Polypeptide Components of Rice Dwarf Virus

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中田昌伸*・福永憲三*・鈴木直治*：イネ萎縮病ウイルスの構成ポリペプチド

Abstract

Polyacrylamide gel electrophoresis of the solubilized protein of rice dwarf virus (RDV), purified from RDV-infected rice plants, showed the virus to be composed of seven structural polypeptides. The molecular weights of these were calculated to be 193,000; 152,000; 131,000; 110,000; 62,000; 46,000 and 45,000 daltons. These were designated I, II, III, IV, V, VI, and VII respectively. Chymotrypsin-treatment of the virus was not effective for removing the outer components of capsid, whereas centrifugation of the virus through preformed cesium chloride gradients separated the preparation into four zones, top(T), upper-middle(M₁), lower-middle(M₂), and bottom (B), the buoyant densities of these being 1.26, 1.39-1.42, 1.43, and 1.46 g/ml respectively. Recentrifugation of M₁ through the cesium chloride gradients yielded three zones, M₁, M₂, and B, indicating that the latter two resulted from M₁ by the shearing effect of cesium chloride centrifugation. Electron microscopy and SDS-polyacrylamide gel electrophoresis of B showed complete loss of II and IV, and a marked decrease of VI and VII, indicating that VI and VII compose the outer capsomeres and II and IV are the amorphous polypeptides that surround the outer capsomeres. The inner core was found to contain I, III, and V, among these V seemed to be the internal protein associated with nucleic acid because this was readily released from virions by freeze-thawing together with nucleic acid.

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Introduction

RDV virions contain double-stranded (ds) RNA and RNA transcriptase that transcribes dsRNA into single-stranded (ss) RNA. In a series of studies on the screening of chemotherapeutants for this virus^{5,11,12}, we have attempted to screen some compounds that selectively inhibit RDV-RNA transcription *in vitro*, but do not inhibit *E. coli* DNA transcription *in vitro*. Skyrin, a red pigment produced by *Endothia parasitica*, was found to fulfil the requirement. Conversely, luteoskyrin, a pigment produced by *Penicillium islandicum* and is structurally related to skyrin, inhibits *E. coli* DNA transcription without inhibiting RDV-RNA transcription. Rugulosin, a yellow pigment produced by *Endothia parasitica* and is also structurally related to skyrin, inhibits *E. coli* DNA transcription strongly and RDV-RNA transcription weakly. The inhibitory effects of the latter two are closely correlated with their ability to bind to and form floccules with *E. coli* DNA or RDV-RNA in the presence of Mg²⁺. Skyrin does not form floccules with the nucleic acids in the

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The following abbreviations are used: RDV, rice dwarf virus; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; TEMED, N, N, N', N'-tetramethylethylenediamine; TCA, trichloroacetic acid; SSC, 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.4; bis, N, N'-methylene bisacrylamide; PTA, phosphotungstic acid; PEG, polyethylene glycol (6,000).

presence of Mg^{2+} .¹²⁾ The result suggests that skyrin inhibits RDV-RNA transcription not by binding to the RNA but by binding to other components of the virus, possibly the polypeptides. This prompted us to examine polypeptide components of the virus, their location in the virion, and their relation to the RNA transcriptase activity.

This paper reports the results of experiments on the electrophoretic analyses of polypeptide components of the solubilized protein of the virus, and effects of different treatments of virions on the integrity and RNA transcriptase activity of the virus.

Materials and Methods

Virus purification. RDV was purified from leaves of rice plants, Norin-8, inoculated with viruliferous leafhoppers, *Nephotettix cincticeps* Uhl., which had been reared on RDV-infected rice plants. The method of purification was as described previously¹²⁾. The procedure involving homogenization of leaf tissues with the addition of CCl_4 in phosphate buffer, pH 6.8, precipitation of virus by low speed centrifugation with the addition of PEG and NaCl, treatment with fluorocarbon (Daiflon S3), and two quasi-equilibrium sucrose gradient centrifugations, was found to be the most successful. Although the fluorocarbon treatment was presumed to remove some of the outerlayer components of the virus as pointed out by Reddy *et al.*⁷⁾, this was necessary because RDV virions are otherwise enveloped in a lipid-like material³⁾.

