Techniques

Composition of Northern Cereal Mosaic Virus and its Detection by Enzyme-Linked Immunosorbent Assay with Anti-Nucleocapsid Serum

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ABSTRACT

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Nucleocapsids (Nc) of northern cereal mosaic virus (NCMV), a plant rhabdovirus, were isolated directly from infected wheat leaves by including Triton X-100 before the first high-speed centrifugation, which was followed by sucrose density gradient centrifugation and CsCl equilibrium density gradient centrifugation. Purified Nc had a tubular structure with a diameter of 28 nm and a pitch of 5.5 nm with uranyl acetate staining. Gel electrophoretic analysis revealed that Nc consisted of N protein with MW 47×10^3 daltons and an RNA with MW 3.5×10^6 daltons, whereas intact virions contained in addition two major and one minor polypeptide with MW 63×10^3, 19×10^3, and 88×10^3 daltons, which probably correspond to G, M, and L proteins, respectively. Antiserum produced against purified Nc had a titer of 1/1,024 determined with Ouchterlony gel double diffusion tests. With the enzyme-linked immunosorbent assay, purified Nc was detectable at a concentration as low as 2×10^{-5} $A_{260\ nm}$ units per milliliter, which corresponds to approximately 10 ng/ml. In vivo-Nc was detected from the homogenates of fresh, frozen, and freeze-dried diseased leaves diluted up to 10^{-5} and also from the homogenate of a single viruliferous planthopper, *Laodelphax striatellus*.

Additional key words: insect-borne virus, serological diagnosis, virion composition.

Rhabdoviruses have a unique complex particle structure, i.e., a tubular nucleocapsid (Nc) enclosed by a lipid-bilayer membrane (with projections on its surface) that forms a large bacilliform particle 50–70 nm in diameter and 200–350 nm long (9,18). The virion consists of a single-stranded RNA with negative polarity and three to five species of polypeptides, i.e., envelope-associated G and M proteins and Nc-associated N, NS, and L proteins (6,9,18). Because of these particle complexities in structure and composition, it is rather difficult to purify intact virions in high yield compared with spherical or elongated plant viruses and it has been impossible to prepare a highly specific, high-titered antiserum. To overcome these inherent difficulties in serology of rhabdoviruses, naked Nc could be used as a more appropriate immunogen than the intact virion (9,19).

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Northern cereal mosaic virus (NCMV) is a cytoplasm-associated plant rhabdovirus that is found infecting cereal plants in central to northern areas of Japan, and is transmitted by the planthopper, *Laodelphax striatellus* Fallen (22). In this paper, we describe the composition of the NCMV virion, a simple purification procedure for isolating its Nc directly from the infected leaves, and the application of the anti-Nc serum to enzyme-linked immunosorbent assay (ELISA) to detect NCMV-Nc in infected plant tissues and in a viruliferous planthopper.

MATERIALS AND METHODS

Virus materials and insects. NCMV-infected wheat leaves were collected from a field in Yamagata Prefecture in June 1983. Viruliferous and aviruliferous planthoppers, *L. striatellus* Fallen, were gifts from Y. Mikoshiba, Tohoku National Agricultural Experiment Station, Iwate, and R. Honkura, Miyagi Prefectural Agricultural Center, Miyagi. The insects were reared on wheat plants (*Triticum aestivum* L. 'Fukuho') at 23–25 C in environmental chambers.

Purification of virions. Twenty grams of fresh, infected wheat leaves in 100 ml of 0.1 M tris-HCl, pH 8.0, containing 0.25% 2-mercaptoethanol were homogenized in a Waring blender. The homogenate was filtered through a double-layer of cheesecloth and centrifuged at 2,700 *g* for 10 min. The supernatant was centrifuged at 156,000 *g* for 30 min and the pellet was suspended in 0.1 M tris-HCl, pH 8.0. The suspension was layered onto a 10–40% sucrose density gradient, which was centrifuged at 60,000 *g* for 30 min. A light-scattering zone at the middle of the gradient was removed and centrifuged at 156,000 *g* for 30 min. The pellet was suspended in 0.1 M tris-HCl, pH 8.0, and Celite (Johns-Manville, No. 545) was added to 10% (w/v). The mixture was filtered through a wet Kimwipe compressed into the barrel of a 10-ml syringe. Celite filtration was repeated until the filtrate became turbid white. The filtrate was centrifuged through a 10–40% sucrose density gradient as described above and the light-scattering zone was removed and centrifuged at 156,000 *g* for 30 min. The final pellet was suspended in 1 ml of 10 mM sodium phosphate, pH 7.2.

