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Wheat Streak Mosaic Virus Cylindrical Inclusion Body Protein

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SUMMARY

A protein of apparent molecular weight 66000 was purified from wheat plants infected with wheat streak mosaic virus. Antiserum to this protein, labelled with gold, specifically stained cylindrical inclusions in ultrathin sections of virus-infected cells. Antiserum to the M_r 66000 protein did not react with capsid protein in Western blots, nor did antiserum to capsid protein react with the M_r 66000 protein. Both antisera reacted with homologous antigens. The concentration of the M_r 66000 protein in extracts from infected leaves was about 100 μ g per g of leaves, which is higher than the usual concentration of virions.

INTRODUCTION

Wheat streak mosaic virus (WSMV) is morphologically similar to potyviruses, but is transmitted by a mite, *Aceria tulipae* Keifer, rather than by aphids as are potyviruses (Brakke, 1971; Hollings & Brunt, 1981). Extracts of WSMV-infected plants contain a M_r 66000 protein not detectable in extracts of uninfected plants (White & Brakke, 1983). This protein resembles proteins found in cylindrical inclusions of potyviruses in two properties, namely apparent molecular weight and presence in a component large enough to be sedimented from plant extracts centrifuged at 12000 r.p.m. (Hiebert *et al.*, 1984).

We report here the partial purification of the M_r 66000 protein, the preparation of an antiserum to it, and evidence of its presence in cylindrical inclusions. A preliminary report has appeared (Brakke *et al.*, 1984).

METHODS

Viruses. The type strain of WSMV (PV 57; American Type Culture Collection, 1980) and a strain collected in the field in 1981 (Sidney 81) were used. Similar results were obtained with either strain. Virus was propagated in wheat plants (*Triticum aestivum* L., cv. Michigan Amber) grown in a greenhouse at 22 to 30°C. Wheat spindle streak mosaic virus was maintained in wheat plants in a growth chamber at 15°C (Brakke *et al.*, 1982). Maize dwarf mosaic virus strain B (MDMV-B) was obtained from Dr Stanley Jensen of this department and maintained in maize (*Zea mays* L., cv. Golden Cross Bantam). Maize inbred SDP-2 was used for mixed infections of WSMV and MDMV-B.

Purification of M_r 66000 protein. Infected leaves were ground in 5 mM-Tris-HCl, 1 mM-KCl, 0.1 mM-EDTA, 1 mM-MgCl₂, 10% glycerol, 5 mM-iodoacetamide, pH 8.0 (standard grinding buffer) using 5 ml per g of leaves. The preparation was kept at about 2°C until it was heated in SDS. The extract was expressed through cheesecloth, and Triton X-100 was added to give a 2% (v/v) concentration. The extract was centrifuged for 2 min at 4000 r.p.m. in the J21 rotor of a Beckman J21-B centrifuge. The pellet was discarded and the supernatant fluid was layered over a 1 cm cushion of 20% sucrose in grinding buffer and centrifuged for 25 min at 12000 r.p.m. in the same rotor. The pellet was suspended in 1 ml of dissociation buffer (50 mM-Tris, 1% 2-mercaptoethanol, 15% sucrose, 2% SDS, 2 mM-EDTA, pH 8.8) per 8 g of leaves. After heating for a few minutes at 100°C, a 5 to 8 mm layer of the solution was layered on a single-well, vertical, 1.5 × 150 × 150 mm 12% polyacrylamide slab gel prepared with the buffers described by Laemmli (1970). After electrophoresis for 1200 to 1500 volt-hours, the slab gel was placed in 0.25 M-KCl at 0°C for 0.25 to 1 h until the protein bands became visible as potassium dodecyl sulphate precipitates. The major band corresponding to a protein of M_r of about 66000 was excised, chopped into 1 mm cubes with a razor

blade, frozen at -20°C , and ground in a mortar with a pestle. The ground gel was extracted twice with 0.1 M Tris-acetate pH 8.3, 0.1% SDS, 0.1% 2-mercaptoethanol with vigorous stirring for 30 min at 55°C and for 1 to 2 h at 25°C . The bulk of the gel was removed by centrifugation and the protein was concentrated by precipitation with 2 vol. ethanol. The precipitate was dissolved in hot 0.1% SDS and stored at 3°C .

