Isolation and Purification of *Rice tungro virus*

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Abstract

Rice tungro disease is caused by two types of virus, namely, *Rice tungro bacilliform virus* (RTBV) and *Rice tungro spherical virus* (RTSV). Their viral carrier is the green leafhopper, *Nephotettix virescens*. Rice tungro viruses were purified from infected plants in order to compare different extraction methods and to calculate viral protein molecular weights. The purification of the two types of virus was studied by preparing virus particles from freeze-ground infected leaves in four ways. In the first three methods, virus particles were released into the supernatant by digesting plant cell walls with a mixture of cellulases at 37 °C for 1, 2 and 3 hours, respectively, whereas the fourth method relied on alternately spinning the ground plant material at high and low speed. The fourth method was the best extraction system for obtaining pure virus. Viral particles were examined by transmission electron microscopy at a magnification of 30 X 10^3. Two types of particles were observed. One was rod-shaped with rounded ends and the other was spherical. These two kinds of viral particle had different sizes. RTBV particles had an average width of 21.30 nm and an average length of 137.57 nm while RTSV particles had an average diameter of 15.03 nm. SDS-PAGE analysis showed that RTBV proteins had molecular weights of 24 and 50 kDa whereas those of RTSV had molecular weights of 22.0, 22.5 and 32.5 kDa.

Keywords: Rice tungro disease, *Rice tungro bacilliform virus* (RTBV), *Rice tungro spherical virus* (RTSV), purification, transmission electron microscopy, and SDS-PAGE

1. Introduction

Rice tungro disease or yellow orange leaf disease, an important rice disease in Asia, is caused by two types of viruses, *Rice tungro bacilliform virus* (RTBV) and *Rice tungro spherical virus* (RTSV). Their viral carrier is the green leafhopper, *Nephotettix virescens*. The symptoms and severity of this disease depends on these two types of viral agents. If rice is co-infected by these two types of virus, it will show the typical severe symptoms of yellow-orange leaf discoloration, plant stunting and reduced yield. On the other hand, if rice is infected only with RTBV, it shows milder symptoms [1]. In contrast, rice plants will show no disease if they are infected only with RTSV. For transmission of full-blown tungro disease by green leafhoppers, both types of virus must therefore be present. Transmission of the tungro disease will succeed if rice is infected first by RTSV, followed by RTBV [2,3]. This suggests that RTSV requires some "helper" function(s) from RTBV to show the severe form of the disease.

The structure of RTSV particles is spherical or icosahedral with a diameter of 30-33 nm. Its capsid comprises three coat proteins, namely CP1, CP2, and CP3. Its nucleic acid is a single-stranded polyadenylated RNA molecule of 12,433 nucleotides [4,5,6] and the virus is classified in the genus *Waikavirus*, family *Sequiviridae* [7]. The other virus, RTBV, has a bacilliform structure with width and length of 38 nm x 200 nm. Its nucleic acid is double-stranded DNA and the virus is classified in the genus *Rice tungro bacilliform-like virus*, family *Caulimoviridae* [7].

In Thailand, the first outbreak of rice tungro disease occurred in 1964 [8] and successive outbreaks transmitted by the green leafhopper increased in severity each year. The productivity of the rice crop in Thailand has since decreased by 50-70% of the 1964 yield. Resistance to rice tungro viruses in different countries is not the same and depends on genetic factors associated with the rice tungro virus, rice plants and the green leafhopper. Cloning and analysing rice tungro viral genes in relation to their expression *in planta* and studying possible interaction(s) with plant gene expression is one route to developing transgenic varieties of tungro-resistant rice with improved crop productivity. A comparison of the severity of rice tungro disease in Thailand with other countries showed that, while RTSV causes no disease symptoms in Thailand, it can attack many rice varieties in other countries [1]. A simple method for the detection of rice tungro virus would be useful in disease diagnosis, in studying viral evolution and in analysing viral mutations leading to tungro-resistant rice plants.

This paper describes the isolation and purification of rice tungro virus particles using four different extraction methods and the subsequent determination of virus purity and viral protein molecular weights. The four isolation methods were compared using five assays: virus yield and purity were estimated (i) spectrophotometrically and were determined (ii) by DIBA, size and shape of virus particles were determined by (iii) transmission electron microscopy (TEM), semi-quantitative comparison of protein components and determination of protein molecular weights were analysed by (iv) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by (v) Western blotting.

2. Materials and Methods

2.1. Preparation of Rice Tungro Virus Infected Rice

The eggs of green leafhoppers, *Nephotettix virescens*, were incubated on young rice plants for 3 days until they matured. Subsequently, the insects were fed on rice isolate Chainat 2000, which is infected with rice tungro disease, for 24-48 hours in order to allow the insects to become contaminated with virus. Before these insect vectors were transferred to feed for 24 hours on ten pots of the rice cultivar TN1, these pots were pre-cultivated for three weeks. After four weeks of incubation and infection, infected rice plants showed rice tungro disease symptoms and were used for the isolation and purification of rice tungro viruses.