Purification of Nc. A "minipurification" procedure (17) was adopted as the first step of the purification. One hundred grams of fresh or frozen, infected leaves were homogenized in 250 ml of 0.1 M ammonium citrate, pH 6.5, 0.1% sodium diethyldithiocarbamate, 0.25% 2-mercaptoethanol, and 1% polyvinylpyrrolidone (PVP) (Wako, K-30) in a Waring blender. The homogenate was filtered through a double-layer of cheesecloth and centrifuged at 15,000 *g* for 10 min. After Triton X-100 was added to 2%, the supernatant was centrifuged at 66,000 *g* for 2 hr over 6 ml of 20% sucrose in 0.1 M ammonium citrate, pH 6.5. The resulting pellet was suspended in 0.1 M ammonium citrate, pH 6.5, and centrifuged at 12,000 *g* for 10 min. The supernatant was centrifuged through a 10–40% linear sucrose density gradient in 0.1 M ammonium citrate, pH 6.5, at 60,000 *g* for 2 hr. The gradient was scanned at 254 nm and fractionated with an ISCO model 185 density gradient fractionator and an ISCO UA-5 absorbance monitor. The Nc fractions were centrifuged at 85,000 *g* for 3 hr and suspended in 0.1 M ammonium citrate, pH 6.5. CsCl was added to the suspension to final density of 1.30 g/cm^3 and centrifuged at 130,000 *g* for 20 hr. A single light-scattering band was removed, diluted with 0.1 M ammonium citrate, pH 6.5, and centrifuged at 85,000 *g* for 2 hr. The pellet was suspended in 10 mM sodium phosphate, pH 7.2. The approximate yield was 7 $A_{260\ nm}$ units per 100 g of infected leaves (not corrected for light scattering).

Electrophoretic analysis of proteins and RNA. Proteins were denatured in 2% SDS, 1% 2-mercaptoethanol, 50 mM tris-HCl, pH 8.8, at 100 C for 5 min and electrophoresed in a 10% polyacrylamide vertical slab gel (14 × 10 × 0.2 cm) by using the discontinuous buffer system described by Laemmli (16). The electrophoresis was run at 40 mA constant current for approximately 3 hr. Gels were stained in 0.1% Coomassie Brilliant Blue R (Sigma) in 50% methanol, 7% acetic acid at 60 C for 1 hr, and destained in 50% methanol, 3.5% acetic acid overnight at room temperature. Phosphorylase *a* (molecular weight [MW] 92.5 × 10^3 daltons), bovine serum albumin (66 × 10^3 daltons), aldolase (39 × 10^3 daltons), carbonic anhydrase (28.8 × 10^3 daltons), tobacco mosaic virus (TMV) coat protein (17.5 × 10^3 daltons), and cytochrome *c* (12.3 × 10^3 daltons) were the MW standards. Viral RNA was extracted, denatured, and electrophoresed as described elsewhere (20). Purified preparations were incubated in 2% SDS, 1% 2-mercaptoethanol, 30 mM sodium phosphate, pH 7.2, containing 200 μg bentonite per milliliter at 60 C for 5 min. Protein was extracted three times with phenol saturated with 30 mM sodium phosphate, pH 7.2, containing 0.1% 8-hydroxyquinoline. The aqueous phase was heated at 65 C for 10 min in 2.2 M formaldehyde, pH 7.0. Samples were electrophoresed in a 2% polyacrylamide, 0.5% agarose (Takara, H14) composite vertical slab gel (14 × 10 × 0.2 cm) at 40 mA constant current for 4 hr. Thirty millimolar sodium phosphate, pH 7.2, was used as gel and reservoir buffers. The gels were stained in 0.005% "Stains-all" (Kodak) in 50% formamide in the dark and destained in water under dim light. For the MW determination, denatured RNA was electrophoresed in 1% agarose tube gels (0.6 cm in diameter × 9 cm in length) at 4 mA per tube constant current for 1 hr. TMV RNA (MW 2.19 × 10^6 daltons for the sodium salt) and *Escherichia coli* ribosomal RNAs (1.01 × 10^6 and 0.53 × 10^6 daltons) were used as the MW standards (1,2,12).

