Purification and serology of barley yellow streak mosaic virus
by Jihad S Skaf

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology
Montana State University
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Abstract:
A new purification procedure has been developed for barley yellow streak mosaic virus (BaYSMV), the causal agent of a disease in barley and wheat. The new procedure utilizes differential and density gradient centrifugation in Percoll gradients and yields highly concentrated virus preparations containing minimal amounts of contaminating host material. These preparations were used to produce polyclonal antibodies so that enzyme-linked immunosorbent assay (ELISA) and immunosorbent electron microscopy (ISEM) tests could be designed for the detection of the virus in barley, wheat, and Nicotiana benthamiana plants as well as in its vector, the brown wheat mite Petrobia latens Muller. These tests allowed us to document the presence of the virus in the Pocatello Valley, Idaho, which was the first report of BaYSMV infecting barley in Idaho and only the second report of the virus occurring in the United States. The concentrated virus preparations were infectious which further suggests that the long filamentous particles associated with the disease are the sole causal agent. It is possible to obtain symptomatic barley plants via the injection of virus extracts into barley leaves using hypodermic needles.

Electron microscopy of negatively stained or detergent-treated virus particles revealed the presence of a finely granular viral nucleocapsid and the existence of a lipid envelope. Internal cross striations characteristic of the rhabdoviruses were not observed. These results, combined with other known biological and morphological characteristics of the virus, led us to propose the extension of the number of enveloped plant virus groups to three. The term barysmovirus group (the barley yellow streak mosaic virus group) has been coined for the newly proposed group.

Our molecular investigation of the viral genome indicated that the virus nucleic acid was not infectious when used to inoculate young tobacco plants. We also verified that the viral nucleic acid consisted of single-stranded (and not double-stranded) RNA that lacked any polyadenylated sequences at the 3' end. However, all efforts to synthesize and clone a cDNA library of the viral genome were unsuccessful.

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of

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APPROVAL

of a thesis submitted by

Jihad S Skaf

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

A new purification procedure has been developed for barley yellow streak mosaic virus (BaYSMV), the causal agent of a disease in barley and wheat. The new procedure utilizes differential and density gradient centrifugation in Percoll gradients and yields highly concentrated virus preparations containing minimal amounts of contaminating host material. These preparations were used to produce polyclonal antibodies so that enzyme-linked immunosorbent assay (ELISA) and immunosorbent electron microscopy (ISEM) tests could be designed for the detection of the virus in barley, wheat, and Nicotiana benthamiana plants as well as in its vector, the brown wheat mite Petrobia latens Muller. These tests allowed us to document the presence of the virus in the Pocatello Valley, Idaho, which was the first report of BaYSMV infecting barley in Idaho and only the second report of the virus occurring in the United States. The concentrated virus preparations were infectious which further suggests that the long filamentous particles associated with the disease are the sole causal agent. It is possible to obtain symptomatic barley plants via the injection of virus extracts into barley leaves using hypodermic needles.

Electron microscopy of negatively stained or detergent-treated virus particles revealed the presence of a finely granular viral nucleocapsid and the existence of a lipid envelope. Internal cross striations characteristic of the rhabdoviruses were not observed. These results, combined with other known biological and morphological characteristics of the virus, led us to propose the extension of the number of enveloped plant virus groups to three. The term barysmovirus group (the barley yellow streak mosaic virus group) has been coined for the newly proposed group.

Our molecular investigation of the viral genome indicated that the virus nucleic acid was not infectious when used to inoculate young tobacco plants. We also verified that the viral nucleic acid consisted of single-stranded (and not double-stranded) RNA that lacked any polyadenylated sequences at the 3′ end. However, all efforts to synthesize and clone a cDNA library of the viral genome were unsuccessful.

INTRODUCTION

Based on all lines of evidence, barley yellow streak mosaic virus (BaYSMV) has been shown to be the causal agent of a disease of barley and wheat (21,22,23,24,25). Until recently, the geographical distribution of the virus was thought to be restricted to the north central and the south central parts of Montana and to southern Alberta, Canada (Carroll, unpublished data).

The virus has a number of unique characteristic features that set it apart from any other known plant virus. The virus is comprised of long filamentous particles of highly unusual dimensions and morphology. The enveloped particles appear to average about 64 nm in diameter and range from 127 nm to an astonishing 4000 nm in length. The virus is transmitted in nature by the brown wheat mite Petrobia latens Muller. Experimentally, BaYSMV is mechanically transmissible from barley or Nicotiana benthamiana to N. benthamiana. Leaves of infected barley plants exhibit a pattern of mosaic and light green to dark yellow streaks, dashes, and stripes that are parallel to leaf veins. An occasional characteristic yellowing of one half of a leaf is also observed (22,23,24).

Routinely, the diagnosis of barley yellow streak mosaic (BaYSM) disease has been made only on the basis of foliar symptom expression and/or thin section electron microscopy

of diseased leaves. However, it has often been difficult to make an accurate identification based on symptom appearance. Moreover, thin section electron microscopy has been time consuming and required special skills and equipment that are not readily available to every research laboratory. The development of a practical and reliable diagnostic method as well as the determination of biological, serological, and molecular characteristics of BaYSMV has been impeded by the lack of a purification procedure that would yield a high concentration of virus particles and a minimal amount of contaminating host material. Thus, this study has been undertaken for the following reasons:

1) to develop a purification procedure for BaYSMV.
2) to produce polyclonal antibodies that could be used to design serological diagnostic tests for BaYSMV, and
3) to further characterize the virus nucleic acid.

LITERATURE REVIEW

The disease

In 1982 plants from a malting barley field in the north central part of Montana showed symptoms suggestive of a virus-like disease that appeared to be different from those known to affect small grain crops in that area (23). These symptoms included leaf mosaic and light green to dark yellow streaks, dashes, and stripes distributed along the parallel axis of leaf veins. In some instances, a characteristic yellowing of only one half of a leaf was observed. The disease was named barley yellow streak mosaic by Robertson and Carroll in 1989 (24).

Disease incidence and severity increased from 1982 to 1989 reaching epidemic levels in six of those years due to drought. Based on the observations of Carroll and Robertson, information from John R. Baringer (Agent and Chair of Pondera County Extension Office), and the personal testimony of farmer Curtis Ries, yield losses have ranged from minimal to 30%. During the 1980's the disease became a source of growing concern, especially in dry land recrop barley.

The pathogen

Electron microscopy of ultrathin sections and crude extracts of symptomatic leaves revealed the presence of highly unusual virus-like particles. The long filamentous

particles appeared to average about 64 nm in diameter and range from 127 nm to an astonishing 4000 nm in length (24). Individual particles seemed to be surrounded by thin (5-10 nm) envelopes of unknown nature and origin. Sometimes, short projections extending from those envelopes and translucent center spaces could be observed (24).

In ultrathin sections, most particles were confined to membrane-bound cytoplasmic cavities(24).

Virus particles were found in leaf, root, and sheath organs and in all cell types of infected plants (24).

The unique size and shape of the particles set this virus apart from any other known plant virus (23). Interestingly enough, the particles seemed to resemble those of some unclassified insect viruses (1,2,5) and human viruses (14). This lack of morphological resemblance to any other known plant virus has allowed some speculation that this virus may belong to a new plant virus group. The pathogen was named barley yellow streak mosaic virus (BaYSMV) by Robertson and Carroll in 1989 (24).

Virus protein and nucleic acid

Partially purified viral preparations were used in a preliminary chemical analysis of the virus. The purification procedure used differential and density gradient centrifugation in sucrose gradients (25).

The viral genome was reported to be composed of two

single stranded RNA segments of apparent molecular weights 11 kb and 13 kb by agarose gel electrophoresis under nondenaturing conditions. Two protein bands of 32 kD and 100 kD were consistently associated with partially purified virus preparations. In addition, a 36 kD band was occasionally observed in virus preparations (25).

Geographical distribution, disease cycle, and host range

In addition to eight counties in north central Montana, barley yellow streak mosaic has been found in the south western part of the state (near Bozeman, MT) and in the south central part of the state (Big Horn county, MT). The disease has also been identified in Alberta, Canada (Carroll, personal communication).

BaYSMV is transmitted in nature by the brown wheat mite Petrobia latens Muller (Acari: Tetranychidae). Although worldwide in distribution (29), the mite is not known to transmit any other plant virus.

Circumstantial evidence indicates that the virus overwinters in and/or on the mite diapausal (white) eggs which are layed on rocks, soil particles, and plant debris late in the summer and early fall. After a dormant period of at least two months and favorable moisture and temperature conditions (15) these eggs hatch producing viruliferous larvae. The larvae develop into mature mites in 7 to 20 days depending on the environmental factors. Hot, dry weather is

conducive to the development of high mite populations. Adult mites live up to a month (7,9,13,16). During the summer the mite produces active (red) eggs. These eggs require about 10 days to hatch. There is also evidence of transovarial passage of the virus through active eggs (Smidansky and Carroll, unpublished data).

BaYSMV has a rather narrow natural host range. Barley (Hordeum vulgare L.), wheat (Triticum aestivum), bristlegrass (Setaria viridis (L.) Beauv.,), barnyard grass (Echinochloa crusgalle Beauv.,), and ryegrass (Lolium persicum Boiss. and Hohen.) were found to be susceptible to the virus (24). Experimentally, BaYSMV is mechanically transmissible from its natural hosts or Nicotiana benthamiana L. to N. benthamiana. There have been no reports of successful mechanical transmission of BaYSMV to barley.

Disease control

To date, there has been no effective pesticide that can provide season-long control of the brown wheat mite in dry land barley. Best results were obtained when granular Di-Syston 15G was used at planting time (11). Foliar sprays of the same pesticide resulted in good mite control for two weeks posttreatment after which the mite numbers began to increase significantly. In irrigated barley fields the reduction in mite numbers has been attributed to irrigation and not to foliar pesticide application (11). Wet conditions

from heavy rain or irrigation are not conducive to the development of large mite populations. However, heavy and frequent irrigation is not always possible in malting barley growing areas.

Crop rotation may also help reduce the mite population as well as virus reservoirs since disease incidence and severity are highest in dry land recrop barley. Unfortunately, it is not always a viable option in most areas where barley is grown.