Assay of RDV-RNA transcriptase activity. The method was as described previously. The standard reaction mixture contained in a final volume of 0.25 ml: 0.2 μ moles each of ATP, GTP, and UTP, 2.0 μ Ci of 3H -UTP, 2.0 μ moles of $MgCl_2$, 1.0 μ mole of phosphoenol pyruvate, 25 μ g of pyruvate kinase, 50 μ g of chymotrypsin, 25 μ moles of Tris-HCl, pH 8.5, and 200 μ g of RDV. In the case of testing effects of chymotrypsin-pretreatment of virions on the enzyme activity, chymotrypsin was omitted from the reaction mixture. After 60 min. at 35C, the reaction was stopped by 5% TCA and the radioactivity of resulting TCA-insoluble fraction was measured as described previously⁴⁾.

CsCl gradient centrifugation. Purified RDV drawn from sucrose gradients was suspended in 0.1 M phosphate buffer, pH 6.8, and centrifuged for 60 min. at 24,000 rpm in a Hitachi RP-40 rotor. The resulting pellet was resuspended in 1 ml of phosphate buffer, pH 6.8, layered on a preformed CsCl gradient (1.3 to 1.5g/ml density range), and centrifuged for 2 hours at 39,000 rpm in a Hitachi RPS-50 rotor. Fractions were collected from the bottom of the tube and the density of each fraction was determined by measuring its refractive index using the formula: $\rho_{CsCl} = 10.860 n_{D25C} - 13.4974$

To remove CsCl, each fraction was taken in a glass tube, both ends of which were sealed with cellophane membranes, subjected to electrophoresis for 6 hr at 2 mA/tube, and then concentrated in a Ultra-Thimble (Schleicher & Schuell). The sample was used for assaying RNA transcriptase activity.

Chymotrypsin treatment of purified RDV. One mg of purified RDV was suspended in 1 ml of 1xSSC, pH 7.4, containing 200 μ g of chymotrypsin and incubated at 25 C or 35 C for 1 hr. The suspension was centrifuged at 24,000 rpm in a Hitachi RP-40 rotor for 1 hr. The resulting pellet was resuspended in appropriate buffers and used for the assay of RNA transcriptase activity and the electrophoretic analysis of polypeptides.

SDS-polyacrylamide gel electrophoresis. Condition for electrophoresis of SDS gels were essentially as described by Smith *et al.*¹⁰⁾ and Reddy *et al.*⁷⁾. Ten percent acrylamide gels, containing 0.267% bis, 0.1% SDS, 0.002 M EDTA, 0.1% TEMED,

and 0.15% ammonium persulfate were prepared in 0.1 M sodium phosphate buffer, pH 7.4. The electrophoresis tank buffer was 0.1M sodium phosphate buffer, pH 7.4, containing 0.1% SDS, 0.002M EDTA. Electrophoresis was performed at room temperature using 6-9 gel columns, 6 mm×100mm, at 5 mA/gel (25-30 V), for 16 to 21 hr. Besides, 8 mm×150 mm gel columns were used for determining the molecular weights polypeptides.

To solubilize the virus protein, samples were suspended in 0.01 M phosphate buffer, pH 7.8, containing 1-2% SDS, 0.002M EDTA, 1-2% mercaptoethanol (v/v), and 10% sucrose, and treated in boiling water for 2min. Fifty to 200 μ g of the solubilized virus in less than 50 μ l was layered on each gel.

