# Mechanical Transmission, Partial Purification, and Preliminary Chemical Analysis of Barley Yellow Streak Mosaic Virus

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**ABSTRACT**

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Barley yellow streak mosaic virus (BaYSMV), shown previously to be transmitted to barley only by the brown wheat mite, *Petrobia latens*, was transmitted to *Nicotiana benthamiana* and *Chenopodium quinoa* by mechanical inoculation with sap from diseased barley leaves. Transmission of BaYSMV also occurred when leaf sap from diseased *N. benthamiana* was used to mechanically inoculate healthy *N. benthamiana*. No transmission occurred, however, when barley was mechanically inoculated with leaf sap from diseased *N. benthamiana*. BaYSMV was partially purified by centrifugation. Two prominent bands formed in sucrose step gradients at the 30/40 and 40/60% interfaces. Electron microscopy revealed that most virus particles were present in the 40/60% interface band. *N. benthamiana* mechanically inoculated with either band became infected. Preliminary chemical analysis of BaYSMV-enriched preparations showed that they contained unique high molecular weight RNAs (11–13 kb) and proteins of 32 and 100 kDa.

In 1982, a viruslike disease was discovered in a field of malting barley in north central Montana. Affected plants had leaf symptoms of streak mosaic and color banding. Ultrathin section electron microscopy of diseased leaves revealed unusual long filaments with variable lengths of 127 to >4,000 nm and a diameter of about 64 nm. The intracellular filaments were designated as viruslike particles because their shape and size somewhat resembled those of viruses (17). Transmission of the agent causing the disease could not be demonstrated when extracts of diseased barley and wheat leaves were used to mechanically inoculate healthy barley and wheat plants (16). Similarly, no transmission of the agent was detected through barley or wheat seed or through soil (16).

By 1986, the disease had become serious in eight counties of the north central region of the state. Field observations of infestations of the brown wheat mite and negative transmission results with insects led us to consider the possibility of mite involvement with the barley disease. Subsequent greenhouse transmission tests confirmed that both the viruslike particles and the mite were intimately associated with the disease (16). Later electron microscopical and cytochemical findings lent further support to the view that the viruslike particles were particles of a novel virus that caused the barley disease. Thus, the name barley yellow streak mosaic virus and the acronym BYSMV were given to the virus.

The original acronym has since been changed to BaYSMV to distinguish it from barley yellow striate mosaic virus (13). That individual particles were surrounded by a membranelike envelope was determined recently by thin section electron microscopy of BaYSMV in gramineous hosts (18). The internal structure of many particles was beadlike and not striated as that of rhabdoviruses (6). BaYSMV particles were found in the cavities of the endoplasmic reticulum and not in nuclei. This paper reports the transmission of BaYSMV to dicotyledonous plants by mechanical means, the partial purification of the virus by ultracentrifugation, and the preliminary chemical analysis of the partially purified virus preparations.

## MATERIALS AND METHODS

**Isolate.** The BaYSMV used in this study originated from diseased field barley near Valier, MT. The virus was maintained in greenhouse-grown barley (*Hordeum vulgare* L. 'Dicktoo,' 'Kearney,' and 'Klages') via transmission by the brown wheat mite, *Petrobia latens* (Muller). Twenty barley seedlings (four per 15-cm pot, five pots) in the one- to three-leaf stage were exposed to viruliferous mites in each of five rectangular nylon-screened cages (38 × 19 × 18 cm). At the end of every 4- to 5-wk period, diseased barley plants were tapped free of mites, removed from the cages, fumigated for 2 hr with Vapona (dichlorvos) pesticide, and placed in a separate greenhouse. Concurrently, new healthy barley seedlings were placed in the cages with the mites to ensure a continued supply of BaYSMV for experimentation. The natural lighting of both greenhouses used in this work was supplemented with fluorescent light for 16 hr per day. The temperature of the greenhouses varied from 15 to 30 C.