Currently, screening experiments for resistance to BaYSMV are underway but so far no resistant barley or wheat cultivars have been found (Carroll, Personal communication). However, two barley introductions, Haua (CIho 734) and Skinless (CIho 1032) screened via mite inoculation of BaYSMV, were found to have some tolerance to the disease (Carroll et al, unpublished data).

MATERIALS AND METHODS

Virus and plants

The BaYSMV isolate used in all experiments was recovered from viruliferous brown wheat mites obtained from an infected barley field near Valier, MT. The isolate has been maintained continuously in the barley cultivar Klages by serial mite transfers in the greenhouse. Tobacco (N. benthamiana) plants were mechanically inoculated with the virus when they had 4 to 6 partially expanded leaves. All plants were grown in the greenhouse where the temperature averaged 24° C. Natural lighting was supplemented with fluorescent (10,000 Lux illumination) lights for 16 hr./day.

Healthy barley and tobacco plants were grown as negative controls under the same conditions.

Mechanical inoculation of tobacco plants

Carborundum-dusted tobacco leaves were mechanically inoculated using leaf extracts obtained from symptomatic barley and/or tobacco plants. The leaves were ground with 100 mM NaH_2PO_4/Na_2HPO_4 phosphate buffer pH 7.2 + 10% sucrose (W/V) in an ice-cold mortar and pestle. Cotton swabs were used to apply the extract onto the upper epidermis of the leaves. Plants were then kept in the greenhouse for 2-3 weeks for symptom observation.

Inoculation of barley by injection

Sterile disposable syringes and hypodermic needles (27 G 1/2 Tuberculin, Becton-Dickinson, Rutherford, NJ) were used to inject leaf extract from barley and tobacco into barley plants at the 2-3 leaf stage. Inoculum was prepared as described previously for mechanical inoculation except that it was filtered through two layers of Miracloth (Calibochem, La Jolla, CA). About 500 ul of inoculum was injected into leaf veins with an average of 5 to 7 inoculation points on each plant.

Virus purification

Most experiments were conducted using N. benthamiana plants 15-20 days after mechanical inoculation. The buffer used in all experiments (unless otherwise indicated) was 100 mM phosphate buffer pH 7.2. The leaves were homogenized in a blender using three volumes of cold buffer plus 10% (W/V) sucrose. The homogenate was filtered through four layers of cheesecloth and a layer of Miracloth before the addition of 5% (W/V) activated charcoal (Darco G 60, MCB, Cincinnati, OH). After stirring for 20 min at 4° C, the mixture was filtered again as described above and centrifuged at 3,000 g for 10 minutes in a Sorvall SS-34 or GSA rotor. In all the following steps temperature was maintained at 4° C. The supernatant liquid was then centrifuged in the same rotor at 27,000 g for 30 min. The resulting dark green pellet was

resuspended in 1/10th of the original volume of the extraction buffer and vacuum filtered through a 47 mm disc of Extra Thick Glass Fiber (Gelman Sciences, Ann Arbor, MI). Eighteen ml of the filtrate was then layered on top of a 5 ml phosphate-buffered 35% (W/W) sucrose cushion and centrifuged at 100,000 g for 30 min in a Beckman Type 30 rotor. Pellets were resuspended in 1/30th of the original volume and vacuum filtered again through a glass fiber filter disc. Five ml of the filtrate (extracted from 50 g green tissue) were thoroughly mixed with 7.5 ml of 100% Percoll (Pharmacia, Upsala, Sweden) and centrifuged at 100,000 g for 20 min in a Beckman 80 Ti rotor. The resulting gradient was fractionated using a Density Gradient Fractionator (ISCO, Lincoln, NE), and fractions enriched with virus particles (as determined by electron microscopy) were pooled, diluted with buffer, layered on top of 5 ml of a 50% (W/W) sucrose cushion, and centrifuged at 100,000 g for 1 hour. The interface was collected, diluted with buffer, and centrifuged at 100,000 g for 30 min. The final pellet, which appeared clear to pale green, was resuspended in 1/300th of the original volume and used in later experiments.

The buoyant density of the virus in Percoll was determined using Pharmacia's density marker beads.

Tissues obtained from healthy plants were processed in the same manner and used as controls.

Treatment of purified BaYSMV with detergents

To determine the nature of the envelope that was believed to surround BaYSMV particles, purified virus preparations were incubated with either Triton X-100 (nonionic detergent) or sodium dodecyl sulfate (SDS) (anionic detergenet) (Sigma, St. Louis, MO). The detergent concentrations used were 0.5% and 1% and the incubations were done at room temperature for 5 min.

Electron microscopy

Formvar coated grids were prepared as described by Davison and Colquhoun, 1985 (6), by placing 15 ul of 0.5% Formvar (Ted Pella, Inc., Redding, CA) in 1,2-dichloroethane onto the surface of clean, double-distilled water. Ethanol-cleaned copper grids 300 mesh were placed on the central region of the almost invisible film and picked up with a clean glass slide. Grids were then allowed to dry at room temperature in a dust-free environment.

Specimens were prepared for electron microscopy by floating formvar coated copper grids on a drop of leaf extract for 5 min. The grids were then rinsed with a few drops of distilled water and stained by floating them for one min on a drop of 4% uranyl acetate (UA) in distilled water. After a final rinse, grids were dried in a Petri plate at room temperature and examined using a Zeiss EM 10 CA electron microscope.

Besides the UA staining described above, 2% phosphotungstic acid (PTA) in water was also used to observe the internal structure of virus particles.

Measurements of virus particles were taken from prints using a Zeiss Interactive Digital Analytical System (ZIDAS).

Antisera production and evaluation

Polyclonal antibodies against BaYSMV were produced in two New Zealand White rabbits. Each animal received four subcutaneous injections administered at biweekly intervals. Each injection contained 0.5 ml purified virus obtained from 50 g of green tobacco leaves. Prior to the first injection, the virus suspension was emulsified in an equal volume of Freund's Complete Adjuvant (GIBCO, Grand Island, NY). Freund's Incomplete Adjuvant was used in subsequent injections. Rabbits were bled 10 and 20 days after the third and fourth injections and the titer was determined using the Ouchterlony double immunodiffusion test as described by Shepard, 1970 (27).

Absorption of the antisera with concentrated healthy host protein

The procedure described by Shepard, 1970 (27) was followed except that the 27,000 g pellet obtained from healthy tobacco tissues using the purification procedure described above was used instead of crude homogenate. That

pellet was resuspended in the purification buffer for several hours and then mixed with an equal volume of chloroform. The emulsion was then centrifuged at 10,000 g for 5 min and the aqueous phase was centrifuged at 100,000 g for one hour. The resulting pellet was resuspended overnight in 1/10th of the original volume in 50 mM Tris-HCl pH 7.2 containing 0.85% NaCl and 10 mM magnesium acetate. To determine the optimal proportions of healthy host proteins necessary to remove all host reactive antibodies from 1 ml of antiserum, serial two-fold dilutions of host protein preparations were made and thoroughly mixed with equal volumes of antiserum and incubated overnight at 4° C. Mixtures were then centrifuged at 3,000 g for 10 min to remove any precipitate and each dilution was tested in double immunodiffusion tests against host proteins for the presence of residual activity. A large scale cross absorption was done after the determination of the appropriate levels of host antigen required. Cross absorbed antisera were then frozen at -20° C until needed.

Immunoglobulin G (IgG) was purified from one of the two absorbed antisera with a protein A column using Immunopure IgG Purification Kit (Pierce, Rockford, IL). Purified IgG was conjugated to alkaline phosphatase (Type VII-S, Sigma, St. Louis, MO) as described by Clark and Adams, 1977 (4).

Direct double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA)

DAS-ELISA was generally done according to the standard protocol described by Clark and Adams, 1977 (4). Several antigen extraction buffers were compared. These buffers included: phosphate-buffered saline (PBS), PBS + 0.05% Tween-20, PBS + 5% dried nonfat milk, PBS + 2% polyvinylpyrrolidone (PVP), and PBS + 5% bovine serum albumin (BSA).

Polystyrene plates (Dynatech immulon II, Dynatech Laboratories, Alexandria, VA) were coated with 200 ul of the IgG preparation SC450 (concentration 1 ug/ml) diluted in a coating buffer pH 9.6 (4) and incubated for 4 hours at 37° C. After incubation, wells were rinsed three times with PBS-Tween-20 (PBST) pH 7.4 and twice with distilled water at three minute intervals. Two hundred ul of sample antigen extracts (at a dilution of 1:10 W/V) were incubated overnight at 4° C or at room temperature. After further rinsing, 200 ul of alkaline-phosphatase-labeled IgG diluted 1:2000 in conjugate buffer were added to the plates and incubated at 37° C for 3 hours. After rinsing, 250 ul of 1 mg/ml p-nitrophenyl phosphate were added to the wells and incubated at room temperature for 1 hour. The extent of virus-antibody reaction was evaluated by measuring the absorbance at 405 nm (A_{405}) using a Minireader II

spectrophotometer (Dynatech Laboratories Inc., Alexandria, VA).

Indirect ELISA (I-ELISA)

Two hundred ul of sample antigen extracts were placed in the wells and incubated overnight at 4° C or at room temperature. Wells were then rinsed as described previously and 200 ul of SC450 (diluted 1:500 in coating buffer) were added and incubated at 37° C for 4 hours. Thereafter, wells were rinsed and 200 ul of 1:1000 goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO) were added and incubated at 37° C for 2 hours. Two hundred ul of enzyme substrate were added after final rinsing and the reaction was evaluated colorimetrically as for direct ELISA.

In all direct and indirect ELISA tests the following controls were incubated in each plate: i) extracts from virus-infected tissues as positive controls, ii) extracts from healthy tissues as negative controls (duplicated in 10 wells), iii) the extraction buffer (in duplicate wells), and iv) extracts from plants infected with barley stripe mosaic virus (BSMV), wheat streak mosaic virus (WSMV), a Montana isolate of barley yellow dwarf virus (BYDV-MT-RMV) all in duplicate wells.

Only samples with (A_{405}) values greater than three times the absorbance of healthy controls were considered positive.

Immunosorbent electron microscopy (ISEM)

The protocol used was based on that described by Derrick and Brlansky in 1976 (8). Formvar-coated grids are floated on a drop of SC450 IgG (diluted 1:500) for 30 min at room temperature. IgG-coated grids were then rinsed by floating them three times on drops of buffer (50 mM Tris-HCl pH 7.2) and three times on drops of distilled water. Grids were then floated on plant extracts for 30 min and then washed again as described above. The grids were stained by floating them on drops of a 5% uranyl acetate in water for 1 min, washed, air-dried, and visualized by electron microscopy as described earlier.

