# Improved Purification of Rice Dwarf Virus by the Use of Polyethylene Glycol

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ABSTRACT

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Purification of rice dwarf virus (RDV) was simplified and greater yield and infectivity of virus were obtained by precipitating RDV with polyethylene glycol 6000 (PEG 6000) from clarified extracts, than by previous procedures employing differential centrifugation. The optimal conditions for use of PEG 6000 were a pH of about 7.0, 0.3 M NaCl, 6% (w/v) PEG 6000, and incubation at 4 C for 4 hours. The optimal ratio of buffer solution to tissue (v/w) was five or six. Yields of purified virus were about 20-30% of that in the clarified extracts. Both clarified extracts and purified preparations contained (5.40-9.18) $\times 10^8$ virions per milliliter at the dilution end point for infectivity. Clarified extracts and purified preparations were quick-frozen in dry-ice ethanol and stored at −72 C for at least 18 months without detectable loss of infectivity.

In 1962, Fukushi et al. (2) first isolated rice dwarf virus (RDV) particles (about 70 nm in diameter) from rice plants infected with RDV and from viruliferous leafhoppers by using differential centrifugation. The electron micrographs published by these authors showed that the particles were enveloped with a membranaceous material. In later studies, Toyoda et al. (12) reported treatment of the virus with the phospholipase of snake venom or pancreatin for removal of the enveloping material and then elution of the virus from DEAE-cellulose columns with 0.20 to 0.25 M NaCl. The virus in the effluent was seen to be highly purified when examined under the electron microscope but preparations obtained by that method yielded virus with low infectivity. Subsequently, purification methods for RDV (9) usually involved treatments with organic solvents, differential centrifugation, and zonal density-gradient centrifugation (1).

Following the work of Hebert (3) on the use of polyethylene glycol 6000 (PEG 6000) for precipitation of four plant viruses, PEG 6000 has been effectively employed to selectively concentrate a number of other plant viruses (10, 11, 13, 14).

This paper reports the use of PEG 6000 in the purification of RDV and storage of the clarified and purified preparations. A preliminary report of part of this investigation has been published (7).

## MATERIALS AND METHODS

**Virus source.**—The RDV isolate used in these experiments was the isolate used by Toyoda et al. (12). It was maintained in rice plants (*Oryza sativa* L. 'Norin No. 29') which were inoculated by viruliferous leafhoppers (*Nephotettix cincticeps* Uhl.). Leaves and leaf-sheaths showing clear symptoms 6-8 weeks after inoculation were selected as sources of RDV.

**Preliminary clarification.**—The selected leaves and leaf-sheaths were cut into small pieces, and then ground in a meat chopper with the addition of 600 ml of 0.1 M phosphate buffer solution containing $Na_2HPO_4$ and $KH_2PO_4$ (pH 7.2) to each 120 gm of leaves and leaf-sheaths. Carbon tetrachloride, at one-half the volume of added phosphate buffer solution, was mixed in the slurry by one or two 2-minute treatments with a Waring Blendor–just long enough to form an emulsion. The emulsion was centrifuged immediately thereafter at low speed (3,000 rpm) for 5 minutes in a Hitachi 20PR centrifuge. To two volumes of clarified supernatant solution from the centrifuge, one of $CCl_2F$-$CClF_2$ (Daiflon $S_3$ from Daikin Co., Oshaka, Japan) was added, and the mixture was homogenized for 1-2 minutes at high speed in the Waring Blendor. Immediately the emulsion again was centrifuged for 5 minutes at low speed. The final supernatant solution is termed the clarified extract below, for example, the clarified extract of Table 4. These procedures were carried out in a cold room (6 C).

**Polyethylene glycol.**—Polyethylene glycol 6000 (PEG 6000) (M.W. 7,500) was used to concentrate the virus from clarified extracts.