**Mechanical transmission tests.** Inocula were prepared by grinding diseased barley leaf tissue (1 g) in 10 ml of 0.1 M potassium-sodium phosphate buffer (PSPB), pH 7.2, plus 10% (w/v) sucrose with a mortar and pestle. Inocula were applied with cotton swabs to leaves of the dicotyledonous indicator test plants (two- to five-leaf stage) dusted previously with Carborundum. Similar inocula were prepared from healthy barley leaves and rubbed onto control indicator test plants. The plant species and the number of each inoculated were as follows: *Brassica rapa* L. 'Pervirdis,' 40; *Callistephus* sp., 27; *Capsicum annuum* L. 'California green pepper,' 16; *Chenopodium amaranticolor* Coste & Reyn., 20; *C. quinoa* Willd., 36; *Cucumis melo* L. 'Golden Montana,' 22; *Datura stramonium* L., 50; *Gomphrena globosa* L., 21; *Nicotiana benthamiana* Domin, 728; *N. clevelandii* A. Gray, 59; *N. debneyi* Domin, 49; *N. edwardsonii* Edwardson, 76; *N. glutinosa* L., 46; *N. megalosiphon* Heurck & Muell., 69; *N. rustica* L., 88; *N. sylvestris* Speg. & Comes, 8; *Petunia* × *hybrida* Hort. Vilm.-Andr., 49; and *Physalis floridana* Rydb., 120. Each test plant was observed weekly for symptom development during a 3-mo period. Ultrathin sections and extracts of leaves from inoculated diseased and healthy appearing plants and from control plants were prepared for electron microscopy as described previously (17,18). Source plants were sampled daily 3, 5, 7–14, 16, 18, 21, and 28 days after inoculation.

**Partial purification of BaYSMV.** The virus was partially purified from systemically infected leaves of *N. benthamiana* 10–14 days after mechanical inoculation following a procedure derived from Jackson and Christie (8) and Lockhart et al (12). Leaf tissue (50 g) was homogenized in 100 ml of 0.2 M sodium citrate, pH 8.5, and 10% sucrose (w/v) for 2 min and filtered through two layers of cheesecloth. The extract was centrifuged at 5,000 *g* for 15 min. The supernatant was

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layered onto step gradients of 30/40/60% sucrose (w/w) dissolved in 0.1 M sodium citrate, pH 7.2 (5 ml/step), and centrifuged in a SW 28 swinging bucket rotor at 141,000 *g* for 2–3 hr at 4 C. The material at each of the two interfaces (30/40 and 40/60%) was removed and treated separately during the remaining steps of procedure.

First, each fraction was diluted with an equal volume of 0.1 M sodium citrate, pH 7.2, and centrifuged again in the SW 28 rotor at 50,000 *g* for 30 min. Next, the two supernatant fractions were centrifuged in the SW 28 rotor at 141,000 *g* for 1 hr and the resulting pellets (141,000-*g* pellets) were resuspended in 0.1 M Tris-HCl, pH 6.8, or in 0.1 M PSPB, pH 7.2. The two supernatant fractions from the 141,000-*g* centrifugation were subjected to 235,000 *g* for 1 hr in an 80 Ti fixed angle rotor, and the resulting pellets (235,000-*g* pellets) were resuspended in 0.1 M PSPB. The 141,000-*g* and 235,000-*g* resuspended pellets were frozen at −20 C. All steps throughout the partial purification were conducted at 4 C or on ice. Fractions of healthy *N. benthamiana* and *N. benthamiana* infected with BaYSMV obtained during centrifugation were assayed by electron microscopy following the same procedure reported for preparing leaf extracts (17,18).

**Preliminary chemical analysis of BaYSMV-enriched preparations.** BaYSMV particle-enriched fractions of the 40/60% interface of sucrose step gradients were extracted for either nucleic acid or protein and subsequently prepared for electrophoresis. The virus fractions were diluted with an equal volume of 0.1 M sodium citrate, pH 7.2, and centrifuged in a Ti 45 fixed angle rotor at 55,000 *g* for 1 hr. For nucleic acid extraction, the resulting pellets were resuspended in sterile distilled water and 0.5 volume of STE (10 mM Tris, 100 mM sodium chloride, and 1 mM ethylenediaminetetraacetic acid). The virus was disrupted by adding 0.05 volume of 20% sodium dodecyl sulfate (SDS), proteinase K (100 μg/ml) and incubating for 1 hr at 37 C (5). The suspension was then emulsified with an equal volume of phenol and centrifuged 10 min at 8,000 *g*.