2.2 Isolation and Purification of Rice Tungro Virus

All four methods are described by Boonnadee et al., [9] and summarized in Figures 1 and 2.

2.3. Estimate of Viral Yield and Viral Purity

The quantities of viruses were estimated by measuring absorbance spectrophotometrically at wavelengths 200-300 nm. Viral purity was estimated by using the ratio between the absorbance values at 260 nm and 280 nm (260/280 ratio).

2.4 Virus Detection by Dot Immunoblot Assay (DIBA) during the Purification Steps

2.4.1 Samples and Controls

The four methods produced the following samples: Method 1-Samples No. 1, 2, 3, 4 and 5; Method 2-Samples No. 1, 2, 3, 4 and 5, Method 3-Samples No.1, 2, 3, and 4, and Method 4-Samples No. 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10. Rice tungro virus-infected rice and uninfected rice were used for positive and negative controls.

2.4.2. DIBA [9]

2µl of all samples were spotted on to nitrocellulose membranes. Cross-sections cut from positive and negative control plant leaves were touched on to the nitrocellulose membranes for 30 seconds to 1 minute to allow transfer of virus particles if present. The nitrocellulose was immersed in phosphate buffer

saline (PBS) pH 7.2, followed separately by immersing in anti-RTBV rabbit antiserum (in-house polyclonal antiserum) at a dilution of 1:1,000 or anti-RTSV rabbit antiserum (in-house polyclonal antiserum) at a dilution of 1:500. Both of which were diluted with 5% skimmed milk in PBS containing 0.05% Tween-20 (PBS-T). Membranes were incubated with gentle shaking at room temperature (RT) for 1 hour. After the nitrocellulose was washed and soaked for 10 minutes with PBS-T for three times, it was incubated in goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma, USA) at a dilution of 1:5,000 for 1 hour at RT. Then, it was washed and soaked in the BCIP/NBT substrate (Sigma, USA) until the pink-violet colour was seen. Afterwards, it was washed with distilled water and blotted dry on tissue paper for viewing.

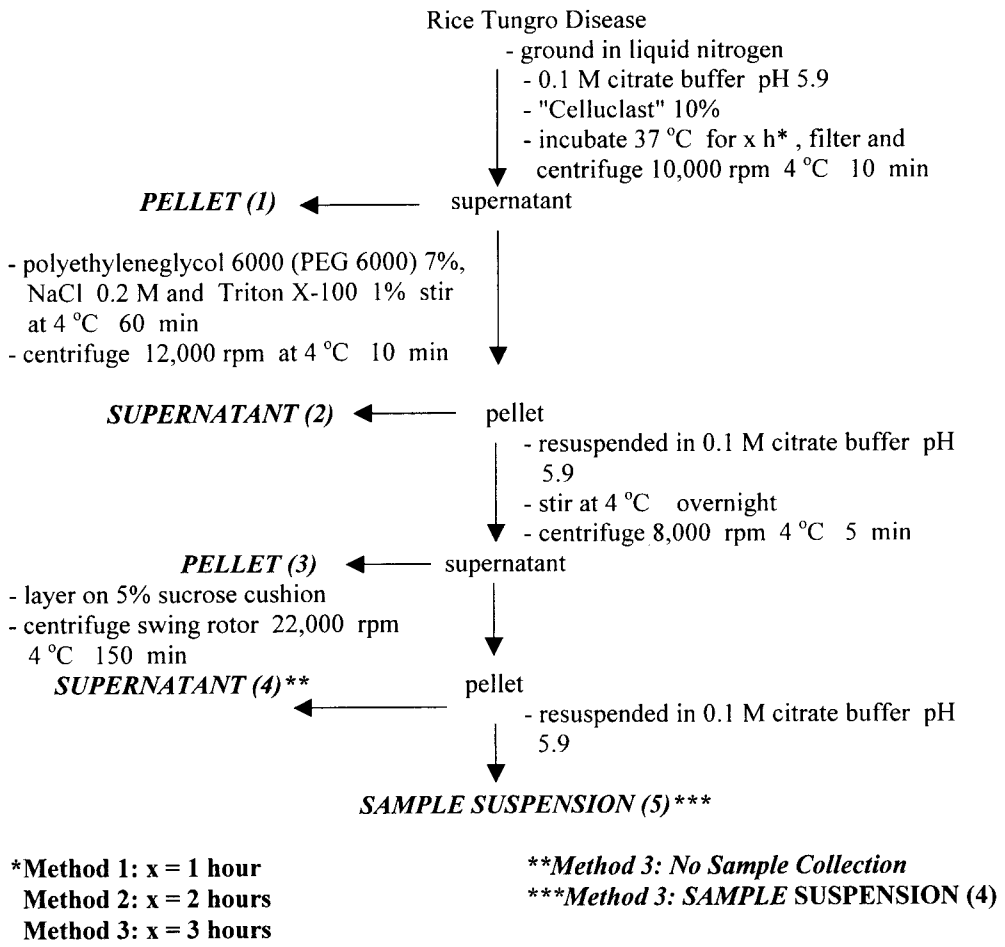


Figure 1. Flow Chart for Isolation and Purification of Rice Tungro Virus Using Methods 1- 3