Antiserum production. A rabbit was injected intramuscularly with purified nucleocapsid suspension emulsified with Freund's complete adjuvant (Difco) (1:1, v/v) four times at biweekly intervals. Two $A_{260\ nm}$ units were given at each injection. One week after the last injection, the rabbit was bled and the serum was collected by the standard methods. Sodium azide was added to 0.1% as a preservative and the serum was stored at −80 C.

Immunodiffusion tests. Immunodiffusion tests were performed in 0.8% agarose (Takara, H14), 0.15 M sodium chloride, 10 mM sodium phosphate at pH 7.2 (PBS), containing 0.05% sodium azide. Plates were incubated at 25 C overnight.

ELISA. The procedure essentially as described by Clark and Adams (3) was followed. Purified γ-globulin was prepared with ammonium sulfate precipitation, Sephadex G-50 (Pharmacia) column chromatography and DE-52 (Whatman) ion-exchange column chromatography. The partially purified γ-globulin was conjugated with alkaline phosphatase (Sigma, type VII) (1:2, w/w) with 0.05% glutaraldehyde at 22 C for 4 hr. Purified Nc was diluted serially with PBS containing 0.05% Tween-20 (Kao-Atlas) (PBST). Infected tissues or planthoppers were homogenized in and serially diluted with PBST containing 2% PVP (PBST-PVP). All the incubation steps were carried out at 25 C. Polystyrene microtitre plates (Nunc) were coated with 1 μg/ml of γ-globulin in 50 mM sodium carbonate, pH 9.6, for 4 hr. *p*-Nitrophenylphosphate at 1 mg/ml in 10% diethanolamine, pH 9.8, was added to each well and incubated for 1 hr. After stopping the reaction by adding 100 μl of 3 M sodium hydroxide, $A_{405\ nm}$ was measured with a Shimazu MPS 5000 spectrophotometer at 1/10 dilution with water. All the assays were duplicated and the average values were calculated.

RESULTS

Composition of intact virion. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of purified virions revealed three major species of polypeptides with MW 63 × 10^3, 47 × 10^3, and 19 × 10^3 daltons and one minor species of polypeptide with MW 88 × 10^3 daltons, probably corresponding to G, N, M, and L proteins, respectively (Fig. 1A). The MW of RNA (22) (sodium salt) was calculated as 3.5 × 10^6 daltons with 1.0% agarose gel electrophoresis after formaldehyde denaturation (Fig. 1B). This value is rather small compared with those reported for other rhabdoviruses (9,18) and may include some error because of lack of an appropriate MW standard in that vicinity.

Isolation and properties of Nc. After centrifugation, the sucrose density gradient was fractionated into 12 parts from the top of the gradient. An ultraviolet-absorbing peak was located at fraction 7 (Fig. 2A). With SDS-PAGE of proteins from 12 fractions, two species of polypeptides with MW 47 × 10^3 and 45 × 10^3 daltons (Fig. 1A, lane 2) were detected from fractions 2 through 12 with the

maximum amount at fraction 7 (Fig. 2B). These two species of polypeptides were indistinguishable in the SDS-PAGE patterns after the partial digestion with *Staphylococcus aureus* V8 protease (5) (Fig. 3), indicating that the 45×10^3 dalton-protein is a partial degradation product of intact N protein with MW 47×10^3 daltons. With the composite gel electrophoresis of RNA extracted from the 12 fractions, a single species of RNA was detected from fractions 6 through 12 with the maximum amount at fraction 7 (Fig. 2C). Only a smear with lower MW was observed from fractions 2 through 5. Electron microscopic observations of each fraction stained with 2% uranyl acetate (UA) revealed tubular particles with 28 nm in diameter and a pitch of 5.5 nm from fractions 3 through 12 with the maximum amount at fraction 2 (Fig. 4). Tubular particles in fractions 3 through 5 were fragmented into short pieces, whereas those in the remaining fractions were mostly longer than 200 nm. When stained in 2% potassium phosphotungstate (PTA), pH 6.0, no tubular particles nor collapsed structures could be observed from any fraction. From these results, fractions 6 and 7 contained the most intact Nc, while fractions 8 through 12 still had a fairly large amount of intact Nc, probably aggregated end to end and fractions 3 through 5 had randomly fragmented short pieces. For antiserum production, fractions 4 through 11 were gathered and further purified with CsCl equilibrium density gradient centrifugation as described in Materials and Methods.