**Rate-zonal density-gradient centrifugation.**—A glycine-$MgCl_2$ buffer solution (0.2 M glycine, 0.01 M $MgCl_2$, pH 7.0) (Gly-Mg solution) was used to make up solutions containing 300 gm and 400 gm sucrose per liter. Gradients were prepared by layering 10 ml of the lighter solution above 10 ml of the heavier solution in tubes fitting the Hitachi RPS-25 rotor. The solutions were allowed to diffuse for at least 20 hours at 4 C. Ten to 15 ml of the extracts concentrated by PEG 6000 were layered on top of each gradient and then centrifuged at 40,695 *g* (average) for 1 hour at 6 C in a Hitachi 55P-2 ultracentrifuge.

**Quasi-equilibrium zonal density-gradient centrifugation.**—Density gradients similarly were

prepared with 10 ml volumes of solutions containing 500 gm and 600 gm sucrose per liter of Gly-Mg solution in tubes of the RPS-25 rotor. Ten to 15 ml of the virus zone removed from the rate-zonal density-gradient tubes was layered onto the top of each quasi-equilibrium density-gradient column and then centrifuged at 26,800 *g* (average) for 16 hours at 6 C in a Hitachi 55P-2 ultracentrifuge. The virus zone (lower zone) was removed by a needle from the bottom of the tube (leaving the upper zone of empty shell) and was then dialyzed against Gly-Mg solution for about 24 hours at 4 C.

**Electron microscope particle counting.**—The particle-counting procedure has been described in detail in an earlier paper (5).

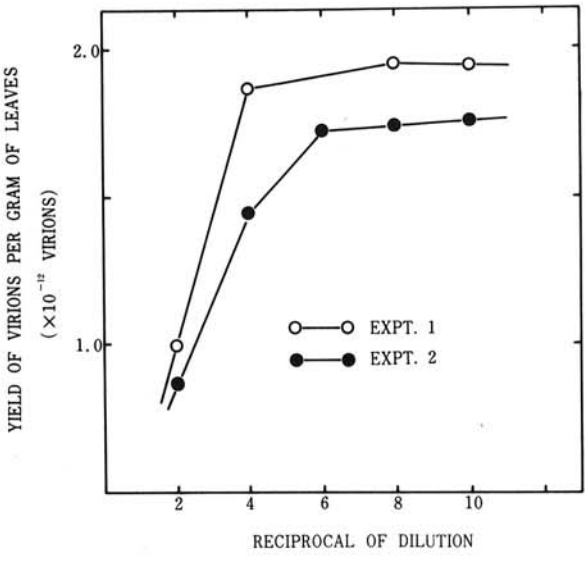


**Fig. 1.** Effect of dilution of leaf extracts on the yield of rice dwarf virus (RDV) from rice leaves. The initial extraction of 2 gm of infected leaves with 2 ml of 0.1 M phosphate buffer solution (pH 7.2) was reckoned to yield a 1/2 dilution.

TABLE 1. Effect of pH in clarified extracts on precipitation of rice dwarf virus (RDV) by polyethylene glycol 6000 (PEG 6000)[a]

| Expt. no. | pH | Yields of the virus[b] (virions × $10^{-12}$/gm) |
|---|---|---|
| 1 | 6.0 | 0.327 |
| | 6.9 | 0.560 |
| | 7.7 | 0.178 |
| 2 | 6.4 | 0.285 |
| | 6.9 | 0.400 |
| | 7.3 | 0.342 |
| 3 | 5.8 | 0.369 |
| | 6.5 | 0.399 |
| | 7.0 | 0.463 |

[a]Precipitation conditions were: NaCl 0.3 M; polyethylene glycol 6000 (PEG 6000) 6%; and an incubation time of 4 hours at 4 C.

[b]The hydrolyzed rice dwarf virus (RDV)-RNA was measured at $OD_{260}$.

**Measurement of total virus yield.**—The virus preparations obtained after the quasi-equilibrium centrifugation were dialyzed for 24 hours against 6 liters of Gly-Mg solution. The virus then was pelleted at 80,730 *g* (average) for 1 hour in a Hitachi RP-40 rotor. The virus was subsequently hydrolyzed in 1 N HCl at 30 C for 24 hours. The RNA hydrolysates were in the supernatant solution after centrifugation of the samples at 3,000 rpm for 10 minutes in a Hitachi 20RP centrifuge and the optical density was measured in Hitachi Model 101 spectrophotometer. One optical density unit of such RNA hydrolysates was considered equivalent to $1.07 \times 10^{12}$ virions per ml (6).

