Purification of rice dwarf virus

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I. Introduction

Fukushi, Shikata, and Kimura¹⁾ isolated characteristic virus-like particles, icosahedrons of 70 m μ in diameter, from both rice dwarf virus (RDV)-infected rice plants and viruliferous leafhoppers by differential centrifugation techniques. The same particles were present in the cells of infected rice plants and of viruliferous leafhoppers (*Nephotettix cincticeps*), sometimes aggregating into a crystal-like arrangement. Fukushi and Kimura²⁾ demonstrated that these particles were infective to the insect vector and also to rice plants, by injecting leafhoppers with particles and feeding them with rice seedlings. From these results, it is concluded that these particles are RDV. The electron micrographs given by these authors¹⁾ show that the particles are enveloped with a membranaceous material. Later studies by Kimura *et al.*^{3,4)} showed that RDV particles isolated by differential centrifugation techniques accompanying chloroform- and phospholipase-treatment possess hollow capsomeres which could possibly be visualized by removing the enveloping material with phospholipase treatment. Similar results were reported by Fukushi *et al.*⁵⁾ by removing the enveloping material with ethylether treatment. The question still remains as to whether the enveloping material is the essential component of RDV or a contamination originating from host responses, and whether the particles present in infected rice plants are identical with those present in infected leafhoppers. Recent electron microscopic studies by Nasu⁶⁾ revealed that particles of a smaller size are present beside the typical RDV particles in viruliferous leafhopper cells where the virus seems to be vigorously multiplying. Are these smaller particles a form of RDV appearing in the process of multiplication? Before answering these questions, further knowledge is required concerning morphological and chemical properties of RDV using purified virus. This paper reports the result of successful purification of RDV from infected rice plants, and the infectivity tests of the purified RDV.

II. Materials and Methods

The RDV isolate used in this study was kindly furnished by Dr. Akira Shinkai who preserved this isolate for past 16 years at the National Institute of Agricultural Sciences, Tokyo. The virus was cultured in rice variety, Norin No. 8. Leaves and leaf-sheaths showing apparent symptoms were harvested and 50 g of fresh weight was used for each purification experiment run. Finally, 300 g was used for the chromatographic separation of RDV employing step-wise elution with NaCl.

Differential centrifugation: Fifty grams of infected leaves and leaf-sheaths were cut into small pieces, ground with the addition of 200 to 500 ml of M/30 phosphate buffer, pH 6.8, then the sap was expressed through cheese cloth and centrifuged at 6,500xg for 30 minutes. The top layer was stirred with 10 or 20 per cent volume of chloroform for 5 minutes and centrifuged at 1,600 xg for 20 minutes. The supernatant was centrifuged at 26,000xg for 60 minutes. After removing the supernatant, the pellet was suspended in M/40 tris buffer, pH 7.2, and centrifuged

* National Institute of Agricultural Sciences, Nishigahara, Kitaku, Tokyo.

at 1,600xg for 20 minutes. The supernatant (fraction A) consisted of high concentration of virus particles when examined under the electron microscope.

Treatment of fraction A with phospholipase: Phospholipase was prepared from snake venom* (*Trimeresurus flavoviridis*) and also from pancreatin. Five mg of venom was dissolved in 1 ml of tris buffer, pH 7.2, and treated in boiling water for 7 minutes, cooled, and centrifuged at 3,000 rpm for 15 minutes. The supernatant was used as crude phospholipase preparation. Five mg of venom was used for each 5 ml of fraction A obtained from 50 g of infected leaves.

Phospholipase from pancreatin was prepared after Hanahan's method⁷⁾. Fifteen grams of pancreatin was dissolved in 100 ml of M/15 phosphate buffer, pH 6.0, stirred for 20 minutes at 4°C, and centrifuged at 12,000 rpm for 45 minutes. The supernatant was adjusted to pH 4.0 with dil. HCl, treated at 70°C for 10 minutes, cooled, and centrifuged at 3,000 rpm for 10 minutes. The supernatant was used as crude phospholipase preparation. Three ml of the enzyme preparation was used for treating 50 ml of fraction A obtained from 300 g of infected leaves.

Fraction A was incubated with phospholipase at 5°C for 48 hours, centrifuged at 26,000 x g for 60 minutes, the precipitate being suspended in 5 ml or 50 ml of phosphate or tris buffer (fraction B). Tris buffer is more suitable than phosphate buffer for the following steps of purification using column chromatography.

Column chromatography: Column chromatography by the use of DEAE-cellulose (Brown Co., 0.93 meq/g) was applied for the separation of RDV from fraction B. Five g of DEAE-cellulose was used for each 5 ml of fraction B obtained from 50 g of infected leaves. The DEAE-cellulose column was conditioned by washing with distilled water, N NaOH, and again with enough amount of distilled water until the pH of effluent fell to 7.2. The column, to which fraction B had been added, was washed with 50 to 100 ml of M/40 tris buffer, pH 7.2, and then with tris buffer mixed with linear gradient of NaCl from 0 to 1.0 M, using two-jar arrangement as the mixing device. The presence of virus in each 5 ml of effluent was examined by measuring the optical density at 260 m μ . After it was confirmed that the virus would be eluted at a definite range of NaCl concentration, step-wise elution method was adopted to step up the purification procedure from larger amount of infected leaves.