Immunodiffusion test. The antiserum produced against the purified Nc formed a single precipitation band up to the dilution of 1/1,024 with 0.5 $A_{260\ nm}$ units per milliliter of homologous antigen. Undiluted antiserum gave no reaction with the soilborne wheat mosaic virus partially purified from wheat plants.

Detection of Nc in leaf homogenates by ELISA. Purified Nc serially diluted with PBST could be detected at a concentration as low as 2×10^{-5} $A_{260\ nm}$/ml, corresponding to approximately 10 ng/ml (Fig. 5A). Nc was detectable from the homogenates of fresh, frozen, and freeze-dried infected leaves up to dilution of 10^{-5} (w/v) with PBST-PVP (Fig. 5B). Freeze-dried leaf homogenate always gave a slightly higher (approximately 10%) $A_{405\ nm}$ value than those of fresh or frozen leaf homogenates. None of the healthy fresh, frozen, and freeze-dried leaf homogenates gave reactions. Inclusion of Triton X-100 at homogenization of the tissue did not give any increase in the $A_{405\ nm}$ value (*unpublished*).

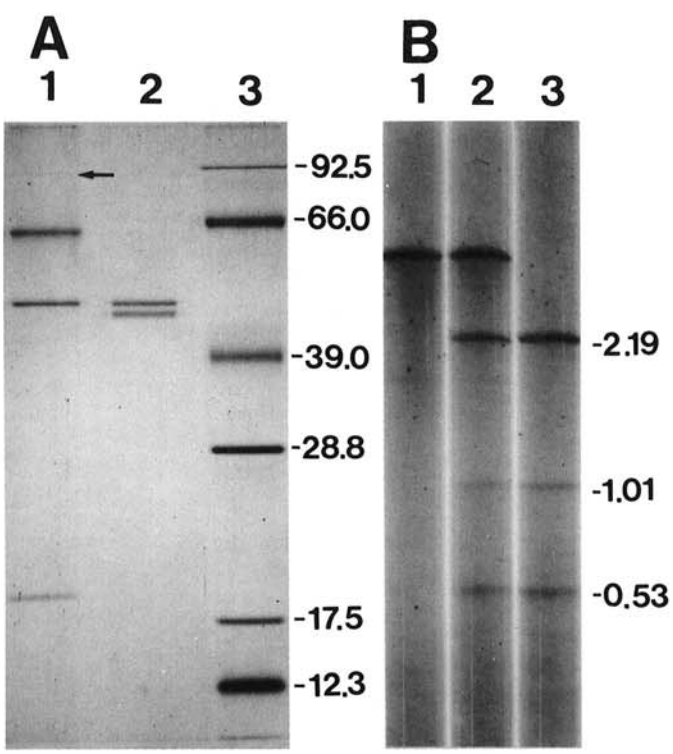

Fig. 1. A, SDS-PAGE pattern of northern cereal mosaic virus virion and nucleocapsid (Nc). (lane 1) Proteins from purified intact virion. Arrow indicates a minor polypeptide with MW 88×10^3 daltons. (lane 2) Proteins from purified Nc. (lane 3) MW standards ($\times 10^{-3}$ daltons) (see Materials and Methods). **B,** Agarose gel electrophoretic pattern of viral RNA denatured with formaldehyde. Lane 1, viral RNA; lane 2, viral RNA with MW standard ($\times 10^6$ daltons) (see Materials and Methods); and lane 3, MW standards only.