The aqueous phase was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with 0.1 volume of 3 M sodium acetate and three volumes of ethanol at −20 C overnight. The mixture was centrifuged at 3,000 *g* for 30 min at 4 C, and the resulting pellet was resuspended in 300 μl of 0.3 M sodium acetate and reprecipitated with 3 volumes of ethanol at −70 C for 15 min. The precipitate was collected by centrifugation (10 min at 4 C) and washed with 70% ethanol, vacuum-dried, resuspended in sterile distilled water, and stored at −70 C (14). Healthy *N. benthamiana* leaf tissue was processed simultaneously in the same manner and stored for parallel controls.

To determine the type of nucleic acid, aliquots from infected and healthy plants were treated with nucleases before electrophoresis on agarose gels. Two to 3 μl of sample was incubated at 37 C with an equal volume of buffer (0.1 M Tris-HCl, pH 7.2, 0.1 M $MgCl_2$, and 1 mM dithioerythritol) and 0.1 μg of RNase A (Sigma Chemical Co., St. Louis, MO) or 200 units of DNase 1 (Pharmacia, Inc., Piscataway, NJ). Brome mosaic virus RNA (a gift from R. C. French) and a 1-kb DNA ladder (Bethesda Research Laboratory, Bethesda, MD) were treated the same way and used as RNA and DNA controls, respectively. A 2-μl mixture of 50% glycerol, 100 mM EDTA, and 0.1% bromo phenol blue was added to each sample and immediately applied to a 1% agarose gel for electrophoresis in 1× TBE (0.089 M Tris, 0.089 M boric acid, and 0.002 M EDTA) running buffer (14).

For protein extraction of the particle-enriched fractions from the 40/60% interface of sucrose density step gradients, the 141,000-*g* and the 235,000-*g* pellets were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11). Samples of the fractions were mixed with sample buffer and heated for 5–10 min at 100 C (7) and electrophoresed on a discontinuous 12% gel at 30 mA for 4 hr. Gels were stained in 0.1% Coomassie Brilliant Blue R250 (Sigma) or in silver stain (Bio-Rad Laboratories, Richmond, CA).

In addition, double-stranded (ds) RNA was extracted from fresh or frozen leaf tissue (3–11 g) of of *N. benthamiana* infected with BaYSMV (15). Frozen leaves of *B. chinensis* infected with turnip yellow mosaic virus (TYMV), *N. benthamiana* infected with tobacco mosaic virus (TMV), and healthy *N. benthamiana* were processed simultaneously for dsRNA controls by the same method. The dsRNAs were analyzed by electrophoresis either in a 1% agarose gel under nondenaturing conditions or in a 5% polyacrylamide gel with TAE (0.04 M Tris, 0.02 M sodium acetate, and 1 mM EDTA, pH 7.2). The gels were stained with ethidium bromide and viewed on a UV light box.

## RESULTS AND DISCUSSION

**Transmission of BaYSMV.** When sap from diseased leaves (Fig. 1A) of mite-inoculated barley plants (Fig. 1B) was used to mechanically inoculate *N. benthamiana* test plants, 624 of 728 (85%) developed symptoms. By comparison, 349 of 390 (89%) *N. benthamiana* inoculated with leaf sap of diseased *N. benthamiana* plants produced the same symptoms. At 5–7 days postinoculation, fully and partially expanded leaves above the inoculated leaves expressed systemic symptoms of irregular chlorotic and necrotic lesions and rings or vein chlorosis and light green mosaics, respectively (Fig. 2).