Field inspection

In July 1991, two barley plants showing yellow green mosaic, streaking, and severe necrosis were sent to the Virology Laboratory at Montana State University by Dr. Robert L. Forster (Extension Plant Pathologist, University of Idaho, Research and Extension Center, Kimberly, ID). These samples were collected from the Pocatello Valley, Oneida County, Idaho near the Utah border. These samples were processed to determine whether or not BaYSMV was the causal agent by using the antiserum SC450 and immunosorbent electron microscopy (ISEM). The ISEM results were confirmed by thin section electron microscopy done by Susan K.

Brumfield (Plant Virology Laboratory, Montana State University, Bozeman, MT).

In July 1992, 125 barley plants exhibiting symptoms characteristic of BaYSMV infection were collected from the same valley and processed as described for 1991 samples.

Extraction of viral nucleic acid

All glassware, plasticware, and solutions were treated with diethylpyrocarbonate (DEPC) as described by Sambrook et al. 1989 (26).

Both the final pellet and the 27,000 g pellet obtained from the purification procedure described previously were used to extract viral nucleic acid. Both pellets were resuspended in 10 mM Tris-HCl pH 7.8, 1 mM EDTA and SDS and Proteinase K (Sigma, St. Louis, MO) were added to final concentrations of 0.5% (W/V) and 100 ug/ml, respectively. The suspension was incubated at 50° C for 1 hour before extracting it three times with phenol:chloroform: isoamylalcohol (25:24:1) and once with chloroform. Each extraction was followed by a centrifugation step at 12,000 g for 10 min. The chloroform extracted supernatant fluid was precipitated with 1/10th volume of 3 M sodium acetate pH 5.5 and 2.5 volumes 95% ethanol at -70° C overnight. After centrifugation (12,000 g for 20 min at 4° C) the resulting pellet was washed three times with 3 M sodium acetate pH 5.5 and centrifuged at 12,000 g for 10 min. The final pellet

was rinsed once with 70% ethanol, vacuum dried, resuspended in DEPC-treated water, and stored at -70° until needed.

To confirm the viral nucleic acid type reported earlier by Robertson and Carroll (25), nucleic acid preparations were treated with either RQ-1 RNase-free DNase (Promega Corporation, Madison, WI) for 20 min at 37° C or with DNase-free RNase (Boehringer Mannheim, Indianapolis, IN) for 20 min at room temperature. Also, to confirm the viral nucleic acid strandedness, nucleic acid preparations were incubated with RNase under either low salt conditions (in distilled water) or high salt conditions (300 mM sodium chloride) as described by Morris and Dodds, 1979 (18).

To determine if a polyadenylated sequence was present on the 3'-end of the viral genome, DNase-treated viral nucleic acid preparations were passed through two cycles of affinity chromatography in an Oligo-dT Cellulose Column (Stratagene, La Jolla, CA). Viral nucleic acid preparations were mixed with an equal volume of 2X loading buffer (1X= 10 mM Tris-HCl pH 7.6, 500 mM NaCl), heated to 70° C for 15 min and then placed immediately on ice for 2-3 min before they were loaded on a 1 ml oligo-dT cellulose column. After washing the column with 1X binding buffer, bound poly A-containing nucleic acid was eluted from the column using elution buffer (10 mM Tris-HCl pH 7.6). Both fractions were precipitated as described previously and analyzed by gel electrophoresis in 1% agarose (SeaKem GTG Agarose, FMC,

Rockland, ME).

The gels were run at 100 V (constant voltage) for two hours and nucleic acid bands were visualized by staining with 5 ng/ml ethidium bromide and then photographed using Polaroid 667 film (Polaroid, Cambridge, MA).

To separate viral bands from other contaminating nucleic acid (e.g. ribosomal RNA), nucleic acid preparations were processed by electrophoresis in a 1.2% low melting temperature agarose (SeaPlaque GTG Agarose, FMC, Rockland, ME) at 25 V (constant voltage) for 18 hours. The desirable bands were cut from the gel with a clean razor blade. Nucleic acid bands were extracted from the low melting temperature agarose using the GELase fast protocol (Epicentre Technologies, Madison, WI) and stored at -70° C until needed.

Infectivity of viral nucleic acid

Nucleic acid preparations extracted (as described previously) from the 27,000 g and final pellets of infected and healthy tobacco plants were used to determine the infectivity of viral nucleic acid. The nucleic acid pellets were resuspended in RNase-free, ice-cold, Tris-HCl pH 7.2 and applied to the surface of young tobacco leaves using DEPC-treated cotton swabs. The presence of the two viral bands in the nucleic acid extracts was confirmed by agarose gel electrophoresis. To confirm that the initial extracts as

well as the 27,000 g pellet (from which the nucleic acid was extracted) contained infectious virions, aliquots from those two fractions were used to inoculate additional tobacco plants as described above.

Double-stranded RNA (dsRNA) analysis

Double stranded RNA from healthy and BaYSMV-infected tobacco and barley plants was extracted as described by Valverde et al. 1990 (30), except that up to 60 g of leaf tissues were used in most instances. Results of dsRNA analysis were confirmed by Dr. R. Valverde (Department of Plant Pathology and Crop Physiology, Louisiana State University Agriculture Center). The type and strandedness of the nucleic acid bands were confirmed by RNase treatments as described above for the viral nucleic acids.

cDNA synthesis

A cDNA Synthesis System Plus kit (Amersham Corporation, Arlington Heights, IL) was used in a number of attempts to synthesize cDNA from viral RNA to be used as molecular probes or in the synthesis of a cDNA library. The first strand was synthesized using avian myeloblastosis virus reverse transcriptase and random hexanucleotide primers. E. coli RNase H was then used to nick the RNA in the RNA-DNA hybrid. E. coli DNA polymerase I then replaces the RNA strand utilizing the nicked RNA as a primer and the 3'-5'

exonuclease activity of T4 DNA polymerase is used to remove any small remaining 3'-overhangs from the first strand cDNA. Both cDNA synthesis steps were monitored by including [α-^{32}P]dCTP and removing small aliquots from the reaction and analyzing them by alkaline gel electrophoresis as recommended by the manufacturer.

Evaluation of cDNA by northern blot analysis

Nucleic acid extracts from healthy and infected barley and tobacco plants were first mixed with formamide and formaldehyde and denatured at 70° C for 15 min as described by Sambrook et al., 1989 (26), before they were blotted on an Immobilon Transfer membrane (Millipore, Bedford, MA) using Posiblotter Pressure Blotter (Stratagene, La Jolla, CA). The membrane was dried at 68 C° and the nucleic acid was cross linked using UV.

Membranes were probed using the cDNA synthesized earlier following the procedure described by Sambrook et al., 1989 (26). X-ray films (X-OMAT AR 5, Kodak, Rochester, NY) were used to record any hybridization between cDNA and denatured nucleic acids.

RESULTS

Virus purification

Virus particles were successfully concentrated from infected plants by the use of differential and density gradient centrifugation in Percoll (Fig. 1). The purification procedure did not result in any significant fragmentation of virus particles (Fig. 2). Virus particle length in the initial extract ranged from 194 nm to 3081 nm with an average of 1155 nm (standard deviation ±574), and the length ranged from 370 nm to 2053 nm with an average of 1006 nm (standard deviation ±367) in the final pellet (Fig. 2).

High magnification view of negatively stained individual virus particles revealed the presence of a putative surrounding envelope, the absence of any internal striation, and a granular appearance of the viral nucleocapsid (Fig. 3).

Percoll was the only suitable centrifugation medium that resulted in any significant concentration of intact virus particles. All attempts to purify the virus using other conventional centrifugation media such as cesium chloride, cesium sulfate, and potassium tartrate failed. Use of the latter media resulted in significant virus particle losses and/or degraded virus unrecognizable as discrete particles by electron microscopy.

The use of activated charcoal was helpful in producing a final pellet that contained minimal amounts of contaminating host material. Also, the addition of 5% celite to the initial extract was helpful too, although the results obtained were inconsistent, especially during large scale purifications where more than 30 g of celite were added .

The virus buoyant density in percoll ranged from 1.06 to 1.07 g/cm^3 (average 1.064 g/cm^3).

The final pellet obtained using this protocol contained infectious virus. Two separate infectivity assays resulted in symptoms characteristic of those caused by BaYSMV in 24 of 40 (60%) and 20 of 40 (50%) tobacco plants inoculated.

Formvar-coated grids were superior to parlodian-coated grids. When samples of the same purified virus preparation were deposited on the two different coating membranes 10 to 20 times more virus particles were observed on formvar-coated grids.

Treatment of purified BaYSMV with detergents

The putative envelope that surrounds individual virus particles was removed by detergent treatment even at the lower concentration (0.5%). Generally, this resulted in total disintegration of the virus particles making them unrecognizable as such by electron microscopy. In a few cases, however, the partially disintegrated virus particles having a beaded appearance were seen (Fig. 4).

Antisera production and evaluation

The antiserum (SC450) elicited by concentrated preparations of BaYSMV had a titer of 1:8 when reacted with virus antigen extracted from barley and tobacco in the double immunodiffusion test using unamended agar. The pattern of the interaction between the native (non-absorbed with concentrated healthy protein) antiserum SC450 and BaYSMV antigen can be characterized by the absence of spur formation and the total merging of precipitin lines. This pattern was observed when virus extracts from infected barley and tobacco plants (Fig. 5) as well as when purified virus from barley and tobacco plants (photo not shown) were reacted with SC450. The antibody-virus antigen precipitin line was formed slightly closer to the antigen depot (Fig. 5).

Antibodies produced against contaminating healthy plant protein (Fig. 5. A) were successfully eliminated by absorbing the native antiserum with healthy tobacco antigen as described in the materials and methods section without affecting the titer (Fig. 5).

Interestingly enough, no precipitin lines were observed when SDS was added to the agar.

The purified IgG from the absorbed antiserum SC450 enabled us to detect BaYSMV in all barley and tobacco plants showing symptoms characteristic of the disease including the barley samples collected from the Pocatello Valley, ID.

These results were confirmed by thin section electron microscopy.