**Infectivity test.**—The infectivity test used in these experiments was the insect injection method by which Kimura and Fukushi (8) succeeded in transmitting RDV to virus-free insect vectors by using fine glass capillaries. These leafhoppers were confined individually in glass test tubes each enclosing a healthy young rice plant. In this case, second- and third-instar nymphs were selected for injection.

## RESULTS

**Effect of diluting the extracts on virus yield.**—The optimal dilution of crude extract which lead to the maximum yield of virus in clarified extract was determined. Two grams of infected leaves were used as starting material to which 0.1 M phosphate buffer solution (pH 7.2) was added to give the desired dilution. The extracts were prepared to make 2-, 4-, 6-, 8-, and 10-fold (w/v) dilutions based on fresh sample weight. Samples were carried through further clarification of organic solvent treatments and PEG 6000 concentration. The precipitates each were resuspended in 2 ml of Gly-Mg solution. Subsequently, virions were counted to

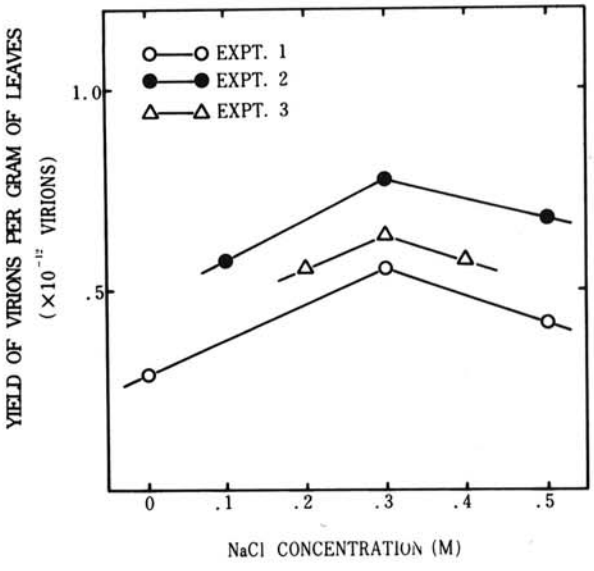


**Fig. 2.** Yields of rice dwarf virus (RDV): effect of NaCl concentration on precipitation of RDV by polyethylene glycol 6000 (PEG 6000). Conditions were: pH 7.0; PEG 6000 6%; and an incubation time of 3 hours at 4 C.

determine the virus yield from each dilution. As shown in Fig. 1, the optimal dilution was 6- to 10-fold.

**Effect of pH on precipitation by polyethylene glycol 6000.**—The pH affected virus precipitation by PEG 6000. Clarified extracts divided into the three samples required in each experiment, were prepared at various pH values (5.8-7.7) with phosphate buffer solutions. NaCl (0.3 M) and PEG 6000 6% (w/v) were added to each sample which was kept for 4 hours at 4 C to permit precipitation to occur. Virus was then further purified and its RNA was measured.

The results from three experiments (Table 1) show that maximum precipitation of RDV is obtained at a pH of about 7.0.

**Effect of NaCl concentration.**—Clarified extracts adjusted to pH 7.0 were divided into the desired number of aliquots and appropriate aliquots were made 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 M with NaCl. Six percent (w/v) PEG 6000 was added and the mixture was incubated for 3 hours at 4 C. Virus then was purified further and its RNA was measured. As shown in Fig. 2, maximal yields of total virus (11) were obtained with about 0.3 M NaCl concentration.

**Effect of polyethylene glycol concentration.**—To determine the optimal PEG 6000 concentration required to obtain maximum virus yield, clarified extracts first were adjusted to pH 7.0 by adding phosphate buffer solution. Salt (NaCl) was added to give 0.3 M and the preparation then was divided into the desired number of aliquots in which various amounts of PEG 6000 were incorporated. After precipitation, the virus was purified further and its RNA was measured. In three experiments in which yields of total virus were measured, 6% (w/v) PEG 6000 gave the best results (Fig. 3).