Symptom severity was usually greatest 14 days postinoculation when leaves displayed a combination of yellow-green mosaic, vein chlorosis, vein banding, puckering, and curling (Fig. 2A). Plant height and leaf size were reduced in diseased plants. This was followed by the formation of small irregular, chlorotic lesions, spots, and rings that enlarged and became whitish (Fig. 2B), and

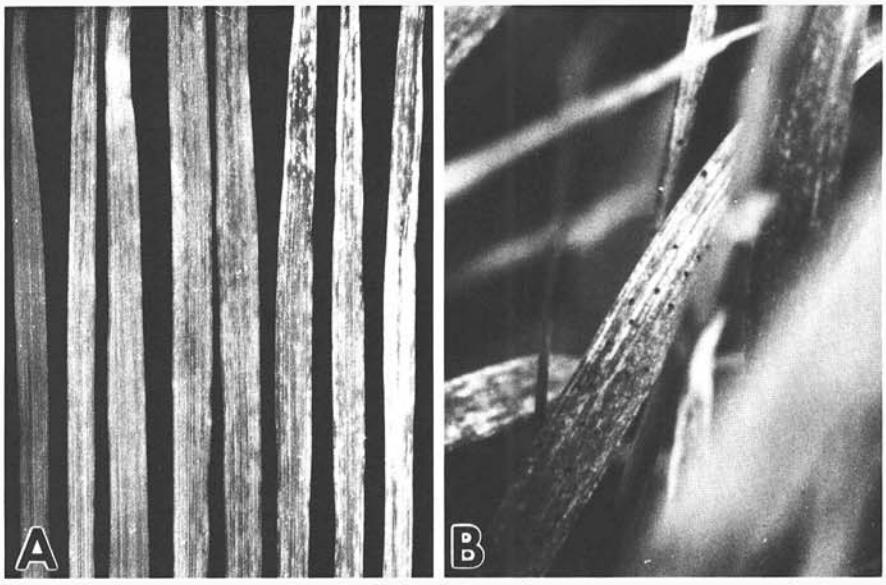


**Fig. 1.** Leaf symptoms caused by the Valier isolate of barley yellow streak mosaic virus in mite-inoculated barley. **(A)** Mosaic symptoms of light green to yellow dashes and streaks. Symptom severity increases from left to right. A healthy control leaf is on the far left. **(B)** Upper surface of a diseased leaf infested by adult brown wheat mites (dark spots). The whitish or silverish dots on the leaf are symptoms of mite feeding.

moderate to severe yellow mosaics (Fig. 2C). By 6–8 wk, postinoculation symptom remission occurred and only faint chlorotic spots or a very mild mosaic were observed.

Electron microscopy of diseased plants (Fig. 3) and partially purified virus preparations suggested that the greatest number of BaYSMV particles were present in systemically infected leaves 7–14 days postinoculation. Nine of 36 plants of *C. quinoa* inoculated with sap from diseased barley developed symptoms only on inoculated leaves. Symptoms consisted mostly of chlorotic lesions (2 mm in diameter, up to 10 per plant). Vein clearing and necrotic lesions occurred on a few leaves. Leaf sap from six of the nine diseased *C. quinoa* plants contained BaYSMV particles. None of the other inoculated plant species expressed symptoms or had detectable BaYSMV particles in their leaf sap.

It was interesting that BaYSMV was transmitted to *N. benthamiana* and *C. quinoa* after mechanical inoculation, especially because mechanical transmission was not achieved earlier with gramineous species (16). Barley, wheat, oats, and other gramineous species tested did not develop symptoms after mechanical inoculation with leaf sap of diseased barley prepared in any commonly used diluent, such as phosphate, citrate, Tris-HCl, or glycine buffer. Similarly, in the present study, barley mechanically inoculated with sap of diseased barley or *N. benthamiana* did not become infected.

The reason for the nonmechanical transmission of BaYSMV to barley and other gramineous species is unknown. However, an analogous transmission phenomenon has been reported for a rhabdovirus that is not mechanically transmissible to its natural gramineous hosts but is mechanically transmissible to a species of tobacco. Rice transitory yellowing virus, infecting only monocotyledonous plants after inoculation by three species of *Neophotettix* leafhoppers, can infect *N. rustica*, a dicotyledonous species, after mechanical inoculation with virus preparations (3). By comparison, BaYSMV infected gramineous species only after inoculation by the brown wheat mite but infected *N. benthamiana* and *C. quinoa* after mechanical inoculation.

Mite transmission of BaYSMV from diseased barley to *N. benthamiana* or from diseased *N. benthamiana* to barley has not been accomplished because the mites do not feed on *N. benthamiana* and die after 2 days of acquisition or transmission access. That the brown wheat mite cotransmits BaYSMV and a second virus seems unlikely because only BaYSMV particles and no other recognizable virus particles have been detected in indicator test plants of mite-inoculated barley or mechanically inoculated *N. benthamiana* or *C. quinoa*.