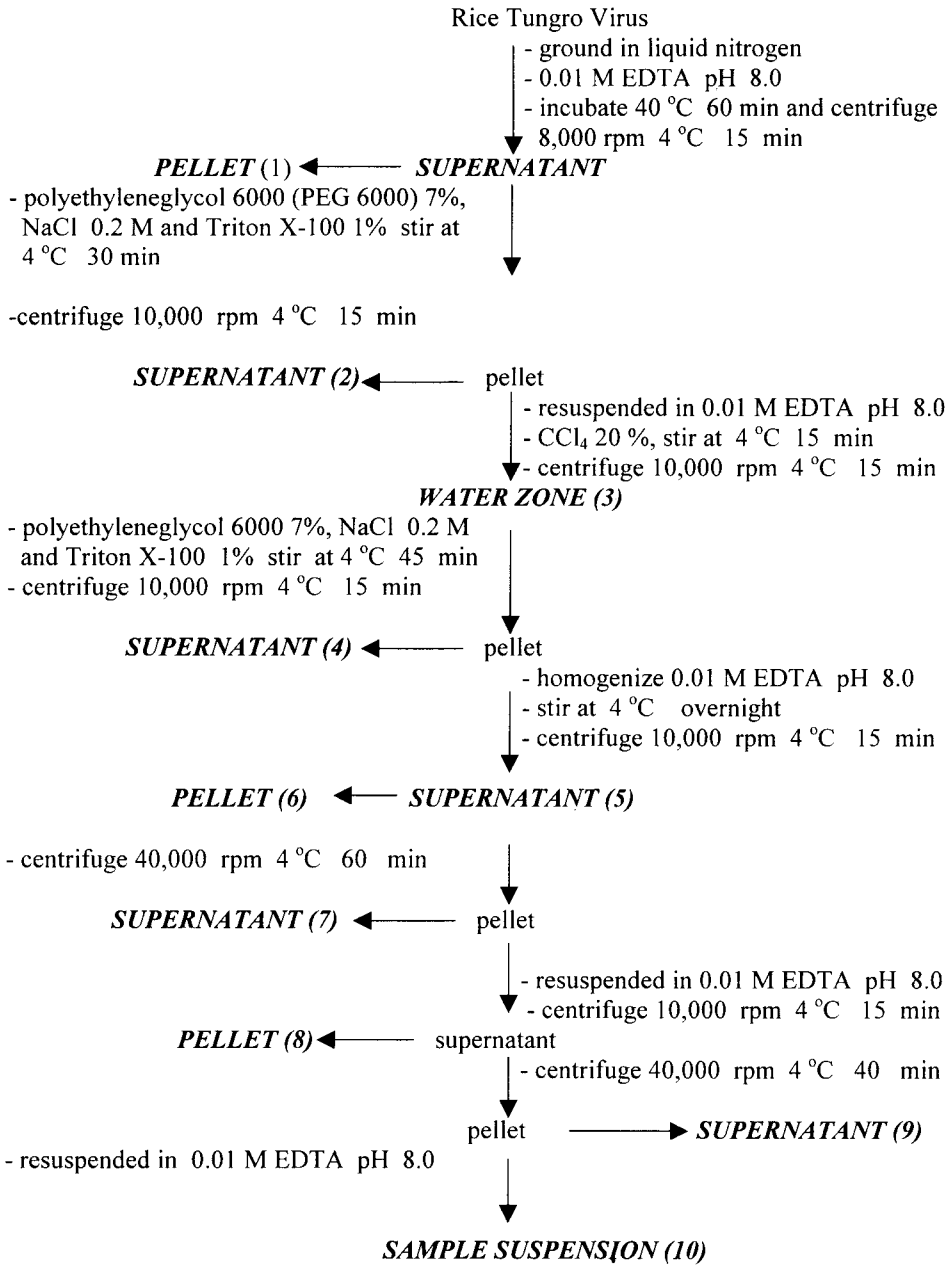


Figure 2. Flow Chart for Isolation and Purification of Rice Tungro Virus Using Method 4

2.5 Determination of Virus Particle Size and Shape by Transmission Electron Microscopy (TEM)

Positive samples from DIBA, Sample No. 5 (Method 1); Sample No. 5 (Method 2); Sample No. 4 (Method 3); and Sample No. 10 (Method 4) were concentrated 10-fold, put on TEM grids and stained as described by Boonnadee et al., [9].