Following the electrophoresis, the gels were stained for 2-4 hr in 0.2% coomassie brilliant blue in a glacial acetic acid: methanol: water (1:5:5) and destained overnight in 20% methanol in 7% acetic acid. The stained gels were scanned at 560 nm in a Yamato-Asuka Ozumor 8-2 Densitometer.

Molecular weight determination. Molecular weights of the RDV polypeptides were determined by the method of Shapiro *et al.*⁸⁾. Chymotrypsinogen A from beef pancreas (25,000; Dickinson & Co.), ovalbumin (45,000; do.), bovine serum albumin (67,000; do.), and gamma globulin (160,000; do.) were used as markers.

Electron microscopy. Aliquots from the various preparations were dialyzed against 0.1 M ammonium acetate, pH 6.0, negatively stained with 2% phosphotungstic acid, pH 7.0, and examined in a JEM 100 electronmicroscope.

Results

SDS-polyacrylamide gel electrophoresis of RDV-polypeptides and determination of their molecular weights

Fig. 1. Structural polypeptides of RDV virions as obtained on polyacrylamide gel electrophoresed: (A) Standard gel (10%) (B) The separation of VI and VII components (7.5% gel).

Fig. 2. Polyacrylamide gel electrophoresis profiles of polypeptide components of RDV virions.

Fig. 3. Molecular weight determination of RDV polypeptides based on electrophoretic migration of assorted marker species. Abbreviation: gG, gamma globulin; BSA, bovine serum albumin; OVA, ovalbumin; Chy, chymotrypsinogen A.

Fig. 4. Isopycnic density gradient sedimentation patterns of RDV in preformed gradient of CsCl ($\rho=1.3$ to 1.5 g/ml) for 2 hrs at 39,000 rpm in a Hitachi RPS-50 rotor: T, top zone; M_1 , upper middle zone; M_2 , lower middle zone; B, bottom zone.

scent. T was the empty shell (Fig. 4). Buoyant densities of T, M_1 , M_2 , and B were 1.26, 1.39–1.42, 1.43, and 1.46 g/ml respectively. Recentrifugation of these components in CsCl gradients showed that only M_1 yielded M_2 and B (Fig. 5), whereas M_2 and B remain unaltered, indicating that the latter two are the degradation products of M_1 by the shearing effect of CsCl gradients.

Electron micrographs of these zones revealed that M_1 contained intact virions (Fig. 6B) and also degradation intermediates from intact virions toward M_2 , M_2

Purified RDV samples drawn from sucrose gradients were solubilized by SDS and subjected to 10% polyacrylamide gel electrophoresis. The profiles show 6 bands, however the most mobile 6th band was separated into two bands when electrophoresis was performed on a 7.5% gel (Fig. 1,2). These were designated I, II, III, IV, V, VI, and VII in the order of molecular weights. Their molecular weights were calculated to be 193,000; 152,000; 131,000; 110,000; 62,000; 46,000 and 45,000 daltons respectively (Fig. 3).

Degradation of RDV in CsCl density gradients

Purified RDV sample drawn from sucrose density gradients were subject to equilibrium CsCl density gradient centrifugation. The samples were separated into four zones, top(T), upper-middle (M_1), lower-middle (M_2), and bottom (B). M_1 was a broad zone, almost continuous to M_2 , but these were distinguishable because M_1 was bluish- and M_2 yellowish-opale-

Fig. 5. Stability of M_1 , M_2 and B zones in CsCl density gradients by reentrifugation.

Fig. 6. Electron micrographs of negatively stained: (A) RDV particles prepared from sucrose gradients, (B) M_1 , (C) M_2 , (D) B particles prepared from CsCl density gradients as shown in Fig. 4. $\times 100,000$.

contained virions on which capsomeres had been loosened (Fig. 6C), and B cores on which a small number of capsomeres still remained attached (Fig. 6D). M_2 virions are very unstable in 2% phosphotungstic acid releasing nucleic acid from virions (Fig. 6C).

Fig. 7. Electrophoresis profiles of RDV polypeptides of zones in CsCl density gradients: Top (A) M_1 (B), M_2 (C), and B (D).