ELISA

Results of the initial work using native, nonabsorbed polyclonal antibodies in standard DAS-ELISA were not satisfactory. A high background reading was obtained with the healthy control. This led to the use of purified BaYSMV-IgG from antiserum SC450 that had been absorbed with concentrated host protein and to the selection of specific extraction buffers for both direct and indirect ELISA (Tables 1 and 2).

For direct ELISA, the best results were obtained when phosphate-buffered saline (PBS) was used as antigen extraction buffer. PBS was considered superior to other satisfactory buffers (e.g. PBS+ 5% bovine serum albumin). The ratio of A_{405} of infected to healthy barley was 16X and 11.5X and for tobacco the ratio was 48.3X and 21.7 for PBS and PBS+ 5% BSA, respectively (Table 1).

Indirect ELISA gave less satisfactory results. The A_{405} of infected to healthy plants was inconsistent between barley and tobacco when the same extraction buffer was used (Table 2). In addition, high buffer readings were observed in the case of PBS and PBS+ 2% polyvinylpyrrolidone.

In both tests there was no difference in the results when extracted samples were incubated at 4° C or at room

temperature.

Satisfactory results were obtained by direct ELISA when mites were extracted with PBS. When one mite was used, the ratio of A_{405} of the viruliferous to nonviruliferous mite was 10X (A_{405}=0.560 and 0.056, respectively). When three mites were used the ratio was 11X (A_{405}=0.610 and 0.054, respectively).

No cross reactivity was observed between the antiserum SC450 and barley stripe mosaic virus (BSMV), wheat streak mosaic virus (WSMV), barley yellow dwarf virus (BYDV-RMV-MT), and tobacco mosaic virus (TMV). Their A_{405} were comparable to those of their respective healthy controls.

Inoculation of barley by injection

For the first experiment, 11 of the 20 Atlas barley plants (55%) inoculated with BaYSMV extracted from barley and tobacco by injection using hypodermic needles showed symptoms characteristic of the disease about six weeks after the inoculation. By contrast, in a second experiment none of the 20 barley plants inoculated by injection developed any symptoms. However, in a third experiment one out of 50 injected barley plants became diseased. In the second and third experiments, plants were injected with virus extracted from infected tobacco plants. In the first and third experiments, only new tillers developed symptoms characteristic of the disease. No symptoms were observed on

injected leaves. The long filamentous virus particles were observed in extracts obtained from all the injected symptomatic plants. Furthermore, these extracts were infectious when used to mechanically inoculate tobacco plants as described in the materials and methods section.

Field inspection

The large filamentous particles unique to BaYSMV were identified in all symptomatic barley samples collected from the Pocatello Valley, ID, in 1991 and 1992. The plants displayed typical mosaic, streaking, and necrosis symptoms. In 1992, at least 400 ha of the barley cultivars Otis, Steptoe, and Korol were affected by the disease. The disease incidence reached 100% in about 30% of the fields inspected with yield loss estimates ranging from 30% to 100%. No differences in disease incidence or severity were observed between the barley cultivars grown in that area. The plants were grown under dry land conditions and the brown wheat mite was abundant in many of the affected fields.

Isolation and partial characterization of viral nucleic acid

Modifying the protocol previously described to extract viral nucleic acid (25) resulted in more consistent results. Washing the nucleic acid pellet with sodium acetate was especially useful. Two prominent nucleic acid bands were

present in most preparations obtained from infected plants but never from healthy ones (Fig. 6). In addition, a third, lower molecular weight band was occasionally observed in nucleic acid preparations extracted from infected plants (Figs. 6 and 7). Large amounts of ribosomal RNAs were always present and attempts to remove them by further purification steps led to the removal of the viral bands as well.

All viral bands were resistant to DNase treatment. However, they were readily degraded by RNase treatments under both low and high salt (NaCl) conditions (Fig. 7).

When passed through an oligo-dT cellulose column the viral bands were always present in the initial eluant indicating the absence of a polyadenylated sequences at the 3'-end of either RNA species (Fig. 7).

Infectivity of viral nucleic acid

BaYSMV nucleic acid was not infectious in our tests. None of the 60 tobacco plants inoculated with viral nucleic acid extracted from either the 27,000 g pellet or from the final pellets showed symptoms of BaYSMV infection. Using the initial extract and the 27,000 g pellet as inocula resulted in symptoms characteristic of BaYSMV infection in 20 of 30 (67%) and 15 of 30 (50%) of the tobacco plants inoculated, respectively. No symptoms were observed in any of the 20 tobacco plant inoculated with nucleic acids extracted from healthy plant tissues.

Double-stranded RNA analysis

A double stranded RNA form of the virus was obtained only in 3 of the 14 extraction experiments (Fig. 8). Large amounts of leaf tissues (up to 60 g) were needed for each extraction. When infected barley plants were used, only traces of ds-RNA were obtained (Fig. 8, and Valverde, personal communication). The type and strandedness of the high molecular weight band were confirmed through RNase treatment under both low and high salt (NaCl) conditions as described previously (data not shown).

cDNA synthesis

All 11 attempts to obtain cDNA synthesis from isolated viral RNA using avian myeloblastosis reverse transcriptase or rTth enzymes failed. No incorporation of any [α-^{32}P]dCTP was obtained, consequently the use of the cDNA synthesis reaction to probe viral RNA blots failed.

Table 1. Comparison of different extraction buffers used for the detection of barley yellow streak mosaic virus (BaYSMV) in leaf extracts of barley and tobacco by direct double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA)[a]

Extraction Buffer	Absorbance[b] at 405 nm						
	Buffer	Barley			Tobacco		
		Healthy	Infected	Ratio	Healthy	Infected	Ratio
PBS[c]	0.000	0.035	0.560	16.0	0.015	0.725	48.3
PBS+ 5% T[d]	0.005	0.100	0.750	7.5	0.053	0.840	15.8
PBS+ 5% DNM[e]	0.001	0.220	0.835	3.8	0.065	0.780	12.0
PBS+ 2% PVP[f]	0.048	0.180	0.625	3.5	0.058	0.633	10.9
PBS+ 5% BSA[g]	0.017	0.065	0.750	11.5	0.035	0.760	21.7

a) Wells were coated with 1 ug/ml SC450 IgG preparation in coating buffer, loaded with 200 ul samples extracted with 10X volume (g/ml) of each extraction buffer, and with 1:2000 dilution of IgG-alkaline-phosphatase conjugate. The IgG preparation was obtained from SC450 antiserum absorbed with concentrated healthy protein.

b) Readings were recorded after 1 hour of enzyme-substrate reaction at room temperature. Each buffer reading is the average of two duplicate wells. Each healthy reading is the average of 10 duplicate wells.

c) PBS= 140 mM NaCl, 1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 3 mM (0.02%) NaN_3 pH 7.4.

d) T= tween-20.

e) DNM= dried nonfat milk.

f) PVP= polyvinylpyrrolidone.

g) BSA= bovine serum albumin.

Table 2. Comparison of different extraction buffers used for the detection of Barley yellow streak mosaic virus (BaYSMV) in leaf extracts of barley and tobacco by indirect enzyme-linked immunosorbent assay (I-ELISA)[a]

Extraction Buffer	Absorbance[b] at 405 nm						
	Buffer	Barley			Tobacco		
		Healthy	Infected	Ratio	Healthy	Infected	Ratio
PBS[c]	0.600	0.010	0.090	9.0	0.083	0.827	10.0
PBS+ 5% T[d]	0.406	0.010	0.150	15.0	0.135	0.707	5.2
PBS+ 5% DNM[e]	0.010	0.000	0.000	0.00	0.010	0.640	64.0
PBS+ 2% PVP[f]	0.228	0.010	0.135	13.5	0.160	0.875	5.5
PBS+ 5% BSA[g]	0.000	0.025	0.080	3.2	0.143	0.780	5.5

a) Wells were loaded with 200 ul samples extracted with 10X volume (g/ml) of each extraction buffer, 200 ul of SC450 IgG preparation diluted 1:500 in coating buffer, and with 1:1000 dilution of goat anti-rabbit IgG-alkaline phosphatase conjugate. The IgG preparation was obtained from SC450 antiserum absorbed with concentrated healthy protein.

b) Readings were recorded after 1 hour of enzyme-substrate reaction at room temperature. Each buffer reading is the average of two duplicate wells. Each healthy reading is the average of 10 duplicate wells.

c) PBS= 140 mM NaCl, 1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 3 mM (0.02%) NaN_3 pH 7.4.

d) T= tween-20.

e) DNM= dried nonfat milk.

f) PVP= polyvinylpyrrolidone.

g) BSA= bovine serum albumin.

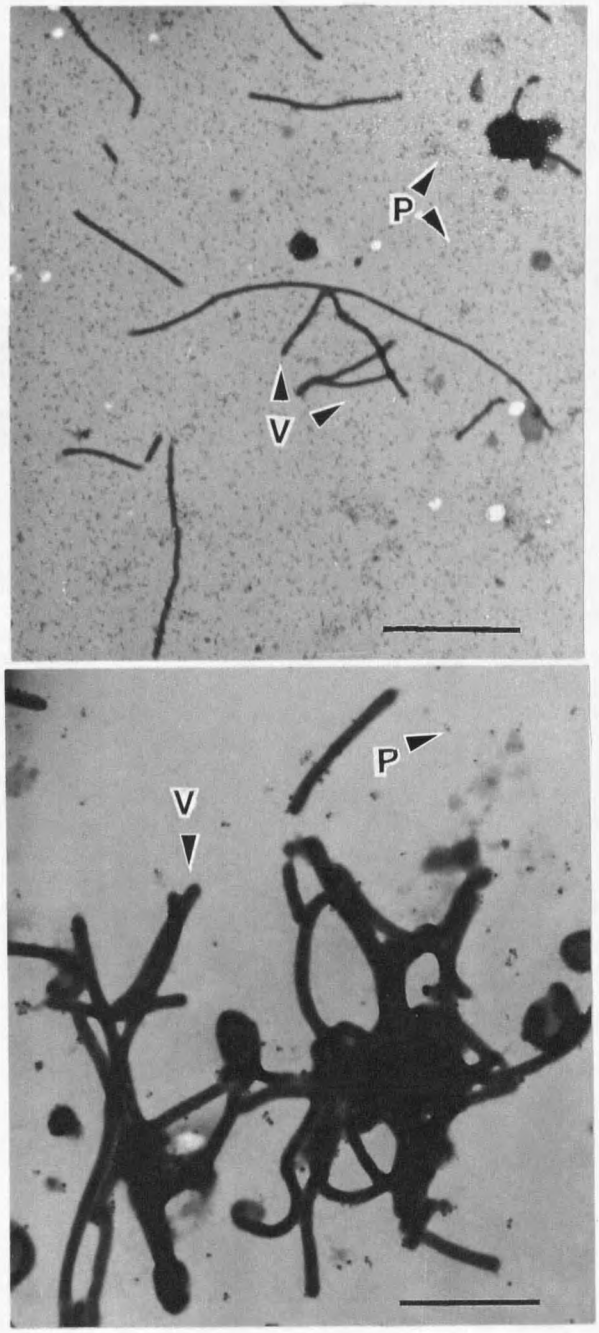

Fig. 1. Electron micrographs of purified preparations of barley yellow streak mosaic virus (BaYSMV) particles (V) positively stained with uranyl acetate. (P) contaminating Percoll particles. Note the various particle lengths in A. A: Bar= 1,000 nm, B: Bar= 500 nm.