2.6.Semi-Quantitative Comparison of Protein Components and Determination of Protein Molecular Weight by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) [10]

SDS-PAGE was carried out following Boonnadee et al., [9] by running 7.5 µl samples as follows: Lane 1-Method 1-Sample No. 4; Lane 2-Method 2-Sample No. 4; Lane 3-Method 3-Sample No. 3; Lane 4-Method 4-Sample No. 8; Lane 5-Method 1-Sample No. 5; Lane 6-Method 2-Sample No. 5; Lane 7-Method 3-Sample No. 4; and Lane 8-Method 4-Sample No. 10. Pre-stained SDS-PAGE standards (Bio-Rad), which contain proteins of MW range 6.5-205 kDa, were used as markers.

2.7. Semi-Quantitative Comparison of Protein Components and Determination of Protein Molecular Weight by Western Blotting [10]

SDS-PAGE gels were blotted on to Hybond-C membrane following Boonnadee et al., [9]. Proteins on the membrane were probed with anti-RTBV rabbit antiserum (in-house polyclonal antiserum) and anti-RTSV rabbit antiserum (in-house polyclonal antiserum), both of which were diluted with 5% skimmed milk in PBS-T. After washing and soaking for 10 minutes with PBS-T for three times, the membrane was incubated in goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma, USA) at a dilution of 1:5,000 for 1 hour at RT. Then, it was washed and soaked in BCIP/NBT substrate (Sigma, USA) until the pink-violet colour was seen. Afterwards, it was washed with distilled water and blotted dry on tissue paper for viewing.

3. Results

3.1. Preparation of Rice Tungro Virus Infected Rice Plants

Infected rice plants showed tungro disease as seen in Figure 3. Their stalks were darker green and shorter than uninfected plants. Their leaves did not spread out and alternated green and yellow in colour from apex to base.

Figure 3. Infected Rice Plants showing Tungro Disease or Yellow Orange Leaf Disease

3.2. Isolation and Purification of Rice Tungro Virus

Samples were collected at various steps in the four methods outlined in Table 1.

3.3. Estimate of Viral Yield and Viral Purity

All final samples from the four methods, namely Samples No. 5 (Method 1), No. 5 (Method 2), No. 4 (Method 3), and No. 10 (Method 4) were measured spectrophotometrically. Because of the higher viral concentration, a 1/10 dilution of Sample No 10 (Method 4) was used. The estimated quantity and purity of viruses from each sample are shown in Figure 4 and Table 2.

3.4. Virus Detection by DIBA during the Purification Steps

RTBV and RTSV were detected by dot immunoblot assay (DIBA) (Figures 5a-5c). For the anti-RTBV DIBA, Sample 5 (Method 1) and Sample 4 (Method 3) showed the darkest violet signal, indicating the highest RTBV concentration in these samples. For anti-RTSV DIBA, Sample 3 (Method 3) showed the darkest violet signal, again indicating the highest concentration of RTSV in this sample. Moreover, the concentration of RTBV was greater than RTSV with isolation Methods 1 and 3 in Figure 5a.

For Method 2 (Figure 5b) Sample 5 probed both with the anti-RTBV antiserum and the anti-RTSV antiserums showed the darkest violet signal, indicating that the highest amounts, of RTBV and RTSV were present in this sample.

For Method 4, Samples 8 and 10 probed with the anti-RTBV antiserum, were the darkest violet (Figure 5c) and had more RTBV than RTSV, which was similar to the results from Methods 1, and 3 above.

Sample No.	Observations during Sample Preparation			
	Method 1	**Method 2**	**Method 3**	**Method 4**
1	dark green precipitate	dark green precipitate	dark green precipitate	dark green precipitate
2	orange supernatant	orange supernatant	orange supernatant	weak orange supernatant
3	dark brown precipitate	dark brown precipitate	dark brown precipitate	weak yellow supernatant
4	gelatinous supernatant	gelatinous supernatant	cream precipitate	colourless supernatant
5	cream precipitate	cream precipitate	--	brown supernatant
6	--	--	--	red-brown precipitate
7	--	--	--	colourless supernatant
8	--	--	--	cream precipitate
9	--	--	--	colourless supernatant
10	--	--	--	cream precipitate