**Partial purification of BaYSMV.** Two prominent bands formed in the sucrose step gradients prepared from diseased plants—a dark green band at the 30/40% interface, and a light green band at the 40/60% interface. Although the particles had a diameter of about 64 nm, most tended to be shorter than those observed in leaf extracts. Short BaYSMV particles could result from fragmentation of apparently long particles or dissociation of two or more particles aggregated end to end. Occasionally, particles were also seen in the 30/40% interface. When the 40/60 and 30/40% bands were used to mechanically inoculate *N. benthamiana* seedlings, eight of 13 plants and two of three plants, respectively, displayed symptoms and contained BaYSMV particles of the same diameter and variable lengths as those previously observed in gramineous hosts. The 30/40 and 40/60% bands from healthy controls did not contain BaYSMV particles, nor did they cause disease in mechanically inoculated *N. benthamiana*.

**Nucleic acid extractions of BaYSMV preparations.** High molecular weight RNAs (approximately $M_r$ 3.6–4.3 × $10^6$ or 11–13 kb; one major and two minor bands) were detected only in the virus-enriched preparations after specific nuclease digestion and agarose gel electrophoresis of partially purified BaYSMV and healthy controls (40/60% interfaces) under nondenaturing conditions (Fig. 4A). Both BaYSMV-enriched and healthy control preparations contained ribosomal RNA (28S and 18S subunits) (Fig. 4A) and host DNA (Fig. 4B).

Double-stranded RNA was not observed in extracts of tissue infected with BaYSMV but was seen in extracts of tissue infected with TYMV or TMV

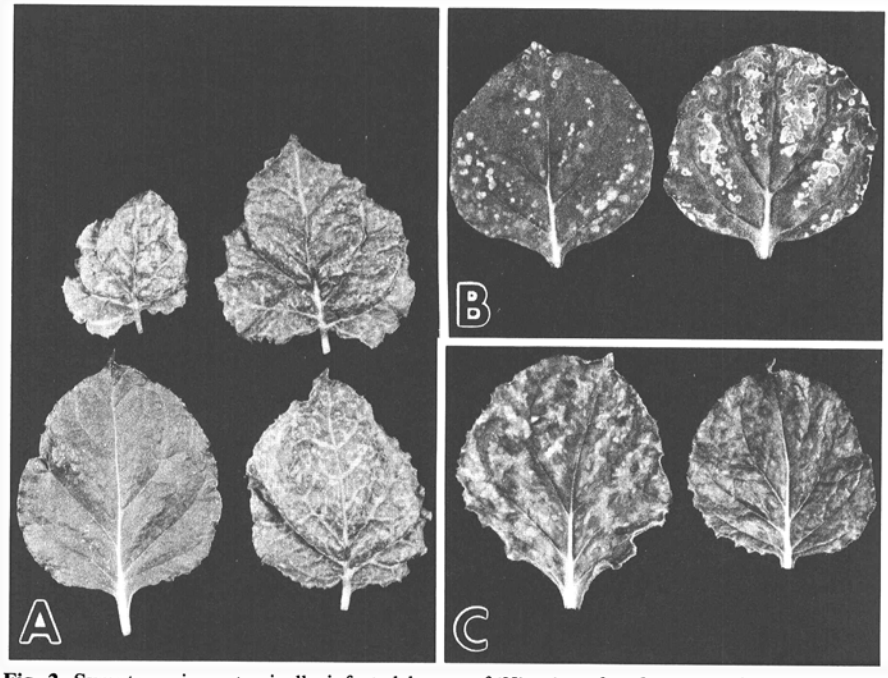


**Fig. 2.** Symptoms in systemically infected leaves of *Nicotiana benthamiana* after mechanical inoculation with barley yellow streak mosaic virus. **(A)** Early symptoms. Yellow green mosaic (upper left), light green mosaic (upper right), and vein chlorosis and vein banding (lower right). Healthy control leaf (lower left). **(B)** Late symptoms. Chlorotic, yellow or white spots and rings (left) and coalescing spots and rings (right). **(C)** Late yellow-green mosaic symptoms, severe (left) and mild (right).