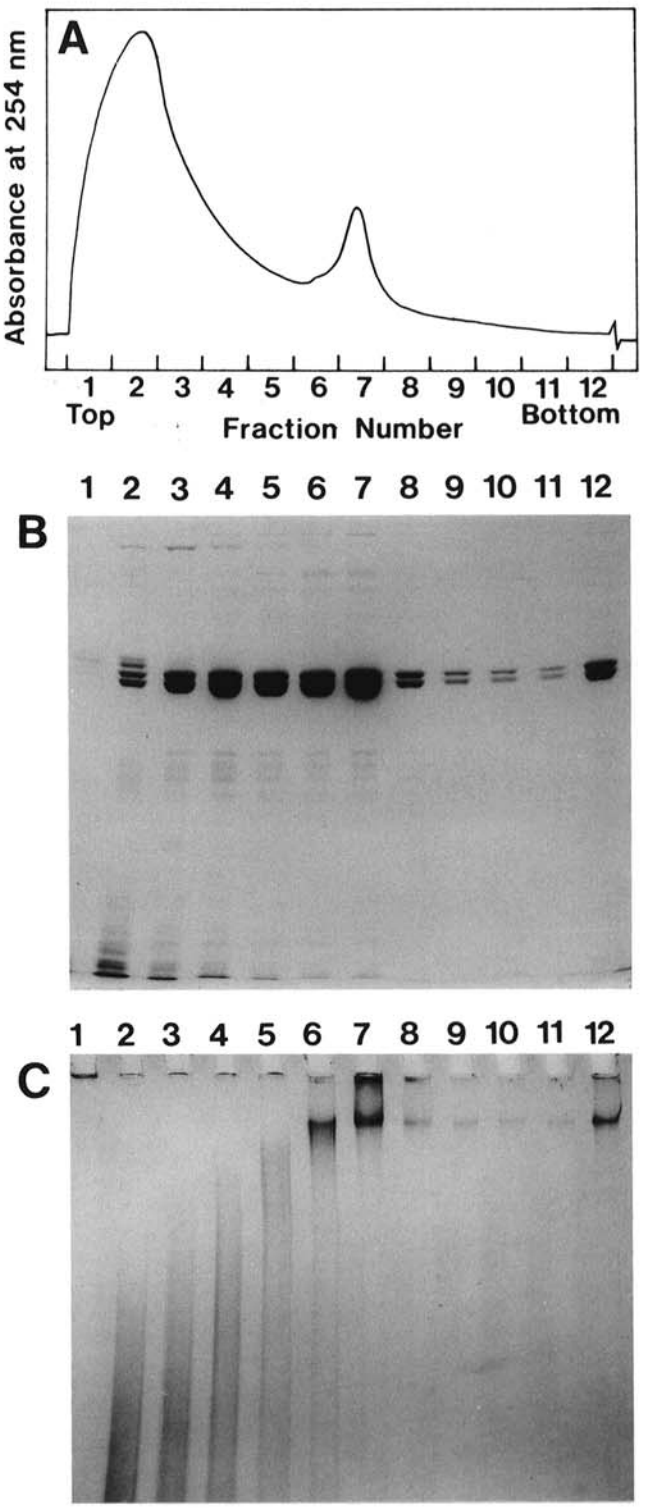

Fig. 2. Sedimentation profile of northern cereal mosaic virus nucleocapsids (Nc) through 10–40% linear sucrose density gradient. **A,** Ultraviolet-absorbing pattern of Nc through the gradient. **B,** SDS-PAGE pattern of protein from 12 fractions from the gradient. **C,** Composite gel electrophoretic pattern of formaldehyde-modified RNA from 12 fractions from the gradient.

Detection of Nc in individual insect homogenates by ELISA. Adult planthoppers (*L. striatellus*) were reared on NCMV-infected or healthy wheat plants. Subsequently, individuals were transferred to healthy wheat seedlings that had been germinated in cheesecloth-covered test tubes for 3 days prior to transfer. After 1 wk of rearing on the seedling, each planthopper was removed and homogenized in 500 μl of PBST-PVP. Each homogenate was assayed by ELISA. Leaves of individual wheat seedlings (approximately 150 mg per seedling) were also removed and homogenized in 2 ml of PBST-PVP and assayed by ELISA. Table 1 shows $A_{405\ nm}$ values of insect homogenates grown on NCMV-infected and healthy wheat plants and their NCMV-transmissibility to healthy wheat seedlings. Of a total of 78 adult planthoppers in two experiments reared on NCMV-infected wheat plants, 40 insects gave strong positive reaction, i.e., 0.66 ± 0.12 $A_{405\ nm}$ in experiment 1 and 0.73 ± 0.14 $A_{405\ nm}$ in experiment 2, seven insects gave weak positive reaction, i.e., 0.28 ± 0.14 in experiment 1 and 0.15 ± 0.01 in experiment 2, and 31 insects gave negative reactions. From all the wheat seedlings fed on by the insects with strong positive reaction, NCMV-infection was readily detected, whereas from those fed on by the insects with weak or negative reactions, NCMV-infection could not be detected. All of 41 insects reared on healthy wheat plants gave negative reactions and also the wheat seedlings fed on by them gave no positive reactions. Thus, ELISA accurately predicted infection of both plants and insects.