Table 1. Isolation and Purification of Rice Tungro Virus by Four Methods

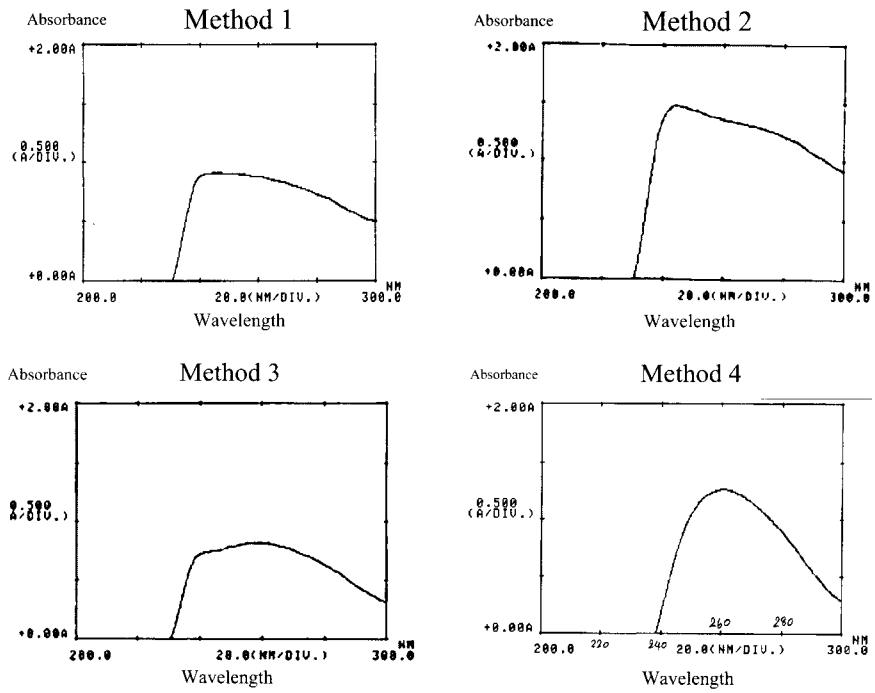


Figure 4. The Quantity of Viruses as Determined Spectrophotometrically Obtained by Methods 1-4

Preparation Method	The Ratio of Absorbance between 260 nm and 280 nm (260/280)
Method 1	0.881 / 0.730 = 1.207
Method 2	1.355 / 1.216 = 1.114
Method 3	0.812 / 0.622 = 1.305
Method 4 (sample diluted 1/10)	1.269 / 0.900 = 1.410

Table 2. The Estimated Purity of Viruses from Methods 1 - 4

Figure 5a DIBA of RTBV and RTSV from Methods 1 and 3

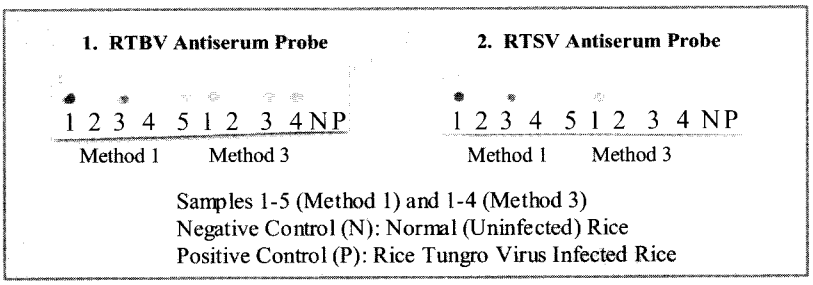


Figure 5b DIBA of RTBV and RTSV from Method 2

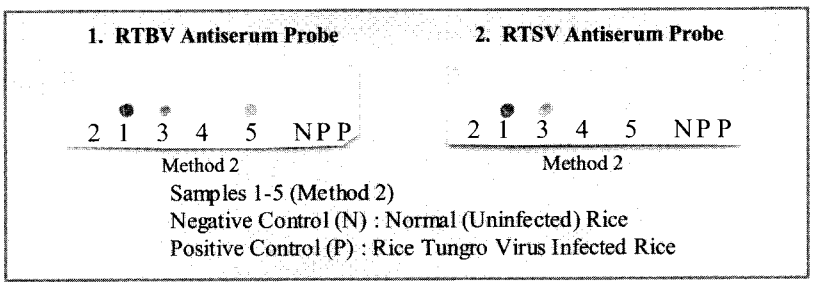
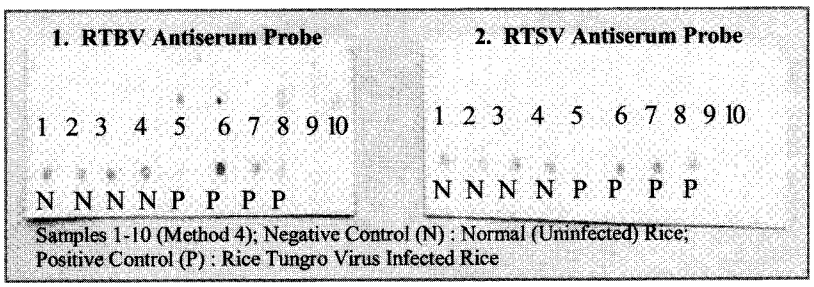


Figure 5c DIBA of RTBV and RTSV from Method 4



Figures 5a.-5c. RTBV and RTSV Were Detected by DIBA

Preparation Method	RTBV		RTSV
	Width (nm)	Length (nm)	Diameter (nm)
Method 1	25.08	143.00	12.22
Method 2	16.35	117.76	9.07
Method 3	20.67	120.00	20.03
Method 4	21.30	137.57	15.03

