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

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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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.

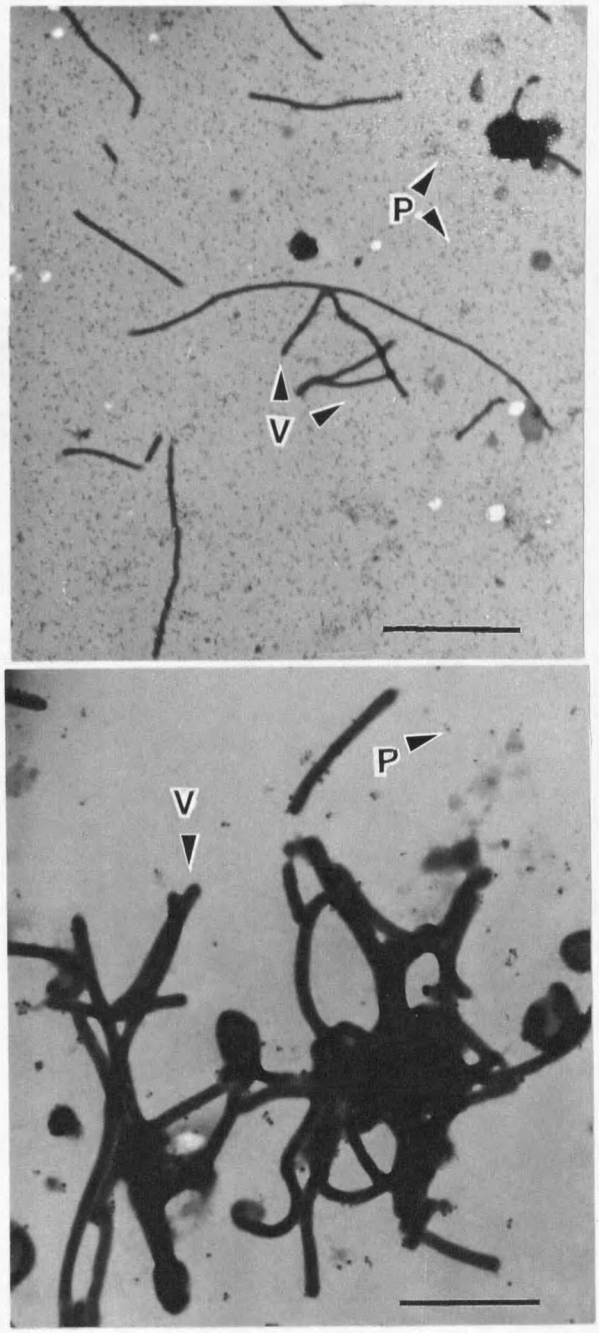

Fig. 1. Electron micrographs of purified preparations of barley yellow streak mosaic virus (BaYSMV) particles (V) positively stained with uranyl acetate. (P) contaminating Percoll particles. Note the various particle lengths in A. A: Bar= 1,000 nm, B: Bar= 500 nm.

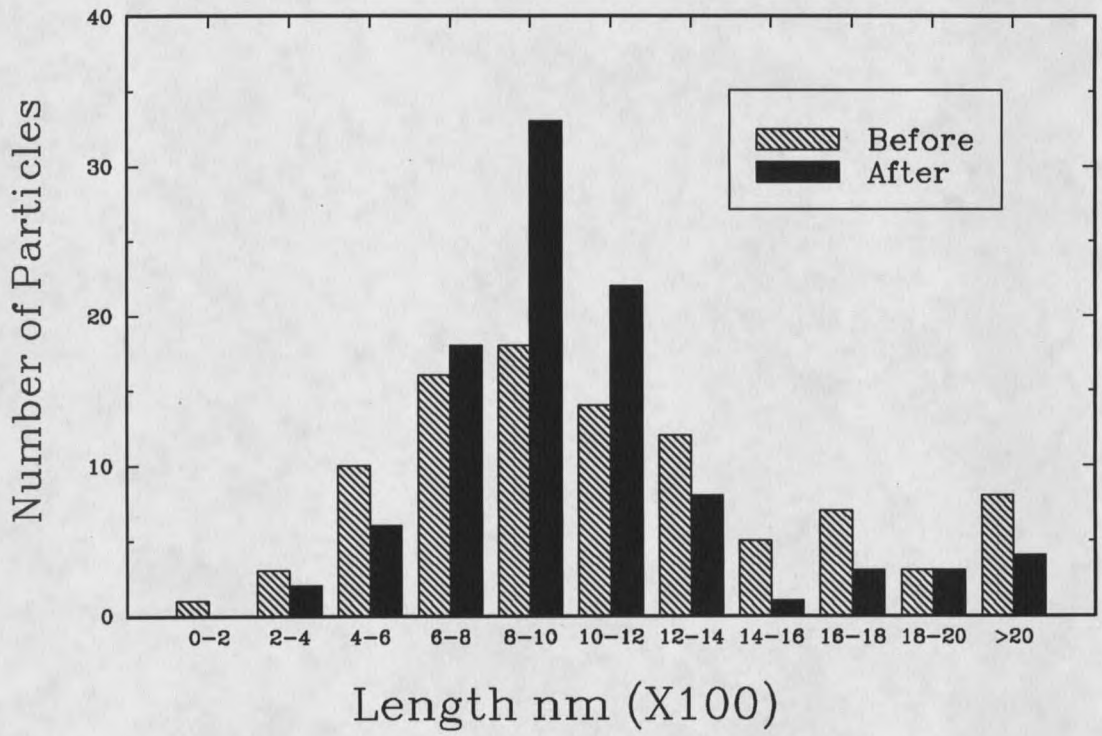

Fig. 2. Particle length distribution of barley yellow streak mosaic virus (BaYSMV) in crude tobacco leaf extracts (before) and in the final pellet obtained by the purification procedure (after).