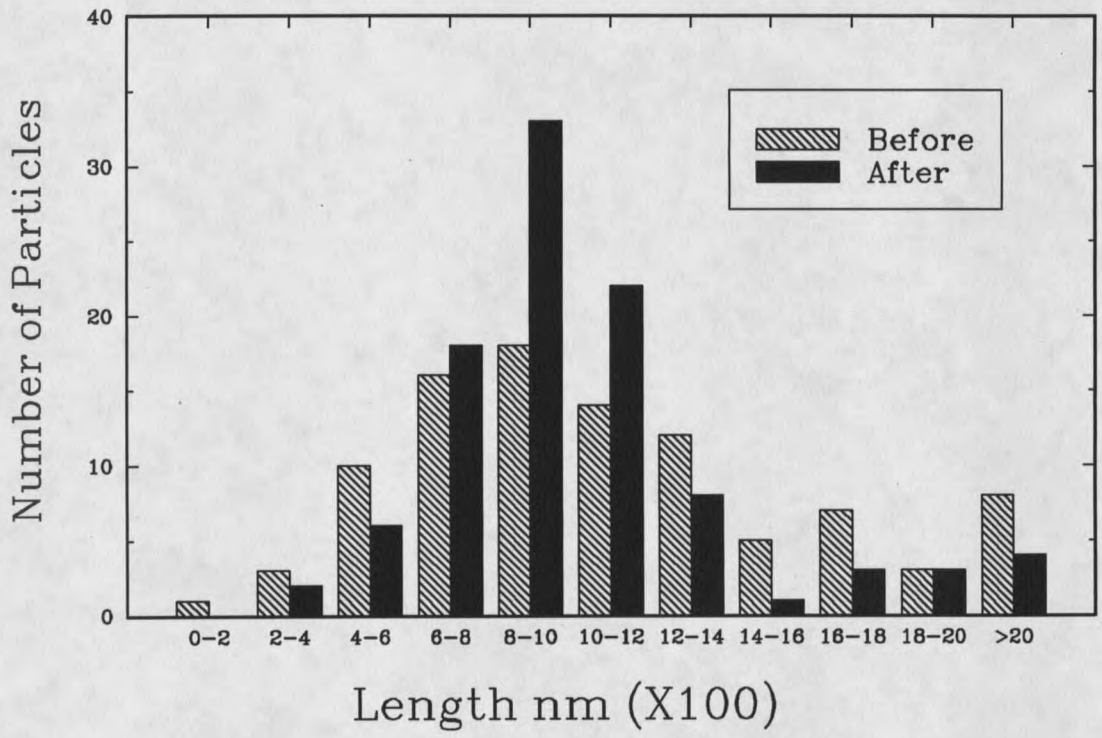

Fig. 2. Particle length distribution of barley yellow streak mosaic virus (BaYSMV) in crude tobacco leaf extracts (before) and in the final pellet obtained by the purification procedure (after).

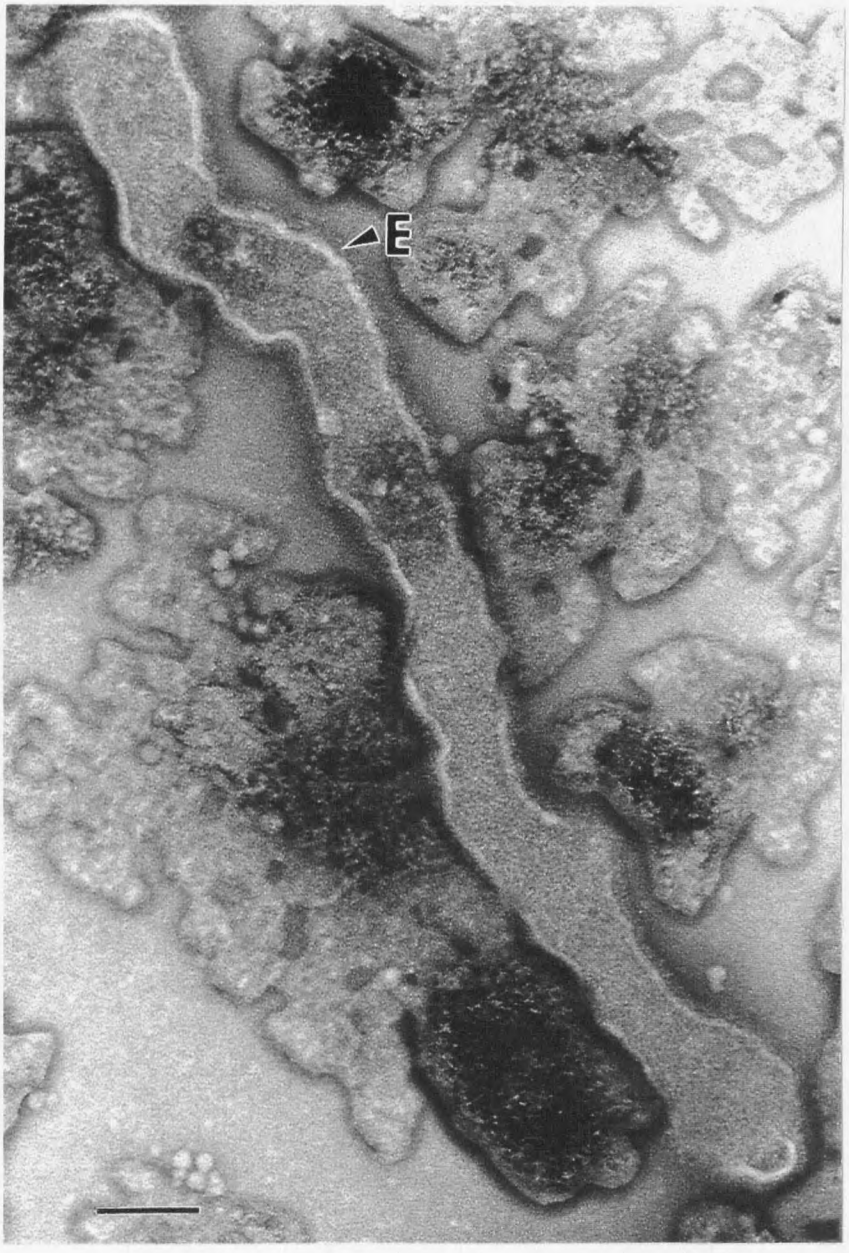

Fig 3. Electron micrograph of a barley yellow streak mosaic virus (BaYSMV) particle negatively stained with PTA. Note the absence of internal cross striations, the finely granular appearance of the viral nucleocapsid, and the putative virus envelope (E). Bar= 100nm.

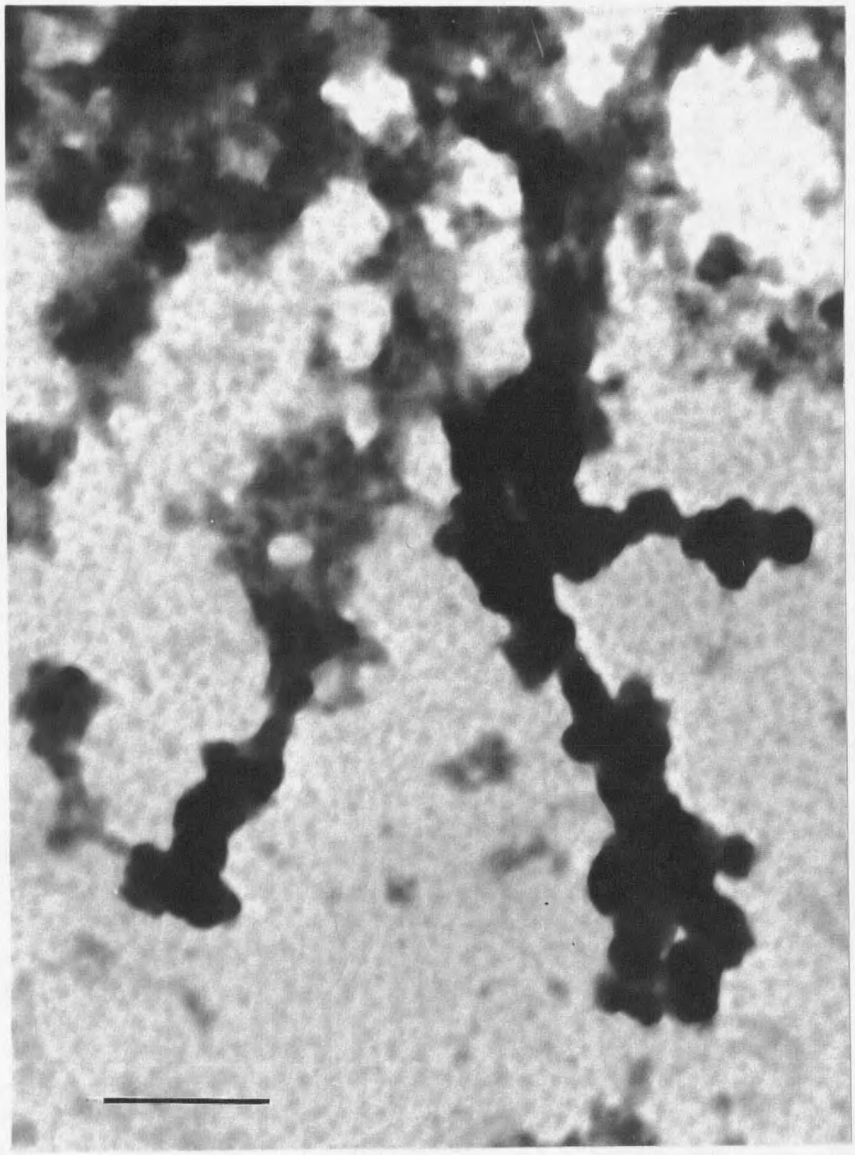

Fig. 4. Electron micrograph of barley yellow streak mosaic virus (BaYSMV) positively stained with uranyl acetate revealing the effect of detergent treatment on the concentrated particles. Note the destruction of particle structural integrity. Bar= 2,000 nm.