Preparation of antiserum. Rabbits were injected intramuscularly with 0.4 mg of purified M_r 66000 protein spread over four sites, on each of three dates at 10 day intervals. After 5 months, a booster series of 0.2, 0.2 and 1.5 mg was injected at 10 day intervals. Antigen was emulsified in Freund's complete adjuvant. Rabbits were bled 6 weeks after the last injection. Antiserum to WSMV virions was prepared as described by Brakke & Ball (1968). Gamma globulin was prepared by ammonium sulphate fractionation.

Serological reactions

Agar diffusion. Agar double diffusion tests were made in 5 cm tight-lid Petri dishes containing 3.2 ml of 0.7% Kallestad agarose dissolved in 0.15 M-NaCl, 0.01 M-phosphate buffer pH 7.0.

Western blot. Antigens were separated by SDS-PAGE and transferred to nitrocellulose paper electrophoretically (Towbin *et al.*, 1979). Antigens were detected by reaction with gold-labelled antibody (Hsu, 1984).

Immunogold staining of thin sections. Pieces of infected leaves were fixed, sectioned and stained as described by Lin & Langenberg (1983). Preliminary tests showed that antiserum to the M_r 66000 protein stained cell walls of healthy wheat. It was, therefore, adsorbed with a preparation of cell walls prepared by grinding uninfected wheat leaves in 1% Triton X-100 in water. The extract was centrifuged for 25 min at 12000 r.p.m. to pellet cell walls which were then washed several times by resuspension in water.

SDS-PAGE. Proteins were solubilized in dissociation buffer and separated on 12%, $1.5 \times 150 \times 150$ mm polyacrylamide slab gels with a discontinuous buffer system (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue and then scanned for absorbance at 550 nm to make quantitative estimates of the amounts of protein in the bands using known amounts of bovine serum albumin in the same gel as standards.

RESULTS

Purification

Purification procedures were evaluated by gel electrophoresis. The procedure specified above was the best of those tested for consistency in yield and purity of the M_r 66000 protein. Fifteen grinding buffers and several centrifugation schedules were tested. Equal yields were obtained from extracts of frozen or fresh leaves, and from frozen or fresh extracts of unfrozen leaves. Preparations from leaves ground in 0.5 M-sodium orthoborate pH 7 to 9, or 0.5 M-potassium phosphate pH 7.0 contained nearly as much M_r 66000 protein as those made in standard buffer, but often contained more contaminating protein. Since a protein of M_r 66000 would not rapidly sediment at 12000 r.p.m. the protein must be a part of a large particle, such as an inclusion body, before SDS treatment. Numerous unsuccessful attempts were made to purify the presumed inclusion body further by centrifugation through cushions or gradients of various salts, combinations of salts and sucrose, sucrose, and combinations of sucrose and urea. A typical SDS-PAGE analysis of a 12000 r.p.m. pellet prepared by the standard procedure is shown in Fig. 1(a), lane 2, and compared to a similar preparation from uninfected plants, Fig. 1(a), lane 1.

The purified protein had an apparent M_r of 65000 to 66000 in 12% polyacrylamide gels. Some preparations contained proteins that migrated as expected for dimers and trimers and which were absent in control preparations from uninfected plants.

The M_r 66000 protein was not detected by SDS-PAGE in uninfected wheat plants nor in those infected with soil-borne wheat mosaic or wheat spindle streak mosaic viruses, nor in barley (*Hordeum vulgare* L., cv. 'Larker') that was uninfected or infected with barley stripe mosaic virus. It was found in extracts of WSMV-infected maize (inbred line N28), and of six WSMV-infected experimental wheat cultivars randomly selected from a resistance-screening nursery. It was also present in plants infected with any of five different isolates of WSMV. The apparent molecular weight was the same for all isolates of virus. Extracts of leaves less than one-third expanded contained less of the M_r 66000 protein than extracts of older leaves. No consistent variation of yield from fully expanded leaves of different ages was noted.

The yield of M_r 66000 protein frequently was approximately 100 μg per g of fully expanded young leaves, based on Coomassie Brilliant Blue staining of SDS-PAGE-separated protein with bovine serum albumin as a standard.