Table 3. The Average Sizes of RTBV and RTSV Particles from Methods 1 – 4

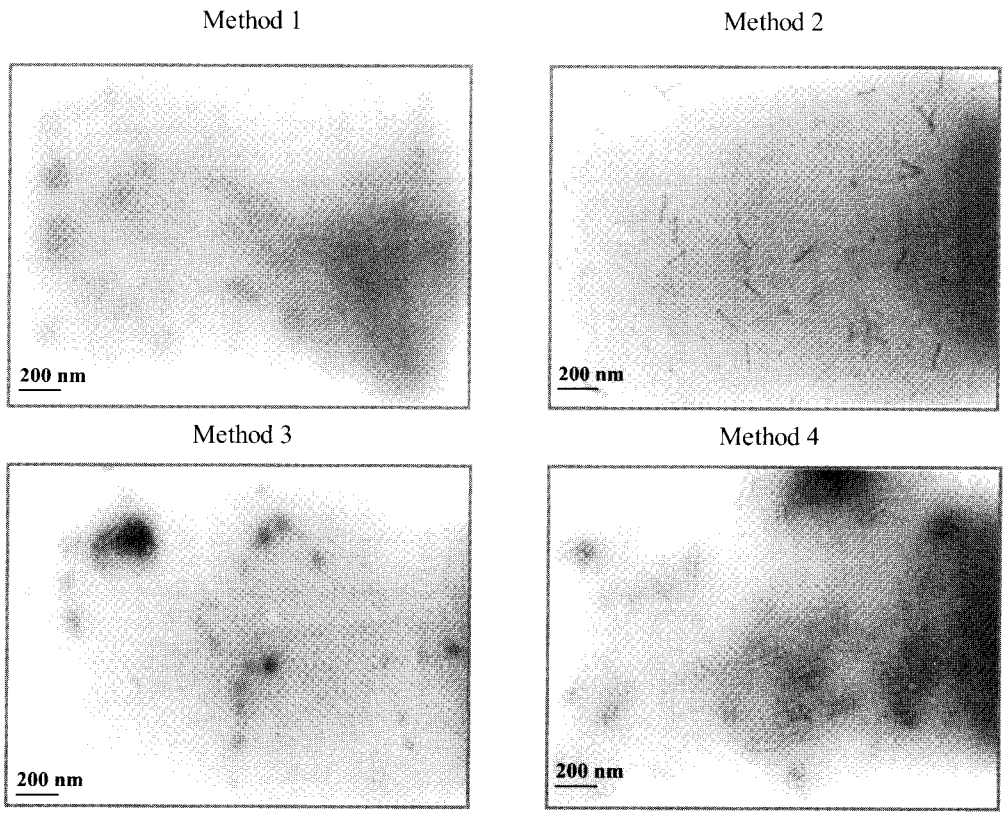
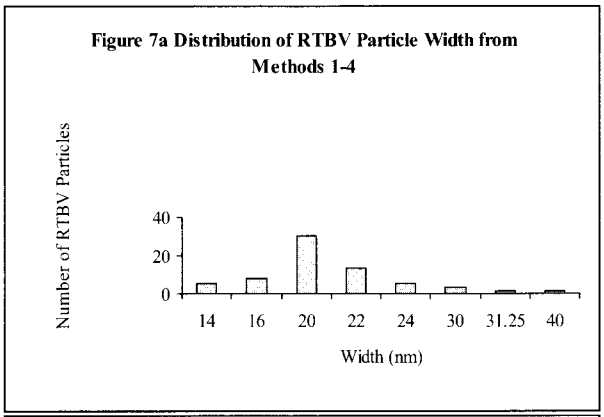


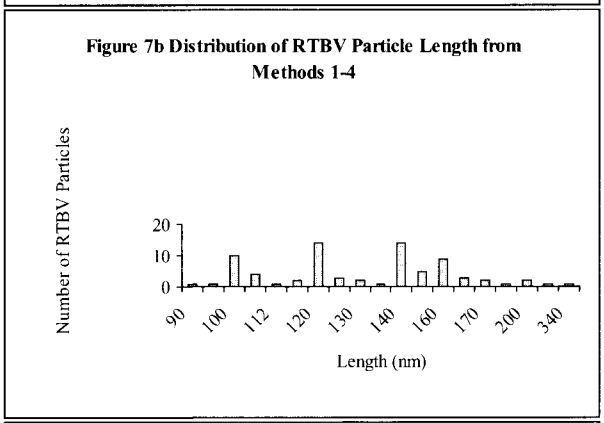
Figure 6 The Shapes of RTBV and RTSV Particles from Methods 1 - 4 as Analysed by Transmission Electron Microscopy (TEM)