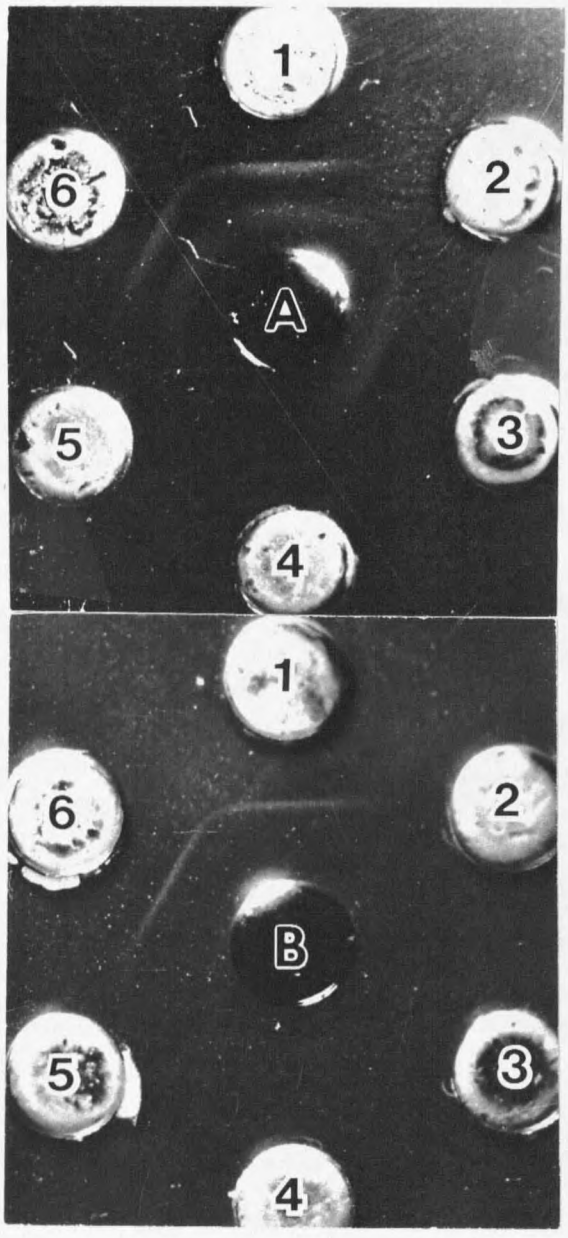

Fig. 5. Photograph showing Ouchterlony double immunodiffusion pattern of the interaction between the antiserum SC450 (center well) elicited against barley yellow streak mosaic virus (BaYSMV) antigen and leaf extract from infected barley plants (1), 100% Percoll (2), leaf extract from healthy barley plants (3), 100 mM phosphate buffer (4), normal serum (5), and infected tobacco plants (6), before absorption with concentrated healthy protein (A), and after cross absorption (B). Well spacings 5mm.

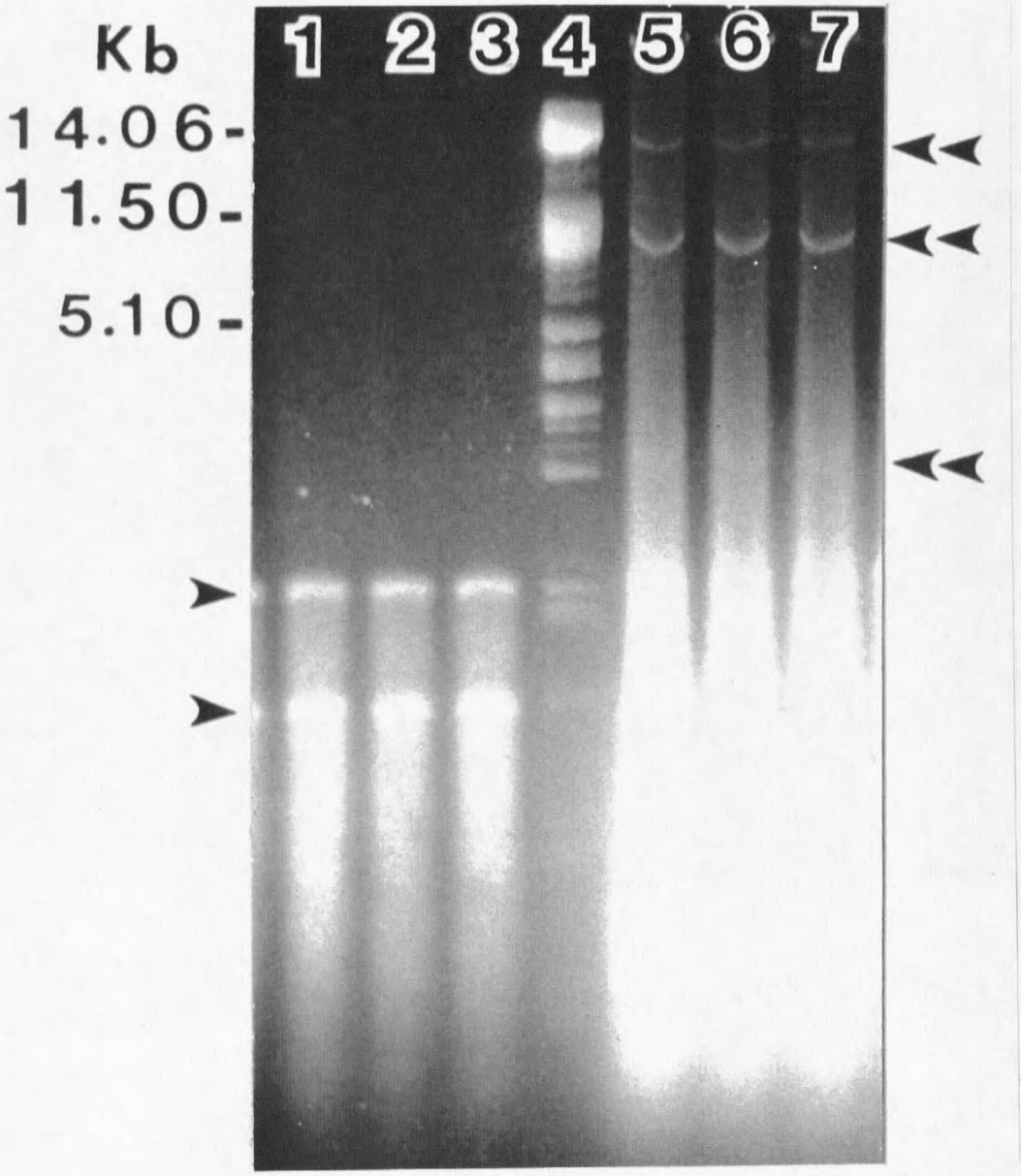

Fig 6. Nondenaturing agarose gel electrophoresis analysis of nucleic acid preparations extracted from concentrated healthy and infected tobacco leaves. Lanes 1-3: RNase-free DNase treatment of nucleic acid extracted from concentrated healthy tobacco leaves. Lane 4: lambda DNA digested with Pst1. Lanes 5-7: RNase-free DNase treatment of nucleic acid extracted from concentrated infected tobacco leaves. Double arrowheads: viral nucleic acid bands. Single arrowheads: 28S and 18S ribosomal RNA.