Electron microscopy: Drops of fraction A, B, and every 5 ml of the effluent showing high optical density at 260 m μ were placed on carbon-coated grids. The grids were shadowed with chromium or platinum, or negatively stained with phosphotungstate and micrographs were taken with Hitachi H.U. -11 electron microscope.

III. Results

1. Column chromatography of fraction A

The DEAE-cellulose column chromatography of fraction A (Fig. 1-A) showed that only a very small amount of virus was eluted with NaCl ranging from 0.2 to 0.25 M, while a much larger amount was firmly adsorbed by DEAE-cellulose without being eluted with NaCl at higher concentration up to 1.0 M and was eluted only with 0.5 M NaOH. This suggests that the virus particles are covered with some strongly acidic substance, presumably the enveloping material shown by Fukushi *et al.*¹³⁾. The treatment of fraction A with 0.5 per cent sodium deoxycholate for 3 hours at 5°C showed no marked change in the chromatographic feature as shown in Fig. 1-B, whereas the incubation of fraction A at 5°C for five days greatly facilitated the elution of virus with NaCl at low concentrations as shown in Fig. 1-C. Electron micrographs of effluents

* The venom was kindly supplied by Dr. T. Omura, Protein Institute, Osaka University.

at 0.2 to 0.25 M NaCl showed that these contained a large amount of typical RDV particles. This may be caused by some impurity which is presumably an enzyme originating from host plants and is able to decompose the enveloping material.

2. Column chromatography of fraction B

The enveloping material seems to be a lipid from its appearance in the electron micrograph shown by Fukushi *et al.*¹³, and is strongly acidic from the result of DEAE-cellulose column chromatography. Phosphorus was actually detected in the chloroform-methanol extract of the dried fraction A. These results suggest that the enveloping material may be a phospholipid.

Fraction B was obtained by treating fraction A with snake venom phospholipase as already described. The DEAE-cellulose column chromatograph of this fraction is shown in Fig. 2-A.

The control, using healthy rice leaves, is shown in Fig. 2-B. The peak appearing at about 0.2 M NaCl in Fig. 2-A is characteristic to fraction B as compared with the chromatographs of fraction A (Fig. 1-A) and the control of fraction B (Fig. 2-B). The effluent corresponding to this peak contained a large amount of typical RDV particles when examined under electron microscope.

3. Column chromatography of fraction B by step-wise elution

Fraction B, 50 ml., was prepared from 300 g fresh weight of infected leaves by the same procedure described above except that pancreatin-phospholipase was used in place of snake venom-phospholipase. Ten ml of the fraction was added to a column containing 6 g of DEAE-cellulose. The column was washed with 200 ml of 0.15 M NaCl, then RDV was eluted with 0.25 M NaCl. Every 20 ml of the effluent was taken in a test tube and the optical density at 260 m μ was determined. The result is shown in Fig. 3. The effluent collected from the 12th and 13th tubes was apparently a dense emulsion with opalescence. This was sedimented by centrifugation at 26,000 $\times$ g for 1 hour and the pellet was suspended in 5 or ml of distilled water (fraction C.)

Fig. 1. DEAE-cellulose column chromatography of fraction A.

A. Fraction A.

B. Fraction A after treatment with 0.5% sodium deoxycholate for 3 hours at 5°C.

C. Fraction A after 5-day incubation at 5°C.

Fig. 2. DEAE-cellulose column chromatography of fraction B which was prepared by treating fraction A with phospholipase.

A: Fraction B from infected plants.
B: Control, from healthy plants.

Fig. 3. DEAE-cellulose column chromatography of fraction C by step-wise elution method.

4. Homogeneity of RDV in fraction C

The homogeneity of the virus in fraction C was examined by electrophoretic and sedimentation patterns. The results are shown in Figs. 4 and 5. An electron micrograph of this fraction is shown in Fig. 6.

Both patterns clearly show that the virus in fraction C is highly homogeneous. Small peaks on both patterns are due to the disintegration product of the virus itself, nucleic acid and empty shells respectively, appearing during the short period of suspending the virus in tris buffer or distilled water (Figs. 7, 8).

The electron micrograph of the shadowed sample of this fraction C shows that this consists of highly concentrated RDV particles (Fig. 6). The micrograph of negatively stained sample shows that the particles are entirely free from the enveloping material and the presence of capsomeres is visualized. (Fig. 7).

5. Infectivity of the purified virus

The infectivity of the virus in fraction C was tested indirectly by proving the transmission of virus from inoculated leafhoppers to rice seedlings. Each virus-free leafhopper was inoculated by injecting about 1/3000 ml of fraction C into its abdomen and was fed with a test rice seedling and the symptoms on rice seedlings were examined

after 20 to 60 days. The results in Tables 1 and 2 show that the purified virus retains infectivity.