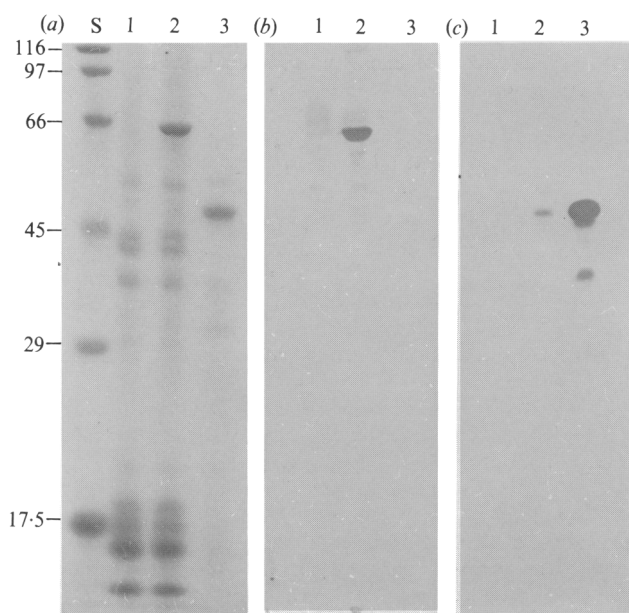


Fig. 1. Detection of proteins separated by SDS-PAGE and detected by Coomassie Brilliant Blue staining and by immunogold staining of Western blots. Lane S contains protein standards. Lanes 1 represent a 12000 r.p.m. pellet from an extract from uninfected plants processed by the procedure given in Methods. Lanes 2 represent a 12000 r.p.m. pellet from an extract from WSMV-infected plants. Lanes 3 represent WSMV virions purified by centrifugation through one sucrose gradient (Brakke & Ball, 1968). Lanes in (a) were stained by Coomassie Brilliant Blue, in (b) by antibody to M_r 66000 protein, and in (c) by antibody to WSMV virions.

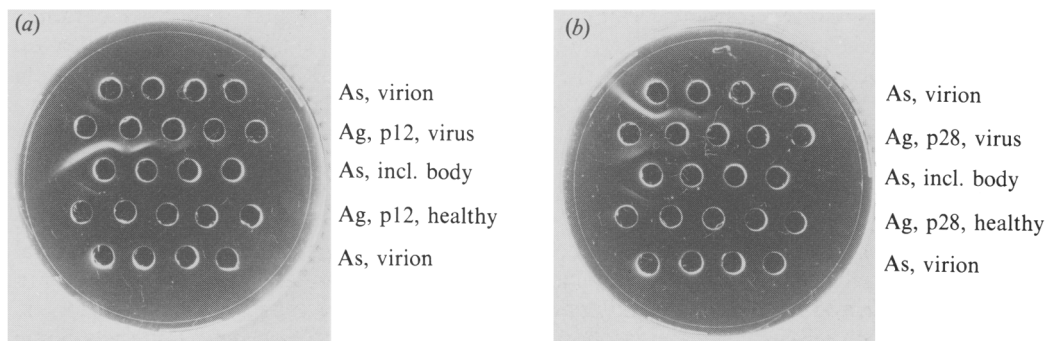


Fig. 2. Agar double diffusion detection of WSMV virion and M_r 66000 proteins. Each well of the top and bottom rows contained 5 μ l of undiluted antiserum (As) to virions and each of the centre wells contained 5 μ l of undiluted antiserum to the M_r 66000 inclusion body protein. In (a) the antigen (Ag) was the 12000 r.p.m. pellet (p12) from either virus-infected or healthy plants. The pellets were suspended in dissociation buffer (see Methods) at 100 μ l per g leaves and heated at 100°C for 2 min. Serial four-fold dilutions were placed in each successive well starting with the well at the left. In (b) the antigen (p28) was the pellet obtained by centrifuging the supernatant of p12 for a further 2 h at 28000 r.p.m. to pellet virions. The pellet was suspended in dissociation buffer, heated, and diluted in fourfold steps. No reaction was observed with normal serum.

