 C for 30 min. The DNA digestion was stopped by phenol–CHCl\textsubscript{3} treatment and the RNAs were precipitated by ethanol. About 10 mg of the low molecular weight RNAs were recovered from 200 g of tissue.

\textit{Column chromatography.} DEAE cellulose (DE–32 : Whatman) column chromatography was carried out at 6 C. The column size was 10×70 mm and flow rate was 50 ml/hr. The low molecular weight RNAs were fractionated by stepwise elution using 0.02 M Tris–HCl, pH 7.6, containing 0.3, 0.5, 0.6, and 0.7 M NaCl.
Sephadex G-100 gel filtration was carried out in a 16×900 mm column at 6°C. The flow rate was 10 ml/hr. The low molecular weight RNAs were eluted in 0.05 M KCl containing 1% methanol.

CF-11 cellulose column chromatography was carried out at room temperature (25°C) according to the procedure of Franklin2). The low molecular weight RNAs were fractionated by stepwise elution using 35% and 15% ethanol followed by ethanol free buffer.

**Polyacrylamide gel electrophoresis.** Polyacrylamide gel electrophoresis under nondenaturing condition was performed in 15% (acrylamide : bis = 30 : 0.8) gel with 0.04 M Tris-0.02 M sodium acetate-0.001 M EDTA, pH 7.2, buffer3) using 15 cm × 19 cm × 1.5 mm slab gel apparatus, at 15 mA constant for 20 hr at 6°C. During electrophoresis, the buffer was recirculated with a peristaltic pump.

Five percent PAGE under denaturing condition was performed in 7 M urea, 0.075 M Tris, 0.045 M boric acid, and 0.001 M EDTA4). Ethanol-precipitated RNA sample was suspended in 0.01 M EDTA, pH 7.0. 16% sucrose, 20% dimethyl sulfoxide, heated at 50°C for 10 min, and electrophoresed, at 5 mA constant for about 7 hr at 40-45°C. After electrophoresis, gels were stained with 0.02% toluidine blue O and destained in distilled water. The viroid band was cut out and homogenized in 0.5 M ammonium acetate, 0.01 M magnesium acetate, and 0.1% SDS5). After stirring with a magnetic stirrer overnight at room temperature, gel slurry was pelleted by a low speed centrifugation and supernatant was collected. The viroid was precipitated with ethanol and dissolved in 0.02 M Tris-HCl containing 0.3 M NaCl, pH 7.6. The viroid was then applied onto a DEAE-cellulose column equilibrated with 0.02 M Tris-HCl containing 0.3 M NaCl, pH 7.6, washed with the same buffer and eluted with 0.02 M Tris-HCl containing 0.6 M NaCl.

**Results**

**Detection of the viroids by polyacrylamide gel electrophoresis**

In early experiments, the low molecular weight RNAs were directly electrophoresed on PAGE. As previously described, HSV- and CPFV-specific RNA were not detected on 5% PAGE, because the migration rate of both HSV and CPFV was identical to that of 7S RNA of host origin. Since it was known that electrophoretic mobility of viroids in gels changes differently from host RNAs at different concentrations of acrylamide, the low molecular weight RNAs extracted from CPFV-infected cucumber were electrophoresed on 5%, 7.5%, 15%, and 20% polyacrylamide gel in Tris-acetate-EDTA buffer. These experiments revealed that CPFV was clearly separated from host RNAs by 15% 20% PAGE. The result of 15% PAGE is shown in Fig. 1. An arrow indicates and CPFV-specific RNA band that was not detected in extracts of healthy cucumber plants. The gel was sectioned into 7 slices as indicated in Fig. 1 and assayed on cucumber plants. Infectivity was mostly associated with the CPFV-specific RNA band.

The concentrated low molecular weight RNA preparation was usually colored and viscous due to contaminated impurities and the bands were frequently disturbed by them and we sometimes failed to detect the viroid band after staining with toluidine blue O. We therefore examined chromatographic procedures for further purification of CPFV.
DEAE cellulose chromatography

Figs. 2 and 3 show the elution pattern of nucleic acid preparation prior to DNase treatment and 15% PAGE analysis of the eluted fractions, respectively. The first peak eluting with 0.3 M NaCl contained mostly DNA and the colored impurities and degra-
ded nucleic acids. The second peak eluting with 0.5 M NaCl contained almost all part of the low molecular weight RNAs including the viroid. Thus most of the impurities were excluded but a large portion of the host RNAs still remained in the eluted viroid preparation.