IV. Discussion

The enveloping material of RDV particles shown by Fukushi *et al*¹¹⁾ raised a question as to whether this was an essential component of RDV or a substance originating from host responses. The present study revealed that this is a strongly acidic substance and is easily removed by treating with crude phospholipase preparation from snake venom or pancreatin, suggesting that this is a kind of phospholipid. The amount of the material adhering to the virus particles varied according to the materials used for isolation of the virus, i. e., the amount was very large with infected rice leaves which grew

vigorously during midsummer, while it was almost negligible with rice leaves harvested in the cool autumn, so that there was no need of phospholipase treatment to elute the virus from DEAE-cellulose column. The infectivity test of the purified virus which had been freed from the enveloping material showed that the enveloping material was not essential for the infectivity.

Fig. 4. Electrophoretic patterns of fraction C in M/20 phosphate buffer of ionic strength 0.2 and pH 8.2, after 1 (upper) and 2 (lower) hours. r: ascending, d: descending

Fig. 5. Sedimentation pattern of fraction C in distilled water, after 4 minutes at 20,410 rpm by Spinco Model E.

Table 1. Infectivity tests made by injecting leafhoppers with the purified virus preparation and feeding them with test rice seedlings.

	Number of leafhoppers		
	inoculated	survived for 20 days	transmitted virus to rice seedlings
I	60	28	7
II	60	21	4

Table 2. Comparison of the infectivity of fractions A and C.

Fraction	Number of leafhoppers			Virus content in fraction
	injected	survived for 20 days	transmitted virus to rice seedling	
A	60	22	5	3.06 mg/ml
C	60	25	2	0.15 mg/ml

All these data indicate that the material is of host origin.

The electron micrographs shown by Fukushi *et al.*¹⁾ indicate that the virus particles are arranged linearly in a bundle of sheaths in infected rice leaf cells. Recent electron microscopic studies by Nasu⁶⁾ on ultrastructure of the RDV-infected cells revealed that the virus particles are arranged linearly in a long line within a sheath and the sheaths gather in a parallel arrangement, finally resulting in formation of crystal-like structure as shown previously by Fukushi *et al.*¹⁾. The chemical nature of the enveloping material and its relationship to the sheath are still obscure.

Summary.

The electron micrograph of rice dwarf virus (RDV) given by Fukushi, Shikata, and Kimura¹⁾ shows that RDV particles are enveloped with a membranaceous material. A fraction containing similar RDV particles was obtained from infected rice leaves by treating the expressed sap with 10 to 20 per cent chloroform and the same differential centrifugation procedure as adopted by Fukushi *et al.*¹⁾. RDV in this fraction was so firmly adsorbed by DEAE-cellulose column that it could not be eluted with increasing concentration of NaCl from 0 to 1.0 M, but was eluted only with 0.5 M NaOH, suggesting that the virus particles were covered with some strongly acidic substance, possibly the enveloping material. This material was easily removed by treating the fraction with phospholipase of snake venom or pancreatin. After this treatment, RDV was eluted from DEAE-cellulose column with 0.2 to 0.25 M NaCl. RDV in the effluent showed typical morphological characteristics, an icosahedron with hollow capsomeres, when examined under electron microscope. Electrophoretic and sedimentation patterns of the effluent showed that the preparation consisted of homogeneous RDV particles. The purified RDV retained infectivity to rice plants when assayed through the insect vector, *Nephotettix cincticeps* Uhler.

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和文摘要

イネ萎縮病ウイルスの純化

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福士・四方・木村¹⁾によるイネ萎縮病ウイルスの電顕像はウイルス粒子が油滴状物質で被われていることを示している。罹病イネ茎葉から分画遠心分離で得たウイルス標品は DEAE セルローズカラムに吸着され NaCl 0~1 M の濃度勾配で溶出されず、0.5 M NaOH でようやく溶出されるところからみて被覆物質は強酸性物質と想像される。蛇毒およびパンクレアチンのホスホリパーゼで 5°C, 2 日処理するとこの物質は完全に除去され、ウイルス粒子は DEAE セルローズカラムから 0.2~0.25 M NaCl で溶出され、この溶出液中のウイルス粒子は電気泳動像、沈降図型および電子顕微鏡観察から純度の高いことが示された。これを無毒ツマグロヨコバイに注射すると、ヨコバイが保毒になりイネを発病させた。このことにより純化されたウイルスが感染力を有することが証明された。同じ方法で、0.15 M NaCl 溶出部を捨て 0.25 M NaCl 溶出部をとると同様のウイルス標品を得る。生葉 300 g から約 30 mg のウイルスが得られる。

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Fig. 6. Electron micrograph of fraction C shadowed with chromium.

Fig. 7. Electron micrograph of RDV particles in fraction C negatively stained with phosphotungstate, showing the presence of capsomeres on each particle and the contamination of empty shells after suspension in distilled water.

Fig. 8. Appearance of RNA-strands from virus particles after freezing followed by thawing (A) and after suspending the virus in distilled water (B).