**Effect of incubation period at 4 C.**—Clarified extracts were adjusted to pH 7.0 by adding phosphate buffer solution. Salt (NaCl, 0.3 M) and PEG 6000 (6%, w/v) were incorporated and the preparations were divided into the desired number of aliquots. Each sample was kept at 4 C for a different incubation period. After precipitation, the virus was purified further, and the RNA in each sample was measured. In three experiments, the optimum incubation period was about 4 hours (Fig. 4).

**Yields of purified virus.**—The particle count method was used to determine the number of virions in the clarified extracts and purified preparations made as described in an earlier paper (5). Yields of purified virus were 21.5-32.4% that of the virus in the clarified extracts (Table 2).

**Infectivity of purified preparations.**—Infectivity could be demonstrated by injecting insect vectors with clarified extracts diluted to $10^{-3}$ or $10^{-3.5}$. Kimura (5) reported that 1 gram of infected leaves contained about $10^{12}$ virions. At the above dilutions of clarified extracts there were still about $10^{8.5}$-$10^{9.0}$ virions per milliliter. As shown in Table 3, both preparations (the clarified and the purified) contained about (5.40-9.18) × virions per milliliter at the dilution end points for infectivity. The infectivity of the virus was not detectably reduced during the purification procedures.

**Freeze-storage of clarified and purified**

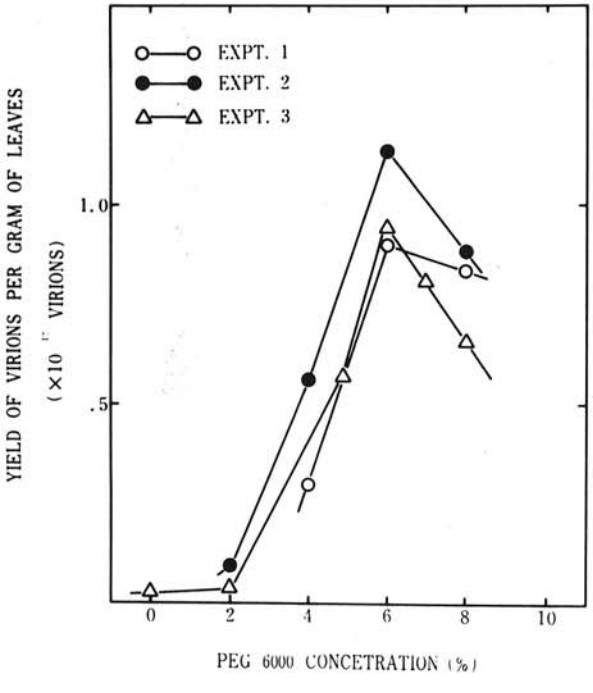


**Fig. 3.** Yields of rice dwarf virus (RDV): effect of polyethylene glycol 6000 (PEG 6000) concentration on precipitation of RDV. Conditions were: pH 7.0; NaCl 0.3 M; with an incubation time of 2.5 or 3.0 hours at 4 C. Solid PEG 6000 was added to give the required percent (w/v) concentration.

