Purification and Serology of Rice Tungro Spherical and Rice Tungro Bacilliform Viruses*

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virus と rice tungro bacilliform virus の純化と血清関係*

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Gálvez¹⁾ reported that spherical particles about 30-33 nm in diameter were purified from rice plants with tungro disease. Saito²⁾ observed bacilliform and spherical particles in most of the specimens with tungro and similar diseases by electron microscopy. These particles were designated as rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV)³⁾. Rice waika virus (RWV) which occurred in Kyushu, Japan, is serologically related to RTSV²⁾. Hibino *et al.*⁴⁾ found that RTSV was transmitted by the vector *Nephotettix virescens*, but that RTBV was transmitted concomitantly only when RTSV was acquired previously or simultaneously, i.e., propagation of RTBV using plants solely infected with RTBV as inoculum was impossible. Since a large quantity of doubly-infected plants were available, such plants were used for virus purification.

A tungro isolate containing both RTBV and RTSV supplied by the International Rice Research Institute, Philippines, was propagated in rice plants (*Oryza sativa* L. cv. Taichung Native 1) by using viruliferous *N. virescens*. Infected plants were harvested about 40 days after inoculation and were stored at -80 C. Clarification and partial purification were carried out according to the method applied for RWV⁵⁾. Leaf extracts in 0.01 M ethylenediaminetetraacetic acid (EDTA), pH 6.0 were heated at 40 C for 1 hr. Virus particles were precipitated with 7 % polyethylene glycol 6,000 plus 0.2 M sodium chloride (NaCl) containing 1 % Triton X-100. The resuspended virus particles were treated with 20 % carbon tetrachloride, and were subjected to differential centrifugation (96,000×*g* for 60 min at 4 C in a Hitachi RP-40 rotor, then 10 min at 3,000×*g*). Supernatant was layered on 10-40 % linear sucrose density gradients (prepared in 0.01 M borate buffer pH 9.0 (BB)) and centrifuged at 60,000×*g* for 3 hr at 4 C in a Hitachi RPS-25 rotor. Tube contents were scanned at A₂₅₄ and fractionated through

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1) Gálvez, G. E. (1968). Virology 35: 418-426. 2) Saito, Y. (1977). Tropical Agriculture Research Series No. 10: 129-135. Tropical Agricultural Research Center. Tsukuba, Ibaraki, Japan. 3) Saito, Y., Hibino, H., Omura, T., and Usugi, T. (1981). Proc. 5th Int. Cong. Virology: 213. 4) Hibino, H., Saleh, N. and Roehchan, M. (1979). Phytopathology 69: 1266-1268. 5) Usugi, T. and Saito, Y. (1975). Ann. Phytopath. Soc. Japan 41: 283 (Abstr.)

Fig. 1. Sucrose density-gradient profiles (A) of partially purified preparation from rice plants infected with both RTSV and RTBV, (B) following repeated sucrose density gradient centrifugations of peak "a" (solid line) and shoulder "b" (broken line).

an ISCO model UA5 ultraviolet analyzer. Typical density-gradient scanning pattern presented in Fig. 1A showed a peak (arrow with "a") containing RTSV, and a shoulder (arrow with "b") on the descending slope containing RTBV. Peak and shoulder fractions were separately collected, virus particles were pelleted by centrifugation, and were subjected to two additional density-gradient centrifugations (Fig. 1B). After three cycles of sucrose density-gradient centrifugation, RTSV was further purified by equilibrium centrifugation in CsCl. When 3.273 g CsCl was dissolved in 4 ml (45 % w/v) of the peak material in 0.01 M phosphate buffer (PB), pH 7.0 and the suspension was centrifuged at $114,000 \times g$ for 40 hr at 4 C in a Hitachi RPS-40 rotor, a single band was formed at about two-thirds of the distance below the meniscus. The peak material was recovered, and CsCl was removed by two cycles of differential centrifugation. Final pellets were resuspended in 0.01 M EDTA, pH 6.0 and such preparations contained isometric particles about 30 nm in diameter (Fig. 2).