Electrophoretic separation of polypeptides from each zone obtained by CsCl density gradient centrifugation

Polypeptide components of the zones, M_1 , M_2 , and B that were separated by CsCl density gradient centrifugation were examined by SDS-polyacrylamide gel electrophoresis. No significant difference in the number of polypeptides and their proportions is seen between M_1 and M_2 (Fig. 7B, C), whereas complete loss of II and IV, and marked decrease of VI and VII can be seen in B (Fig. 7D). Results indicate that II, IV, VI, and VII are the components of outer capsid, while I, III, and V are those of core. The decrease of the amounts of VI and VII coincides well with the partial loss of capsomeres as seen in the electronmicrograph of B, indicating that VI and VII compose the capsomeres. II and IV are the minor elements, the amounts of which vary according to the virus samples purified from different materials, indicating that these are unstable in the purification processes, possibly the polypeptides that compose the amorphous protein layer surrounding the capsomeres.

Table 1. Decrease of RDV-RNA transcriptase activity after CsCl gradient centrifugation

Viral Component	Transcriptase Activity (cpm)*
Intact virion	31,278
M ₁	10,440
M ₂	9,680
B	6,090

*CsCl was removed by electrophoresis as described in Materials and Methods.

Fig. 8. Electrophoresis profiles of polypeptides of RDV virions after treatment with chymotrypsin.

Table 2. Transcriptase activity of RDV-virions after treatment with chymotrypsin

Incubation* time (min.)	Incubation temperature (C)	Activity** (cmp)
0	25	22,415
30	25	21,258
I 60	25	21,308
120	25	18,196
150	25	21,338
0	35	18,688
30	35	18,226
II 60	35	17,928
120	35	20,731
150	35	19,342

*200 μ g of RDV was incubated in 1 ml 1 $\times$ SSC, pH7.4 containing 200 μ g chymotrypsin.

**Activity was measured as described in Materials and Methods

thought to compose the core, was easily released from virions by freeze-thawing together with RNA, suggesting that the polypeptide might be intimately associated with RNA in the core. No change in the polypeptide components was found with the virions incubated in Tris-HCl-Mg²⁺ buffer, pH 8.5, and also in the reaction mixture that devoid of chymotrypsin. The results indicate that the presence of Mg²⁺ in the incubation solution stabilizes attachment of capsomeres to the core surface.

RNA-transcriptase activities of M₁, M₂ and B

The zones, M₁, M₂, and B, separated by CsCl density gradient centrifugation, were subjected to electrophoresis to remove CsCl and then assayed for their RNA transcriptase activities. Although a great loss of activity occurred in each zone as a result of CsCl centrifugation, about 1/4 of the original activity was still retained even in B, the cores (Table 1). This suggests that I, III, and V, that compose the core, play important roles in the RNA transcription.

Effects of chymotrypsin treatment on RNA transcriptase activity and polypeptide components of virions

It has been reported by Silverstein *et al.*⁹⁾ and Astell *et al.*¹⁾ that RNA transcriptase activity of reovirus virions is exhibited only when the major outer capsomere protein, σ_3 , is removed by chymotrypsin *in vitro* or by hydrolases contained in the lysosomal compartment *in vivo*. This is not the case with RDV because the chymotrypsin treatment of RDV virions did not remove any of the polypeptides (Fig. 8) nor enhance RNA transcriptase activity (Table 2). The results are consistent with that of Kodama *et al.*⁴⁾ who reported that the presence of chymotrypsin in the reaction mixture did not enhance the RNA transcriptase activity but the optimum pH, 8.5, was the most effective factor to activate the enzyme.