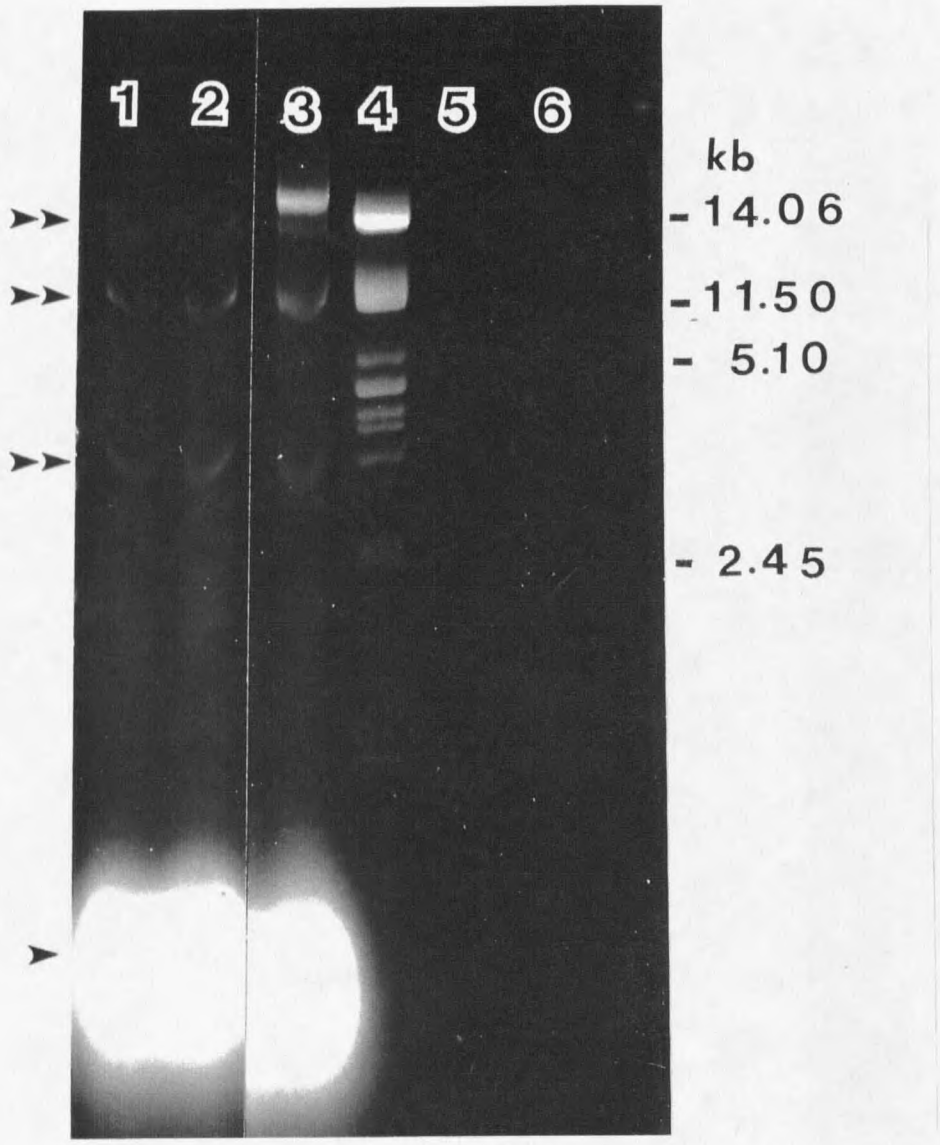

Fig 7. Analysis of barley yellow streak mosaic virus (BaYSMV) nucleic acid preparations by nondenaturing agarose gel electrophoresis. Lane 1: RNase-free DNase treatment of nucleic acid from concentrated virus preparations, lane 2: poly A (-) fraction eluted from oligo dT-cellulose column loaded with the same nucleic acid extracts as in lane 1, lane 3: native nucleic acid from concentrated virus preparations, lane 4: Lambda DNA digested with Pst1, lane 5: RNase and DNase treatment of nucleic acid from concentrated virus preparations under low salt (NaCl) conditions, lane 6: RNase and DNase treatment of nucleic acid extracted from concentrated virus preparations under high salt conditions. Note the large amounts of ribosomal RNA (single arrowheads). Double arrowheads: viral nucleic acid.

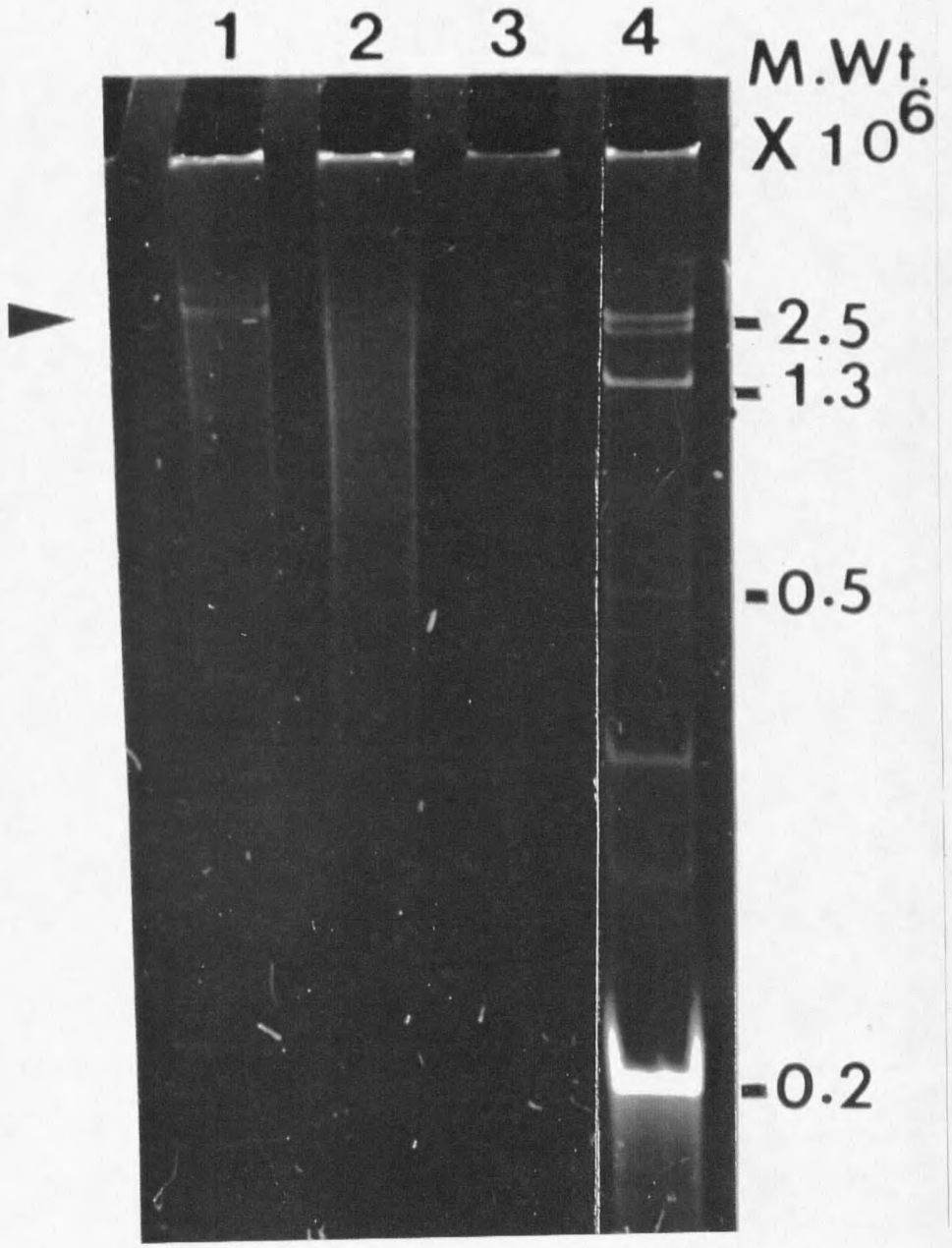

Figure 8: Polyacrylamide gel electrophoresis of dsRNA extracted from tobacco plants infected with BaYSMV (lane 1), healthy tobacco plants (lane 2), infected barley plants (lane 3), and tobacco plants infected with cucumber mosaic virus and satellite RNA (lane 4). All lanes are treated with DNase and RNase under high salt (300 mM NaCl) conditions.

DISCUSSION

For almost ten years, all efforts to further determine biological, serological, and molecular characteristics of barley yellow streak mosaic virus have been impeded by the lack of a purification procedure that consistently yielded a high concentration of virus particles and low amounts of contaminating plant material. That problem has been solved by the purification procedure described in this dissertation. Although a procedure to partially purify BaYSMV had been published (25), no photographic evidence of the partially purified virus particles was presented. Additionally, efforts to elicit antibodies against the contents of the final pellet obtained using that procedure were unsuccessful.

A critical step in the new purification procedure was the pelleting of the virus particles using a relatively low centrifugal force for a short time (27,000 g for 30 min) in the early steps of the purification. The 27,000 g supernatant fluid contained very few (if any) virus particles as determined by electron microscopy. In the previously published protocol (25), pellets obtained using a higher centrifugal force (50,000 g for 30 min) were discarded resulting in a serious loss of the virus.

Another critical step in the new protocol was the use of Percoll. Percoll was a suitable centrifugation medium

because of the very short time required to obtain a linear gradient (20 min). All attempts to purify the virus using other conventional centrifugation media (e.g. cesium chloride, cesium sulfate, sucrose, and potassium tartrate) failed because of the relatively long centrifugation times required to reach linear gradients (6 to 24 hours). Such long centrifugation times resulted in the disintegration and/or the possible alteration of the virus surface properties which made the virus particles unrecognizable by electron microscopy. Preserving the structural integrity of virus particles was extremely important because electron microscopy was the only way to monitor the concentration of the particles throughout the various steps of the purification procedure. Using two cycles of centrifugation in percoll gradients helped reduce the amount of contaminating host materials but it also led to a reduction in the yield of virus particles which often necessitated combining virus preparations obtained from several purifications.

The most serious problem associated with the new procedure was that of being unable to quantify the virus yield in mg/kg terms. The reasons for this are as follows: i) there is no known sensitive local lesion host that can be used in infectivity assays to determine the yield, ii) the amount of Percoll left in the final concentrated virus pellet interfered with the use of spectroscopy to determine

the yield, and iii) this procedure resulted in highly concentrated virus particle preparations and not in highly purified virus preparations; contaminating host material was present in the final pellet as evidenced by electron microscopy and the presence of antibodies against healthy plant antigen. This contaminating host material interfered with the use of spectroscopy to determine virus yield.

Activated charcoal used in the initial step of the purification procedure was helpful probably due to its ability to adsorb certain substances that would otherwise result in the disintegration and/or aggregation of the virus.

Celite, which is commonly used to purify the enveloped rhabdoviruses (10), was not satisfactory especially in large scale purifications when more than 30 g were added to the initial extract. Virus particles were irreversibly trapped by the celite during the subsequent filtration step which led to a significant reduction in the yield. When less celite was used, the loss of virus particles was reduced but that was associated with an increase in contaminating healthy materials.

The use of polyvinylpyrrolidone did not result in any appreciable reduction of contaminants.

The partial removal of Percoll from the virus preparations by centrifugation through a 50 % (W/W) sucrose pad presented a novel convenient alternative to the methods

recommended by the manufacturer (20). High speed centrifugation resulted in loss of virus particles in the Percoll pellet. Also, gel filtration is recommended by the manufacturer but it failed to totally remove percoll from rice transitory yellowing virus (RTYV) preparations (3).

The dimensions of virus particles obtained from the purification procedure described here are consistent with the previously published dimensions (25) indicating that the new protocol did not cause any significant fragmentation of the long filamentous virus particles. The apparent selection of virus particles of certain lengths (Fig. 2) can be attributed to the need to avoid certain gradient fractions that may have contained virus particles but they also contained a lot of contaminating healthy materials.

High purity of virus preparations had to be compromised by avoiding harsh protocols and additives (e.g. chloroform, 2-mercaptoethanol, detergents, etc.) so that structural integrity of the particles could be preserved. Avoidance of such additives has been recommended for enveloped viruses such as members of the bunyaviridae family (28).

This report documents that the concentrated long filamentous virus particles obtained using the new purification protocol are infectious. Their infectivity provides reasonable proof that those particles are actually the causal agent of barley yellow streak disease and

suggests the absence of a "second hitchhiking agent" that might be the actual causal agent of BaYSM. It is highly unlikely that such an agent would be copurified along with BaYSMV particles using this particular procedure.