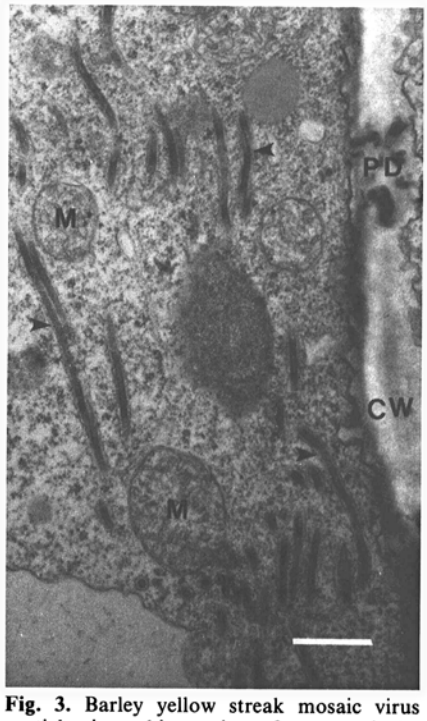


**Fig. 3.** Barley yellow streak mosaic virus particles in a thin section of a systemically infected leaf from a mechanically inoculated plant of *Nicotiana benthamiana*. The virus particles occur in near longitudinal (arrowheads), oblique, and cross-sectional orientations. Also visible are the cell wall (CW), plasmodesmata (PD), and mitochondria (M). Bar = 430 nm.