Change of polypeptide components after various treatment of virion

RDV virions were incubated (1) in Tris-HCl, pH 8.5, at 35 C for 1 hr, (2) in Tris-HCl-Mg²⁺, pH 8.5, at 35 C for 1 hr, (3) in standard reaction mixture for the assay of RNA transcriptase activity (minus chymotrypsin), at 35 C for 1 hr, or (4) suspended in distilled water and treated with 3 repetitions of freeze-thawing. Polypeptide components of the samples were examined by SDS-polyacrylamide gel electrophoresis. Results indicated that VI and VII, that compose capsomeres, are readily removed from virions at a high pH, 8.5, in the absence of Mg²⁺ (Fig 9, C, D). V, that was

Fig.9. Polyacrylamide gel electrophoresis profiles of polypeptides of RDV virions after treatment with Tris-HCl buffer or freeze-thawing: The sample was treated with freeze-thawing, then centrifuged at 24,000 rpm for 1 hr. supernatant (A) and pellet(B); The sample was incubated in Tris-HCl, pH 8.5, for 1 hr, then centrifuged at 24,000 rpm 1 hr. Supernatant (C) and pellet (D).

Fig.10. Polyacrylamide gel electrophoresis profiles of polypeptides of RDV empty shells.

Polypeptide components of empty shells

Polypeptide components of the empty shells drawn from CsCl gradients (1.26 g/ml) and also from sucrose gradients were examined by SDS-polyacrylamide gel electrophoresis. Results indicate that all the polypeptide components of intact virions are present in empty shells, except that the proportion of V is much smaller than that in intact virions (Fig. 10).

Discussion

This study was undertaken to gain some insight into the polypeptide components of RDV virions and their location in the virion structure. Just like reovirus and wound tumor virus, RDV virions contain seven species of polypeptides. These were tentatively designated I, II, III, IV, V, VI, and VII in the order of molecular weights. Although it was reported by Reddy *et al.*⁷⁾ that the purification procedure of wound tumor virus by using Freon-CCl₄ resulted in a loss of some outer shell components, the use of fluorocarbon and CCl₄ was necessary for the purification of RDV because RDV virions were otherwise enveloped by a lipid-like material³⁾. The components II and IV were thought to be comparable with 131,000 and 96,000 dalton components of wound tumor virus that surround the capsomeres, because their proportions often varied according to virus samples purified from rice leaves of different ages and those collected in different seasons, possibly due to their susceptibility to fluorocarbon-CCl₄ treatment. In this study, the RDV sample purified by fluorocarbon-CCl₄ treatment, PEG sedimentation, followed by sucrose density gradient centrifugation were used as the original material for polypeptide analyses.

Unlike reovirus, chymotrypsin could not remove capsomeres of RDV nor enhance RNA transcriptase activity of virions. The only way to remove the outer shell components efficiently was to utilize the shearing effect of CsCl density gradient centrifugation.

In CsCl gradients, RDV virions separated into four zones, T, M₁, M₂ and B, of which M₁ was a broad zone almost continuous to M₂ but was easily distinguished because of its bluish fluorescence in contrast to the yellowish fluorescence of M₂.

Recentrifugation in CsCl gradients showed that M₁ yielded M₂ and B, indicating that the degradation of virions proceeded from M₁ via M₂ to B. Prolonged centrifugation,

e. g., for 12 hr, in CsCl gradients resulted in the increase of M_2 and B, and the disappearance of M_1 as reported by Reddy *et al.*⁷⁾ for wound tumor virus. Centrifugation in CsCl gradients in Tris-buffer, pH 8.5, showed the same results. Electron micrographs of these zones showed that T was empty shells, M_1 consisted of intact virions and intermediates of various degrees of disruption toward M_2 , which buoyant densities varying from 1.39 to 1.42, M_2 the virions on which outer capsomeres were loosened, and B the cores on which capsomeres had been lost not completely but to a great extent.