_Sephadex C100 chromatography_

Fig. 4 and 5 show the elution pattern and 15% PAGE analysis of the eluted fractions, respectively. The peak in exclusion volume contained viroid RNA. Some cellular RNA species of similar sizes to the viroid and highly viscous impurities that remained at the top of the gel. Such impurities frequently disturbed the electrophoretic migration of RNAs and made further purification of the viroid difficult.
**CF11 cellulose chromatography**

Fig. 6 and 7 show the elution pattern and 15% PAGE analysis of the eluted fraction, respectively. The first peak eluted with 35% ethanol contained a large portion of tRNA, degraded nucleic acids and impurities (Fig. 7). The second peak eluted with 15% ethanol contained viroid RNA, small amount of tRNA, 5S RNA, and some minor species of cellular RNAs. The third peak eluted with the buffer alone scarcely contained nucleic acids. The bioassay showed that almost all of viroid RNA was eluted with 15% ethanol (Fig. 6).

**Polyacrylamide gel electrophoresis of the purified viroid under denaturing condition**

After 15% PAGE and elution of the viroid from the gel, purity of the viroid preparation was assayed by 5% PAGE under denaturing condition. Two major bands (toluidine blue O stain) were consistently detected after electrophoresis. The bioassay indicated that both bands were highly associated with infectivities (Fig. 8).

It was assumed that the slower migrating and faster migrating bands were circular and linear molecules of the same viroid, respectively, as has been reported for other viroids. However, in some preparation, several minor bands whose migration was identical to RNAs of healthy tissue were detected indicating a trace of contamination of cellular RNAs.

**Discussion**

Sänger and his co-workers purified CPFV from tomato cv. Rentina. However, purification from the tomato plants had two disadvantages. First, the infected tomato cv. Rentina did not produce distinct symptoms under greenhouse conditions. This makes an additional procedure to assure that inoculated tomato plants are indeed infected. Cucumber plant is an obvious choice of starting materials, because CPFV produces distinct symptoms on this host. Second, low molecular weight RNAs preparations from tomato contained much more colored substances than from cucumber. Although Sänger's attempts had been unsuccessful, we were able to purify CPFV from cucumber as shown in this paper.

It was found that CF-11 cellulose chromatography was the most effective for purification among chromatographic procedures, because it could separate not only the impurities but also a large portion of tRNA from the viroid. DEAE cellulose chromatography could remove the impurities, particularly highly colored materials, but a large portion of host RNAs eluted together with the viroid. Sephadex G-100 chromatography separated the viroid almost completely from both tRNA and 5S RNA but unable to remove the viscous impurities which sometimes made further purification of the viroid by PAGE impossible. Alternatively, two cycles of 15% PAGE effectively purify CPFV. Ohno et al. also obtained satisfactory results using two cycles of 10% PAGE for purification of HSV. The combination of CF-11 cellulose chromatography and 15% PAGE, or two cycles of 15% PAGE yielded 3-6 µg/200g of tissue of purified CPFV.

The use of 15% PAGE was found to be effective to separate the viroid from the cellular RNAs of the similar size. Hop stunt viroid migrated almost identically to CPFV.
in 15% PAGE, and was purified by the method described here. Yoshikawa and Takahashi\(^{23}\) used 7.5% PAGE for the purification of HSV in which HSV migrated between 7 S and 9 S cellular RNAs. However, the RNAs banded sharper in 15% PAGE than in 7.5% PAGE and were more effectively separated from each other. The migration pattern of HSV in 10% PAGE\(^{29}\) was similar to that in our 15% PAGE. But we preferred 15% PAGE because of the same reasons for 7.5% PAGE. Twenty percent PAGE also effectively separated CPFV and HSV. However, it was difficult to make gels due to a substantial shrinkage occurred during gel formation in our electrophoreosis apparatus.

Our purified viroid preparations still contain a trace of cellular RNAs as revealed by 5% PAGE under denaturing condition. Further purification by 5% PAGE under denaturing condition will be necessary when viroids were used in the direct sequencing or hybridization experiments where the highest purity is required.

**Literature cited**

12. Sänger, L.H. (Personal communication).
和文摘要

Cucumber pale fruit viroid の純化

Cucumber pale fruit viroid を根病キュリ茎葉より純化した。核酸はフェノール・クロロホルム・SDS法で抽出し、エタノールで沈澱させた。多糖類は、エチレングリコールモノメチルエーテル処理、またフェノール物質は、セチルトリメチルアンモニウムプロミド処理で除去した。更に 2 M LiCl 可溶分画を DNase I で処理して、低分子 RNA 分画を得た。ウイルスはその分画より CF-11 セルロースクロマトグラフィーと 15％ポリアクリルアミドスラブゲル電気泳動を用いて純化した。ウイルスの収量は、3 〜 9 μg/200g 茎葉であった。尿素を含む、5 ％ポリアクリルアミドスラブゲル電気泳動で、純化ウイルスは 2 本のバンドに分かれ、両者に感染性があった。