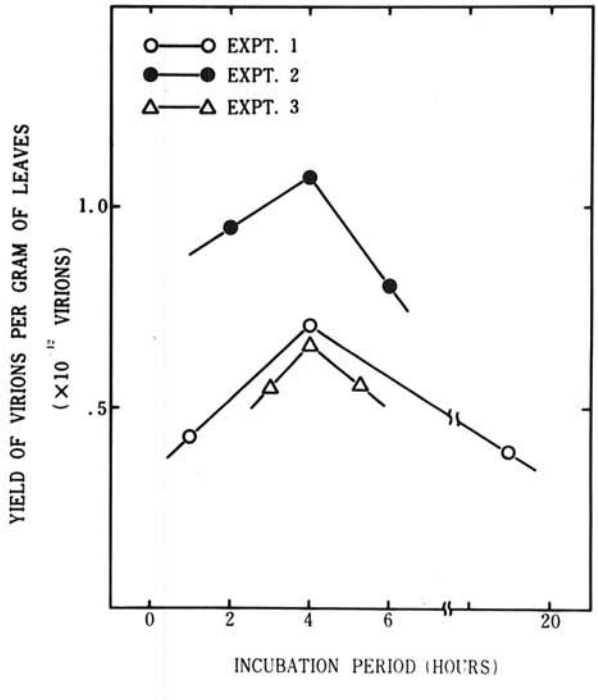


**Fig. 4.** Yields of rice dwarf virus (RDV): effect of incubation period at 4 C on precipitation of RDV by polyethylene glycol 6000 (PEG 6000). Conditions were: pH 7.0; NaCl 0.3 M; and PEG 6000 6% (w/v).

**preparations.**—Samples of clarified extracts and purified preparations were frozen quickly in small ampules at −72 C in an ethanol-dry ice bath. They were then stored at −72 C and later, at intervals, the samples were melted quickly in a water bath at 40 C, and infectivity tests and particle counts were done. As shown in Tables 4 and 5, virus infectivity and concentration of the clarified extracts and purified preparations were almost constant for 18 months.

## DISCUSSION

Purification of RDV was by organic solvent treatments, PEG 6000 concentration, rate- and equilibrium-zonal density-gradient centrifugation. Purification was improved by use of PEG 6000 for concentration and fractionation of the virus. Previously it was difficult to concentrate virus from bulky crude virus extracts only by differential centrifugation. Therefore, the amount of buffer solution that could be added was restricted. Furthermore, extraction of the virus from tissues was incomplete and consequently yields of virus were low. The amount of buffer solution for extracting RDV was optimal at 6 to 10 times the fresh weight of leaves for the best concentration of RDV by PEG 6000.

In using PEG 6000, its concentration, the concentration of NaCl, the pH, and the incubation period all are important. The yield of virus was determined directly as number of virions per gram of leaves by

TABLE 2. Yields of rice dwarf virus (RDV)[a] in the clarified extracts and in the purified preparations

| Expt. no. | Clarified extracts[b] (virions × $10^{-12}$/gm) | Purified preparation[c] (virions × $10^{-12}$/gm) | Percentage of virions purified from clarified extracts |
|---|---|---|---|
| 1 | (1.47 ± 0.14) | (0.36 ± 0.02) | 24.3 |
| 2 | (1.81 ± 0.16) | (0.46 ± 0.09) | 25.5 |
| 3 | (2.61 ± 0.24) | (0.82 ± 0.14) | 31.5 |
| 4 | (1.66 ± 0.17) | (0.54 ± 0.11) | 32.4 |
| 5 | (2.33 ± 0.22) | (0.50 ± 0.09) | 21.5 |

[a]The numbers of virions were measured by particle counting in the electron micrographs.
[b]These preparations were obtained after using procedures for virus extraction and organic solvent treatments.
[c]These preparations were obtained after using procedures for virus extraction, organic solvent treatments, polyethylene glycol 6000 (PEG 6000) concentration, and rate- and equilibrium-zonal density gradient centrifugations.

TABLE 3. Infectivity of rice dwarf virus (RDV) at dilution end point in clarified extracts and purified preparations

| Expt. no. | Preparation | Concentration (C)[a] (virions/ml) | Infectivity test at dilutions[b] $C \times 10^{-2.5}$ | $C \times 10^{-3.0}$ | $C \times 10^{-3.5}$ | $C \times 10^{-4.0}$ | Concentration at dilution end point (virions × $10^{-8}$/ml) |
|---|---|---|---|---|---|---|---|
| 1 | Clarified | (1.88 ± 0.20) × $10^{12}$ | ... | 4/16 | 2/17 | 0/19 | 6.02 |
| | Purified | (5.40 ± 0.52) × $10^{11}$ | 2/18 | 1/17 | ... | ... | 5.40 |
| 2 | Clarified | (2.15 ± 0.21) × $10^{12}$ | ... | 3/15 | 1/18 | 0/20 | 6.88 |
| | Purified | (7.52 ± 0.75) × $10^{11}$ | 4/19 | 1/11 | 0/15 | ... | 7.52 |
| 3 | Clarified | (2.87 ± 0.28) × $10^{12}$ | ... | 4/20 | 1/21 | ... | 9.18 |
| | Purified | (9.00 ± 0.93) × $10^{11}$ | 3/17 | 1/20 | ... | ... | 9.00 |

[a]The numbers of virions were measured by particle counting in the electron micrographs.
[b]Infectivity was tested by injecting leafhopper with the preparations. The denominators represent the numbers of leafhoppers surviving more than 10 days; the numerators represent numbers of leafhoppers that infected the test plants.