Since polypeptides II and IV had been completely, and VI and VII partially lost in B, these must be the components of the outer shell. II and IV appear to compose the amorphous protein layer that surround the capsomeres because their proportions vary according to the leaf materials of different ages and seasons. VI and VII are the major components that decrease in parallel with the disruption of virions indicating that these are the components of capsomeres. The remaining I, III, and V compose the core, of which V is readily released from virions by freeze-thawing suggesting that this is the internal protein intimately associated with nucleic acid.

Although CsCl density gradient centrifugation greatly reduced RNA transcriptase activity of virions, M_1 , M_2 still retained about 1/3, and B about 1/4 that of the original intact virions, indicating that the components of core, i. e., I, III, and V must play important roles in the enzyme action.

Incubation of virions in Tris-HCl, pH 8.5, for 1 hr at 35C caused loss of VI and VII, whereas incubation in Tris-HCl-Mg²⁺, pH 8.5, caused no change of polypeptide components. This was also the case with the incubation of virions in the reaction mixture in which 8mM of Mg²⁺ was present and essential for the enzyme reaction. These results indicate that capsomeres remain attached on the virions that are producing RNA in the reaction mixture where Mg²⁺ is present. This is a striking contrast to reovirus, in which RNA transcriptase is activated only when virions are

Table 3. Correspondence of genome segment size of RDV to the size of virus-polypeptide

RDV No.	genome segment M. W. ($\times 10^6$)*	RDV polypeptide species			
		Molecular weight		No.	Location
		Calculated**	Observed		
1.	3.10	194,000	193,000	I	core
2.	2.50	156,000	152,000	II	outer protein layer
3.	2.20	137,500	131,000	III	core
4.	1.80	112,500			
5.	1.76	110,000	110,000	IV	outer protein layer
6.	1.05	65,600			
7.	1.02	63,700	62,000	V	core
8.	0.78	48,800	46,000	VI	underlying capsomere
9.	0.70	43,000	45,000	VII	underlying capsomere
10.	0.67	41,900			
11.	0.48	30,000			
12.	0.48	30,000			

* Molecular weights were taken from Reddy *et al.* (1974).

** Mol. W. of polypeptide = $\frac{\text{Mol. W. of RDV genome segment} \times \text{Mol. W. of amino acid (120)}}{\text{Mol. W. of RDV base pair (640)} \times 3}$

converted into subviral pariticles *in vivo* or into cores by the action of chymotrypsin *in vitro*.

RDV-genome has been reported to consist of 12 dsRNA segments of known molecular weights^{2,6)}. If we assume that the mRNA that is transcribed from each of the dsRNA segment is monocistronic, the molecular weight of polypeptide translated from each mRNA can be calculated from molecular weight the respective dsRNA segment. The relationship between molecular weights of genome segments and those of virion-polypeptides are shown in Table 3. The table also indicates the location of each polypeptide in the structure of virion.

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和 文 摘 要

イネ萎縮病ウイルス粒子の構成ポリペプチド

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イネ萎縮病ウイルス感染葉から分離したウイルス標品は SDS-ポリアクリルアミドゲル電気泳動によって 7 種のポリペプチドに分離された。泳動距離の短い方から順に I~VII とした。それぞれの分子量は 193,000 ; 152,000 ; 110,000 ; 62,000 ; 46,000 ; 45,000 ダルトンであった。キモトリプシン処理によって外殻ポリペプチドは除かれなかった。CsCl 濃度勾配平衡遠心によって M₁, M₂, B の三成分に分かれ、それぞれの浮遊密度は 1.39~1.42, 1.43, 1.46g/ml であり、CsCl 内での遠心による shearing force を受けて粒子は M₁→M₂→B へと変化したものと考えられる。電子顕微鏡観察と電気泳動から B は II と IV が完全に、VI と VII が一部除かれたものであり、II と IV は最外殻を形成する不定形のポリペプチド、VI と VII はカプソメアを形成するポリペプチドと考えられる。I, III, V はコアを形成するポリペプチドと推測され、V は凍結融解によって核酸と共に粒子から漏出することから核内のポリペプチドと考えられる。