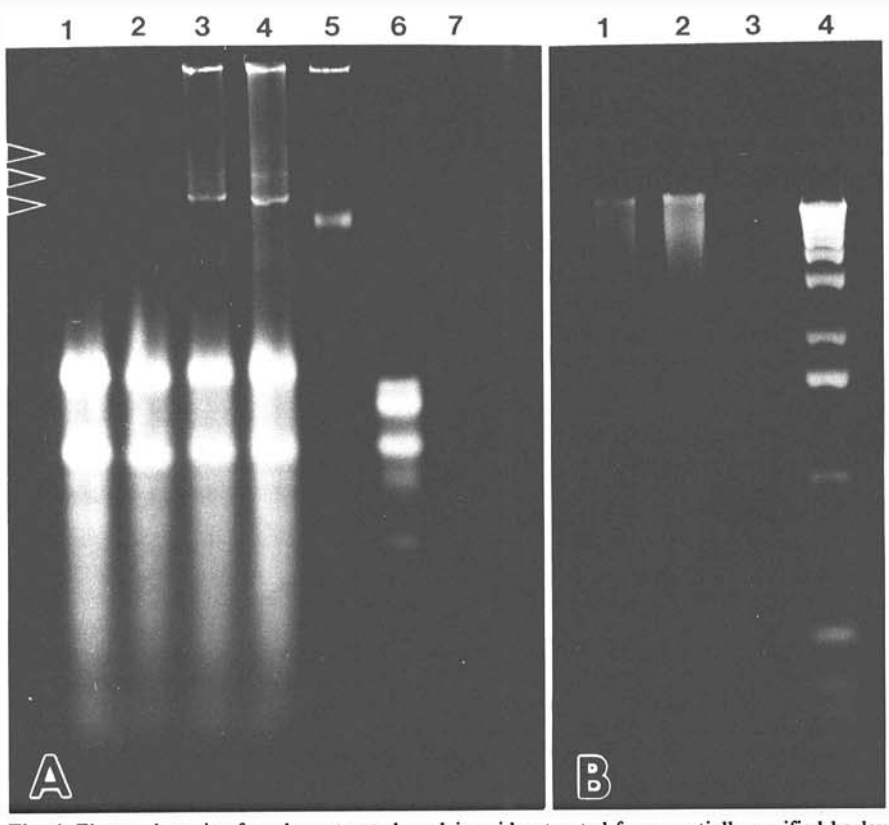


**Fig. 4.** Electrophoresis of nuclease-treated nucleic acid extracted from partially purified barley yellow streak mosaic virus (BaYSMV) from infected *Nicotiana benthamiana* tobacco or from its counterpart in healthy *N. benthamiana* (1% agarose gel under nondenaturing conditions). **(A)** DNAse-treated nucleic acids. Lanes 1 and 2, ribosomal RNAs (28S and 18S) from noninfected tobacco; lanes 3 and 4, unique RNAs (11–13 kb) from partially purified BaYSMV (arrowheads); lane 5, wheat streak mosaic virus RNA (10 kb) marker; lane 6, brome mosaic virus RNA marker with descending molecular weights of 3.2, 2.9, 2.2, and 0.9 kb; and lane 7, 1-kb DNA ladder (BRL). **(B)** RNase treated nucleic acids. Lane 1, DNA from nucleic acid extracted from healthy *N. benthamiana*; lane 2, DNA from partially purified BaYSMV; lane 3, degraded brome mosaic virus; and lane 4, 1-kb DNA ladder.

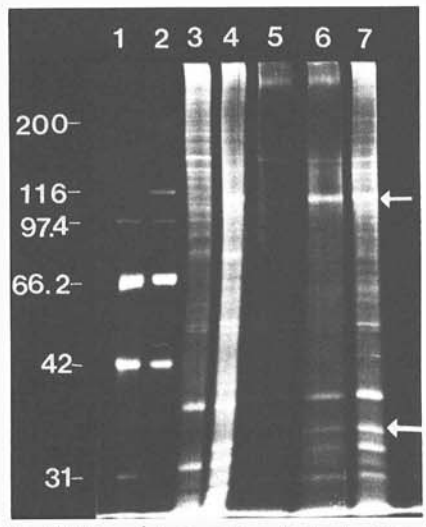


**Fig. 5.** Silver-stained sodium dodecyl sulfate-polyacrylamide gel (12%) electrophoresis profiles of proteins from partially purified barley yellow streak mosaic virus (BaYSMV) or from its counterpart in healthy *Nicotiana benthamiana*. Lanes 1 and 2, molecular weight markers myosin (200 kDa), B-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbuminn (42.7 kDA), and bovine carbonic anhydrase (31 kDa); lane 3, extract from the 141,000-*g* pellet of noninfected tobacco; lane 4, extract from the 141,000-*g* pellet of partially purified BaYSMV; lane 5, extract from 235,000-*g* pellet of uninfected tobacco; lane 6, extract from 235,000-*g* pellet of partially purified BaYSMV; and lane 7, extract of concentrated BaYSMV particles from the 40/60% sucrose interface of step gradients. Arrows indicate two unique protein bands of about 32 and 100 kDa that were obtained from tobacco plants infected with BaYSMV.

under the same conditions of column chromatography and electrophoresis (*data not shown*). Interestingly, dsRNAs have not been detected in tissues infected by the plant rhabdoviruses, which also have enveloped, elongated virus particles.

Assuming that all bands seen on the gels of DNase-treated preparations consisted of RNA and, conversely, that all bands observed on the gels of RNase-treated preparations consisted of DNA, it appears that the nucleic acid of BaYSMV particles is RNA. In addition, nondetection of dsRNA in tissue infected with BaYSMV suggests that the virus particle RNA is not double stranded. However, further RNase and DNase treatment and agarose gel electrophoresis of BaYSMV preparations will be required to confirm the type and strandedness of the nucleic acid of BaYSMV particles. For example, RNase should be used at several different concentrations under low and high salt conditions.

**Protein extraction from BaYSMV preparations.** Two unique protein bands of 32 and 100 kDa were observed consistently by SDS-PAGE (Fig. 5) in preparations from partially purified BaYSMV but not in preparations from controls. Another unique band of about 36 kDa was occasionally observed in preparations from tissue infected with BaYSMV (*data not shown*). It is not known if the three protein bands are three different molecular species or if any of them is composed of lipoprotein. Full characterization of the proteins associated with BaYSMV particles awaits further experimentation.

The results of this work and previous studies (17,18) suggest that BaYSMV is a novel virus having unique particles that do not resemble any of those in presently classified groups or families of plant viruses. Indeed, BaYSMV particles are similar in morphology to those of some unclassified viruses that infect insects (1,2,4,9,10).


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