Equilibrium centrifugation in CsCl was not appropriate for the purification of RTBV, because the particles were degraded during the two cycles of

differential centrifugation applied to remove CsCl after the equilibrium centrifugation. After three cycles of sucrose density-gradient centrifugation, RTBV was further purified using antiserum to RWV whose titer was 1: 640 in the precipitin ring interface test. Partially purified RTBV fraction was mixed with equal volume of the serum which was diluted to 1/100 with 0.01 M PB, pH 7.0 containing 0.85 % NaCl. The mixture was incubated for 1 hr at 37 C, followed by over night incubation at 4 C. Preparations were negatively stained with neutralized phosphotungstic acid (PTA) and were examined under a Hitachi H-500 electron microscope. As shown in Fig. 3 contaminating RTSV particles were clumped and easily sedimented by low speed centrifugation. After two additional serum treatments final supernatant was layered on sucrose density-gradient, centrifuged, and fractionated as mentioned above. Virus was concentrated by high speed centrifugation and pellets were resuspended in 0.01 M EDTA, pH 6.0. Purified RTBV particles were 30-35 nm in width with varying length (Fig. 4). The length of the particles was measured and compared to that of particles in leaf dip preparations

Figs. 2-6. Electron micrographs of RTSV and RTBV negatively stained with 2% phosphotungstate pH 7.0.

Fig. 2. Purified preparation of RTSV. Bar represents 200 nm.

Fig. 3. Reaction of RTSV with the serum against RWV. Bar represents 300 nm.

Fig. 4. Purified preparation of RTBV. Bar represents 200 nm.

Fig. 5, 6. Immuno-electron microscopy preparation of diseased leaves on a grid previously mixed with serum of RTSV (Fig. 5) or RTBV (Fig. 6). Bars represent 300 nm.

Fig. 7. Particle length distribution of RTBV in purified preparation (A) and in leaf dip preparation (B).

using the method of Brakke⁶⁾. Buoyant densities of RTSV and RTBV were 1.551 g/cm³ and 1.312 g/cm³, respectively. The fact that RTBV formed a single band indicated that the buoyant density of RTBV was even, though the virus contained particles of various sizes.

Rabbits were immunized separately against RTSV and RTBV by an intramuscular injection with purified preparations emulsified with an equal volume of Freund's complete adjuvant as well as by repeated intravenous injections. In the precipitin ring interface test, antisera titers were 1/320 and 1/2048 against RTSV and RTBV, respectively. For double gel diffusion test, 0.8% agar in 0.01 M PB, pH 7.6 containing 0.85% NaCl, 0.001 M EDTA and 0.05% sodium azide was used. No heterologous reactions occurred between these antisera and the two kinds of particles. Absence of serological relationship between these viruses was also confirmed by clumping technique⁷⁾ of immunoelectron microscopy. When antiserum was mixed with sap from doubly infected plant, only homologous reactions showed clumped particles with halos (Fig. 5, 6).

Hibino *et al.* reported that RTSV and RTBV multiplied independently, though the presence of RTSV was indispensable for the transmission of RTBV⁴⁾. In the present study, it was demonstrated that there was no serological relationship between RTSV and RTBV. These results further indicate that RTSV and RTBV are independent virus, rather than multiparticulate or satellite viruses⁸⁾.

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6) Brakke, M. K. (1967) Methods. in Virol. 2: 93-136. 7) Milne, R. G. and Luisoni, E. (1977). Methods. in Virol. 6: 265-281. 8) Lane, L. C. (1980). Nucleic Acids in Plants (Hall, T. C. and Davis, J. W. eds.). Vol. 2: 65-110. C. R. C. Press, Florida.

(Fig. 7A, B). Majority of the particles (97%) were included in a major peak at 100-220 nm in purified preparations. There was no significant difference in the distribution pattern of the length between purified and leaf dip preparations.

Ratios of absorbance (260/280) for purified RTSV and RTBV were 1.75 and 1.11, respectively. Buoyant densities of the particles were estimated by equilibrium centrifugation in CsCl. For RTBV, 2.154 g CsCl was dissolved in 4 ml (35%, w/v) of the purified preparation in 0.01 M PB, pH 7.0 and centrifuged at 114,500×*g* for 40 hr at 4 C in a Hitachi RPS-40 rotor. A single band was formed at about one-third of the distance below the meniscus. The density of the fraction was determined from the refractive index