The failure to obtain any symptomatic plants using viral nucleic acid as inoculum can be attributed to i) the degradation of the viral nucleic acid by nucleases on the surface of tobacco leaves and/or ii) the viral coat protein, envelope and/or other nonstructural proteins are required for infectivity. In the literature, there are only a few cases where coat protein-deficient variants of a small number of mechanically transmitted viruses have been successfully transmitted to plants (12).

The envelope surrounding virus particles was readily removed when purified virus particles were treated with anionic or nonionic detergents suggesting that the envelope has a lipid nature. The presence of the envelope was essential for preserving the structural integrity of the particles. The removal of the envelope resulted either in the partial or the total disintegration of the long filamentous particles giving them a beaded appearance (Fig. 4).

The use of PTA as a negative stain also caused the removal of the viral envelope and the disintegration of most virus particles. However, a few, apparently intact, negatively-stained virus particles were observed. The

negative stain revealed a finely granular nucleocapsid and no cross striations (Fig. 3) indicating that BaYSMV is not a rhabdovirus. Therefore, the number of enveloped plant virus groups could be extended to three: rhabdoviruses, tospoviruses (17), and a new group having BaYSMV as the type member. It is proposed that the new group be named the barysmovirus group (the barley yellow streak mosaic virus group). Some of the defining characteristics of the barysmovirus group are as follows: 1) like members of the rhabdoviruses and tospoviruses groups, BaYSMV particles are surrounded by a lipid membrane, 2) the vector of BaYSMV is a true spider mite (the brown wheat mite) whereas the vectors of the other two enveloped plant viruses are insects, and 3) the morphology of BaYSMV, specifically the lack of internal cross striation and particle dimensions, sets it apart from the rod-like or bullet-shaped particles of rhabdoviruses and from the spherical particles of tospoviruses. Undoubtedly, further biological and molecular characterization of BaYSMV is needed before the above mentioned classification can be submitted for consideration by the International Committee on the Taxonomy of Viruses (ICTV).

The experiments that demonstrated that it is possible to obtain symptomatic barley plants via the injection of extracts from infected plants are the first reported for mechanical transmission of BaYSMV to barley. Although this

method is not as efficient or consistent as using the brown wheat mites, it provided an important step toward completing Koch's postulates. This approach should be further investigated to determine all the factors affecting the infectivity. Obtaining higher and more consistent percentages of infection can be very helpful in screening genotypes for resistance to BaYSMV.

This report describes the production of the first antiserum ever elicited against BaYSMV antigen. The presence of antibodies that reacted with healthy plant antigens in double immunodiffusion tests was expected due to the mild nature of the purification procedure used to obtain the virus preparations for inject immunogen. These antibodies were successfully eliminated by absorbing the SC450 antiserum with concentrated healthy tobacco protein. The unique virus antigen-antibody precipitin line was produced in double immunodiffusion tests only in the absence of SDS from the agar indicating that the antibodies react with epitopes that are changed upon treatment with detergents. Since the pore diameter of agar gels at low concentration is slightly greater than 300 nm (19), it is extremely unlikely that intact BaYSMV particles would diffuse through the agar matrix. Thus, it can be concluded that the precipitin line is produced by a reaction between antibodies and a diffusible viral antigen. The molecular weight of the viral antigen is likely to be slightly greater than that of the

antibody IgGs since the precipitin line formed between them is slightly closer to the virus extract well. The complete fusion of the precipitin lines formed between SC450 and virus antigen (both from barley and tobacco plant extracts and purified preparations) indicate that the antibodies are reacting with the same virus antigen regardless of the host from which the virus was extracted.

The purified IgGs from the absorbed SC450 antiserum were used to design practical and reliable ISEM and ELISA protocols with which to detect the virus in both infected plants and viruliferous mites.

Until July 1991, BaYSMV incidence in the USA was believed to be limited to ten counties of the state of Montana but the above described serological tests allowed us to positively confirm the presence of the virus in the Pocatello Valley, ID for the first time.

In that valley, barley is grown under dry land conditions which are conducive to the development of the disease. Also, the brown wheat mite was abundant in many of the fields visited which explains the high disease incidence in many of the fields. In 1992, at least 400 ha of the barley cultivars Otis, Steptoe, and Korol were affected by the disease. Farmers in that area indicated that it is common practice to plant barley in the same fields for many consecutive years. They also indicated that they have been seeing the symptoms for the last five years and the brown

wheat mite for the last ten years. The symptoms, however, were never attributed to BaYSMV until 1991 mainly due to the lack of a reliable method to diagnose the disease in barley.

An interesting observation was that the disease incidence in the barley fields planted to safflower in the previous season was almost 60% lower than in the fields planted to barley. In the Pocatello Valley safflower may very well be used in rotation with barley to reduce the mite populations and virus reservoirs especially if environmental conditions conducive to high disease incidence persist. It is important, however, that scientifically designed experiments be conducted before recommending such a control measure.

Beyond its importance as a diagnostic tool, the antibodies and the ELISA should be helpful in other areas such as studying virus-vector interactions, virus movement, and breeding for disease-resistant culitvars.

Sodium acetate helped remove contaminating polysaccharides which was essential in obtaining cleaner and more consistent preparations of viral nucleic acid.

In addition to the two viral bands reported by Robertson and Carroll (25), a third, lower molecular weight band was occasionally observed in our experiments (Fig. 7). This third band could either be a discrete segment of the viral genome or a different configuration of one of the two other bands. Nucleic acid analysis under denaturing

conditions and\or sequence data are needed before any final conclusions could be made. Robertson and Carroll (25) reported that the viral nucleic acid consisted of RNA. However, RNase treatments under high salt conditions were not included in their experiment. Our results confirm the type of viral nucleic acid and since all viral bands were digested with RNase under both low and high salt condition it can be said with certainty that these bands consist of single-stranded and not double-stranded RNA.

The dsRNA form of BaYSMV was obtained from infected barley and tobacco plants only occasionally. Up to 60 g of plant tissues had to be used in order to obtain dsRNA that was detectable by gel electrophoresis. That made any use of that nucleic acid form in other experiments (e.g. cDNA synthesis and cloning) both impractical and unreliable. Also, the molecular weight of dsRNA could not be determined due to the lack of dsRNAs that can be used as molecular markers.

The lack of any polyadenylated sequences at the 3′ end of the viral nucleic acid and the unavailability of any sequence information about the viral genome impeded all efforts to synthesize and clone a cDNA library. The set of random hexamer primers used in the cDNA synthesis experiments did not seem to anneal to the viral nucleic acid. One possible solution is to try different commercially available sets of random primers to attempt to obtain genome

sequence information via direct RNA sequencing. The importance of obtaining a cDNA library of the viral genome is multi-fold: 1) sequences obtained via sequencing various clones can be used to compare BaYSMV at the molecular level with other viruses. Such comparisons can help in establishing the relatedness of BaYSMV to other virus groups such as the rhabdovirus group which has been suggested to be a taxonomic home for BaYSMV (25), 2) obtaining cDNA probes and/or genomic sequences will allow the detection of the viral nucleic acid via Northern hybridization or PCR-based tests. Beside its importance in diagnosis, such a detection method will help answer many of the lingering questions in the area of the relationship between BaYSMV and its vector.

SUMMARY

The principal objective of this study was to further characterize barley yellow streak mosaic virus (BaYSMV), the causal agent of a disease in barley and wheat. To accomplish this a new purification procedure that yielded a high concentration of virus particles and a minimal amount of contaminating host material had to be developed.

The results of this study demonstrated that it is possible to obtain infectious, highly concentrated virus particles using a purification scheme that includes differential centrifugation and Percoll density gradient centrifugation. The final pellet contained concentrated virus particles that were infectious. This finding provided sufficient evidence to suggest that the long filamentous particles that have always been associated with the disease are indeed the causal agent. The nature of the new purification procedure makes it highly unlikely that a second agent is involved in disease induction.

Concentrated virus particles were used to produce the first ever antiserum against BaYSMV. The antiserum SC450 was used to design DAS-ELISA (double antibody sandwich-enzyme-linked immunosorbent assay) and ISEM (immunosorbent electron microscopy) tests. The tests allowed the reliable and accurate detection of the virus in infected tobacco and barley plants as well as in viruliferous Petrobia latens

Muller (the brown wheat mite, the vector of BaYSMV). Consequently, the virus has been documented to infect barley cultivars grown under dry land conditions in the Pocatello Valley, Idaho. This was the first report of the virus infecting barley in Idaho and only the second report of the virus occurring in the United States. The antiserum should prove useful in large scale surveys to document the incidence of the virus in previously unsurveyed areas of Montana and in other states especially when electron microscopy facilities are not available.

It has also been demonstrated for the first time that it is possible to obtain symptomatic barley plants by injecting virus extracts into barley leaves using hypodermic needles. This method is not as consistent as using the brown wheat mites to inoculate barley plants but, if perfected, it could provide a useful tool in the efforts to screen barley cultivars for resistance to BaYSMV.

Based on electron microscopic evidence obtained from negatively stained or detergent treated virus particles as well as other data available on the biology of BaYSMV, it can be proposed that the number of enveloped plant virus groups be extended from two to three: the rhabdovirus group, the tospovirus group, and the new barysmovirus virus group (the barley yellow streak mosaic virus group). The basis of this proposal is that BaYSMV particles are surrounded by a lipid envelope but lack the cross striations characteristic

of the rhabdovirus group. In addition, the vector and the unique dimensions of BaYSMV particles clearly distinguish this virus from members of the other two virus groups. Obviously, this proposal is preliminary and needs to be supported by obtaining more molecular and biological data on the virus.

Our efforts to further characterize BaYSMV at the molecular level were not successful. All attempts to synthesize and obtain a cDNA library of the viral genome failed. However, we have confirmed that the viral genome consists of single-stranded RNA and demonstrated that it lacks any polyadenylated sequences at the 3′ end. Future work should center on cloning and sequencing the viral genome by utilizing several cloning strategies and direct RNA sequencing methods. Genome sequences should be very useful in designing cDNA probes as well as PCR-based diagnostic tests to detect the virus and study relationships with its vector. In addition, molecular data combined with biological, morphological, and serological data will help study the relatedness of the virus to other viruses and find a taxonomic home for BaYSMV.

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