DISCUSSION

NCMV-Nc was successfully purified directly from the infected wheat leaves by using a procedure adapted from those used for rabies virus (19) and wheat striate mosaic virus (23). A non-ionic detergent, Triton X-100 (2%, v/v), was included at the first high-speed centrifugation, which was followed by sucrose density gradient centrifugation and CsCl equilibrium density gradient centrifugation. This procedure for Nc isolation is time-efficient and also probably gives a higher yield compared with that from the purified intact virion preparation, for which fairly empirical clarification steps should be included. Although apparently labile to PTA staining under the electron microscope, Nc was shown with UA staining to be quite stable, even tolerant to freezing and thawing treatments that often disrupt intact virion structure.

Intact rhabdovirus preparations are weakly immunogenic and behave as the mixture of several distinct antigens (8,14,21,23) due to the structural instability and complex protein composition. This makes serological diagnosis of rhabdoviruses difficult. However, the naked Nc with simple and stable structure was shown to serve as an appropriate immunogen, producing high-titered, highly specific antiserum. The anti-Nc serum could be effectively used for ELISA

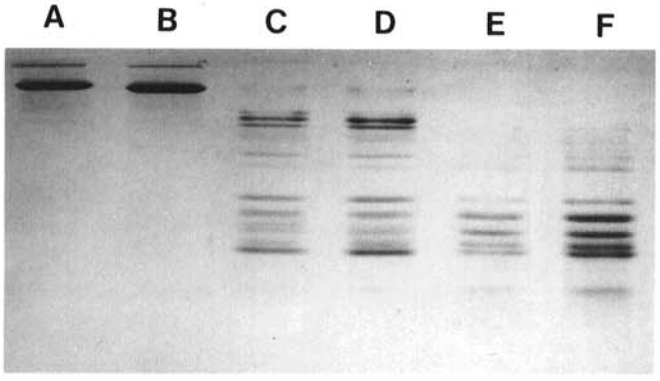

Fig. 3. SDS-PAGE pattern of 47×10^3 dalton- and 45×10^3 dalton-proteins derived from Nc, treated with *Staphylococcus aureus* V8 protease. (lanes A, C, and E) 47×10^3 dalton-protein. (lanes B, D, and F) 45×10^3 dalton-protein. (lanes A and B) Control. (lanes C and D) Treated with 0.05 μg of protease. (lanes E and F) Treated with 0.5 μg of protease.

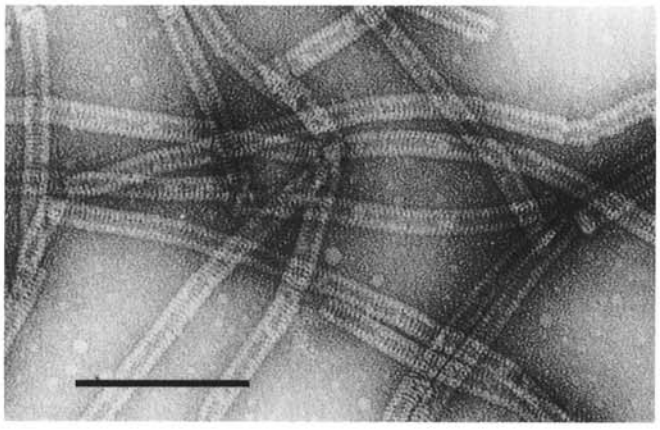

Fig. 4. Electron micrograph of purified Nc stained with 2% uranyl acetate. Bar represents 200 nm.