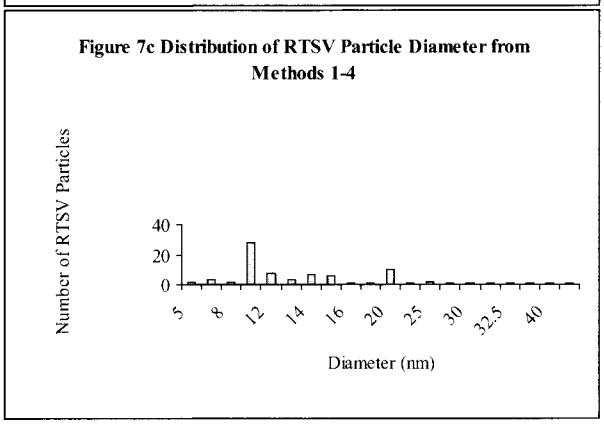
3.5. Determination of Virus Particle Size and Shape by TEM

The average sizes of RTBV and RTSV particles from Methods 1-4 are shown in Table 3 and their shapes are shown in Figure 6.

The size distributions of RTBV and RTSV particles are shown in Figures 7a-7c. The average width and length of RTBV were 21.30 nm and 137.57 nm, respectively. In addition, the average diameter of RTSV was 15.03 nm. These results were different than the results of Hibono et al., [11], which gave an average width and length of 30 nm and 200 nm, respectively, for RTBV and an average diameter of 30 nm for RTSV.





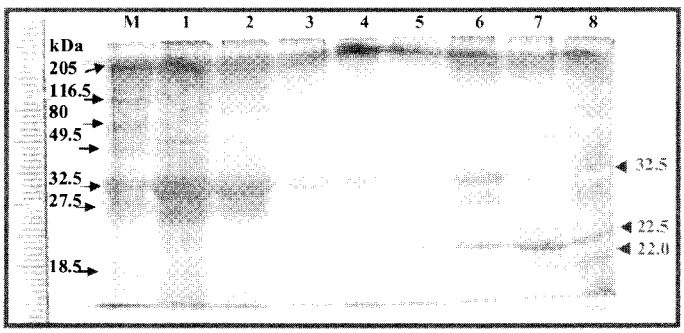


Figures 7a-7c. The Size Distributions of RTBV and RTSV Particles

3.6 Semi-Quantitative Comparison of Protein Components and Determination of Protein Molecular Weight by SDS-PAGE

Samples from the four isolation and purification methods are shown in Figure 8, the final samples from each method as shown in lanes 5 – 8.

Lanes 1, 2, 6, 7, 8 had more than two major bands. Moreover, the more intensely staining bands ran towards the middle of the gel around a size of 30 kDa. There were about eight major proteins in each of lanes 1, 2, 6, 7 and 8, with MW of 18.5, 32.5, 49.5, 205, between 18.5-27.5, 49.5-80.0 and 80.0-116.5 kDa, respectively. Proteins in other lanes had MW of 32.5 kDa (lane 3) and 32.5 kDa and 22.0 kDa (lanes 4 and 5). Many fainter bands were also visible in each lane, which may or may not be virus-derived, so Western blotting was carried out to specifically identify RTBV and RTSV proteins.



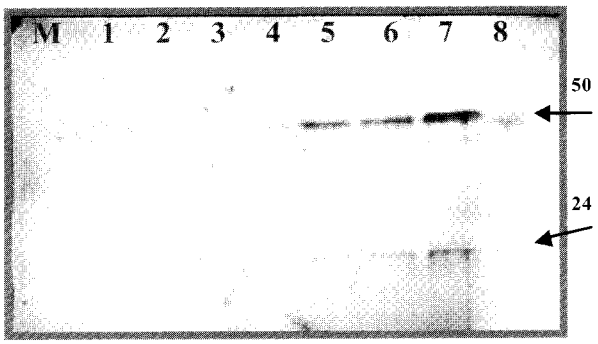
M = pre-stained MW markers

Lane 1-Method 1-Sample No. 4; Lane 2-Method 2-Sample No. 4;
Lane 3-Method 3-Sample No. 3; Lane 4-Method 4-Sample No. 8;
Lane 5-Method 1-Sample No. 5; Lane 6-Method 2-Sample No. 5;
Lane 7-Method 3-Sample No. 4; and Lane 8-Method 4-Sample No10.

Figure 8. Semi-Quantitative Comparison of Protein Components and Determination of Protein Molecular Weights by SDS-PAGE Following Staining with Coomassie Blue

3.7. Semi-Quantitative Comparison of Protein Components and Determination of Protein Molecular Weight by Western Blotting