TABLE 4. Frozen-storage of clarified rice dwarf virus (RDV) extracts

| Duration of storage at −72 C | Concentration (C)[a] (virions × $10^{-12}$/ml) | Leafhopper injection[b] infectivity assay $C \times 10^{-3.0}$ | $C \times 10^{-3.5}$ |
|---|---|---|---|
| Control (unfrozen) | (1.13 ± 0.11) | 4/20 | 2/20 |
| 1 week(s) | (1.29 ± 0.13) | 3/19 | 2/18 |
| 2 weeks | (1.10 ± 0.10) | 11/21 | 4/21 |
| 1 month(s) | (1.13 ± 0.11) | 5/18 | 4/14 |
| 3 months | (1.09 ± 0.10) | 6/17 | 3/13 |
| 6 months | (1.24 ± 0.12) | 3/21 | 2/21 |
| 18 months | (1.23 ± 0.12) | 4/21 | 2/21 |

[a]The numbers of virions were measured by particle counting in the electron micrographs.
[b]The denominators represent the numbers of leafhoppers surviving more than 10 days; the numerators represent the numbers of leafhoppers that infected plants.

TABLE 5. Frozen-storage of purified rice dwarf virus (RDV) preparations

| Duration of storage at −72 C | Concentration (C)[a] (virions × $10^{-11}$/ml) | Leafhopper injection[b] infectivity assay | |
|---|---|---|---|
| | | $C \times 10^{-2.0}$ | $C \times 10^{-2.5}$ |
| Control (unfrozen) | (2.14 ± 0.22) | 5/26 | 4/29 |
| 1 week(s) | (2.11 ± 0.20) | 6/27 | 3/24 |
| 2 weeks | (1.98 ± 0.19) | 4/17 | 2/22 |
| 1 month(s) | (2.09 ± 0.20) | 4/28 | 2/26 |
| 6 months | (2.05 ± 0.21) | 2/20 | 1/24 |
| 18 months | (1.95 ± 0.19) | 4/20 | 2/19 |

[a]The numbers of virions were measured by particle counting in the electron micrographs.
[b]The denominators represent the numbers of leafhoppers surviving more than 10 days; the numerators represent the numbers of leafhoppers that infected plants.

particle counting and indirectly by conversion of $OD_{260}$ measurements of hydrolyzed RDV-RNA. One OD unit was equivalent to $1.07 \times 10^{12}$ virions per ml (6).

Crude extracts from 1 g of young infected rice leaves contained from $(1\text{-}3) \times 10^{12}$ virions (5). After clarification of extracts and concentration of the virus by PEG 6000, there were about 80% and 60% as many virions, respectively, and final purified preparations contained about 20% of the virions in the crude extracts. Infectivity of purified preparations was of the same order as that of clarified extracts, that is $(5.40\text{-}9.18) \times 10^8$ virions per milliliter at the dilution end points for infectivity. Therefore, the virus in the process of purification retained its infectivity without detectable loss. With a previous procedure (9), the yield of purified virus was given as about 3-5% of the virus in the crude extracts. About 90% of the active virus was lost during the purification procedure. Low virus yield and low infectivity of the purified preparation have been serious handicaps in studies of RDV.

Hsu and Black (4) found different optimal concentrations of PEG 6000 for precipitation of two field varieties of potato yellow dwarf virus, which were serologically related but also had serological differences. RDV and wound tumor virus (WTV) are similar in many ways, but the conditions for PEG 6000 concentration of RDV and WTV (11) are different.

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