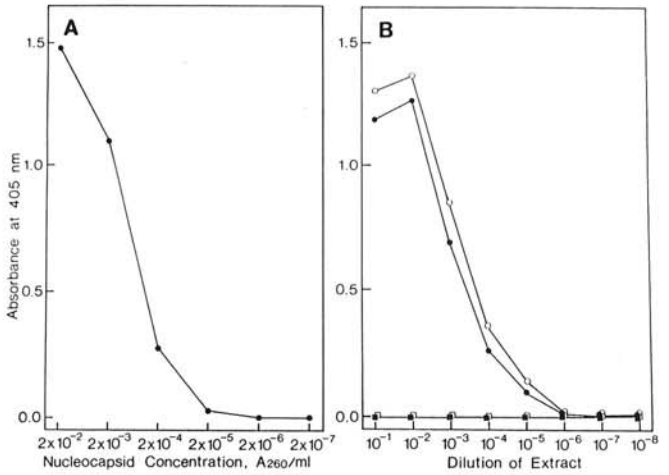

Fig. 5. A, ELISA $A_{405\ nm}$ values for purified Nc serially diluted with PBST. **B,** ELISA $A_{405\ nm}$ values for wheat leaf homogenates serially diluted with PBST-PVP. Homogenates of 1 g of tissue with 9 ml of PBS-PVP was regarded as 10^{-1} dilution. (●——●) Fresh, infected leaves. (o——o) Freeze-dried, infected leaves. (■——■) Fresh, healthy leaves. (□——□) Freeze-dried, healthy leaves.

TABLE 1. $A_{405\ nm}$ values for the homogenates of insects grown on northern cereal mosaic virus (NCMV)-infected and healthy wheat plants and of wheat seedlings fed on by the individual insects

Experiment	Wheat plant fed on by insects[a]	Number of insects[b]	ELISA $A_{405\ nm}$ value: Insect homogenate[c]	ELISA $A_{405\ nm}$ value: Plant homogenate[d]
1	NCMV-infected	26/42	0.66 ±0.12[e]	0.46 ±0.33
		5/42	0.28 ±0.14	0.00 ±0.00
		11/42	0.00 ±0.00	0.00 ±0.00
	Healthy	22/22	0.00 ±0.00	0.00 ±0.00
2	NCMV-infected	14/36	0.73 ±0.14	0.63 ±0.09
		2/36	0.15 ±0.01	0.00 ±0.00
		20/36	0.00 ±0.00	0.00 ±0.00
	Healthy	19/19	0.00 ±0.00	0.00±0.00

[a] Planthoppers were reared on NCMV-infected and healthy wheat plants separately. Only the adult planthoppers were used for experiments.
[b] Ratios of the number of the insects with strong positive, weak positive, and negative reactions to the number of the total insects.
[c] Insects were reared on wheat seedlings individually for 1 wk and homogenized in 500 μl of PBST-PVP. For each insect, assay was duplicated and the average value was calculated.
[d] After a week-rearing of the insects, leaves of individual wheat seedlings were removed and homogenized in 2 ml of PBST-PVP. Assay was duplicated and average value was calculated.
[e] Mean ± standard deviation.

without any modification from the original method (3). The inclusion of Triton X-100 in the homogenates did not increase the $A_{405\ nm}$ value. Thus, the homogenization of the plant tissue or insect bodies in PBST-PVP at room temperature and/or incubation of the homogenates at 25 C overnight seem to be enough for dissociation of the outer viral envelope. The high sensitivity of ELISA also enabled the detection of NCMV-Nc from a single viruliferous planthopper as shown with other insectborne viruses (4,10,11,13). Failure of insects with weak positive ELISA reaction to transmit virus might be explained by the low NCMV-titer in the insect bodies or the absence of virus in the salivary glands.

The procedure for Nc purification from the infected plant materials may be adopted to other plant rhabdoviruses, with the concentration of non-ionic detergent at the first high-speed centrifugation depending on the virus species (6). In addition to the antiserum production and its application to various serological studies, purified Nc could be used for the source of RNA extraction for studying rhabdoviral RNA and for grouping of rhabdoviruses based on the peptide mapping of N proteins (7,15).

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