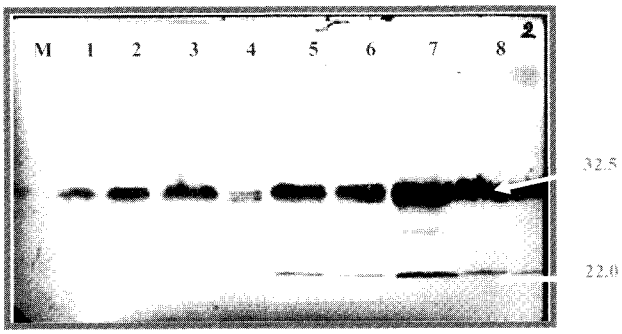
When probing specifically for RTBV proteins (Figure 9), the final samples from each isolation method (Sample No. 5, Method 1-lane 5); Sample No. 5, Method 2-lane 6; Sample No. 4, Method 3-lane 7; and Sample No. 10, Method 4-lane 8), each gave two positive RTBV bands of MW 24 kDa and 50 kDa. On the contrary, Sample No. 8 from Method 4 (Figure 9, lane 4) only gave a single positive band of MW 50.0 kDa. This sample was derived from a pellet two steps prior to the final Sample No. 10 in this method (Figure 2) and therefore shows differential purification of this 50 kDa protein.



M = pre-stained MW markers

Lane 1-Method 1-Sample No. 4; Lane 2-Method 2-Sample No. 4;
Lane 3-Method 3-Sample No. 3; Lane 4-Method 4-Sample No. 8;
Lane 5-Method 1-Sample No. 5; Lane 6-Method 2-Sample No. 5;
Lane 7-Method 3-Sample No. 4; and Lane 8-Method 4-Sample No.10.

Figure 9. Western Blot of Samples Taken during the Isolation and Purification of Rice Tungro Disease Viral Particles Probed with Anti-RTBV Antiserum



M = pre-stained MW markers

Lane 1-Method 1-Sample No. 4; Lane 2-Method 2-Sample No. 4;
Lane 3-Method 3-Sample No. 3; Lane 4-Method 4-Sample No. 8;
Lane 5-Method 1-Sample No. 5; Lane 6-Method 2-Sample No. 5;
Lane 7-Method 3-Sample No. 4; and Lane 8-Method 4-Sample No.10.

Figure 10. Western Blot of Samples Taken during the Isolation and Purification of Rice Tungro Disease Viral Particles Probed with Anti-RTSV Antiserum

Figure 10 shows the detection of RTSV proteins in various samples. Samples in lanes 1- 4 only gave one positive band at 32.5 kDa. On the contrary, samples in lanes 5, 6 and 8 contained RTSV proteins of MW 22.0 kDa and 32.5 kDa. In addition, lane 7 had three specific RTSV bands of MW 22.0, 22.5 and 32.5 kDa.

4. Discussion and Summary

Almost every preparation of rice tungro virus infected rice plants showed severe tungro disease. The isolation and purification of RTBV and RTSV by the four methods described in this work resulted in cream-coloured precipitates. The fourth method, which relied on alternately spinning at high and low speed, gave the highest concentration of virus and the highest purity as estimated by a 260 nm/280 nm ratio of 1.41. It can be concluded that the fourth method was the best extraction system for obtaining pure viruses. On the contrary, the other three methods, in which virus particles were released into the supernatant by digesting plant cell walls with a mixture of cellulases at 37 °C for 1, 2 and 3 hours, respectively, gave less pure virus preparations.

All four purification methods resulted in both RTBV and RTSV as determined by DIBA, but the concentration of RTBV was always higher than RTSV.

From TEM studies, the average width and length of RTBV particles were 21.3 nm and 137.57 nm, respectively. In addition, RTSV particles on average were 15.03 nm in diameter. These results were different from those described by Hibono et al., [11], perhaps because of different characteristics of individual virus strains isolated from different locations.

SDS-PAGE analysis indicated that during the isolation and purification of rice tungro virus several protein bands were visible, some of which were viral in origin and others of which were more likely to be plant-derived. Western blotting was used to identify specific RTBV and RTSV proteins. RTBV had two major proteins of MW 24 kDa and 50 kDa, which are thought to comprise the viral capsid or possibly regulate viral gene expression and were very similar to those described by Hay et al., [12]. These proteins are translated from ORF1 and ORF4 respectively [6,7]. RTSV specific proteins were 22.0, 22.5 and 32.5 kDa in size which were almost identical to the capsid proteins of MW 22.0, 22.5, and 33 kDa described by Druka [5]. These capsid proteins are translated from a small ORF near the 3′ end RNA of RTSV {4,7]. All four methods can therefore isolate and purify both RTBV and RTSV particles.

Western blotting and probing for RTSV proteins showed that Sample No. 4 (Method 1), Sample No. 4 (Method 2), Sample No. 3 (Method 3) and Sample No. 8 (Method 4) all had a specific RTSV protein of MW 32.5 kDa, in addition to being present in the final sample preparations. This differential purification of an RTSV-specific protein at an earlier stage in the purification process suggests that RTSV and RTBV particles may be partially separated at different steps and that this overestimated the amount of RTBV particles compared with RTSV in the final samples. It indicates that none of the methods is optimal for the isolation and purification specifically of RTSV but that all methods still give rise to a mixture of RTBV and RTSV.

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