Serology

Antiserum to the M_r 66000 protein reacted strongly in agar diffusion tests with both a 12000 r.p.m. and a 28000 r.p.m. pellet of an extract from infected plants but only weakly with similar extracts from healthy plants (Fig. 2). Both the 12000 r.p.m. pellet and the 28000 r.p.m. pellet

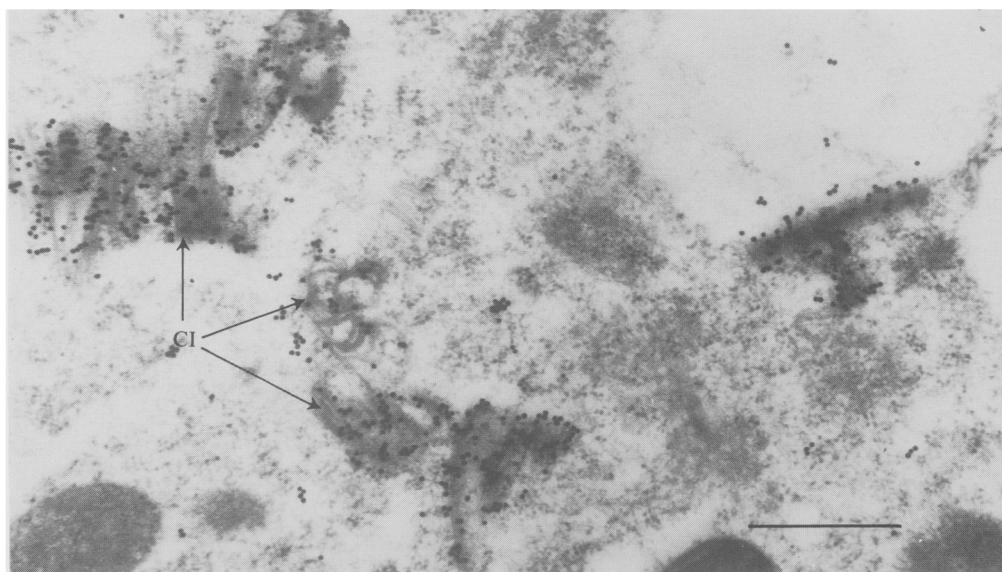


Fig. 3. Thin section of wheat cell infected with WSMV after staining with gamma globulin from antiserum to M_r 66000 protein which had been adsorbed with a cell wall preparation from uninfected wheat plants. The gamma globulin bound to antigen in the section was detected by gold-labelled goat antibodies to rabbit globulin. The cylindrical inclusions (CI) are stained, showing the presence of M_r 66000 protein. Bar marker represents 500 nm.

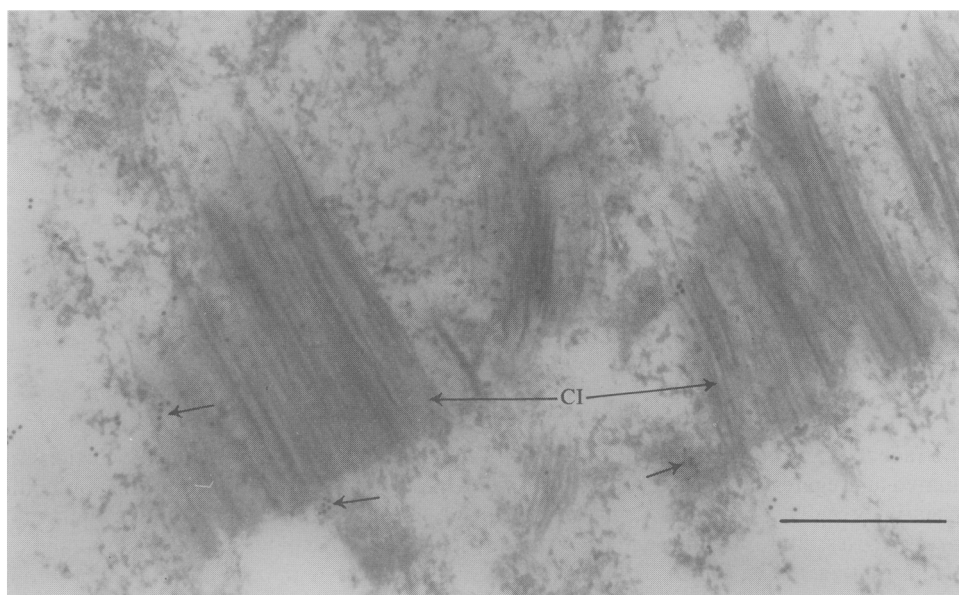


Fig. 4. Thin section of a wheat cell infected with wheat spindle streak mosaic virus after staining with gamma globulin from antiserum to WSMV M_r 66000 protein which had been adsorbed with a cell wall preparation from uninfected wheat plants. The gamma globulin bound to antigen in the section was detected by gold-labelled goat antibodies to rabbit globulin. A few gold particles (arrows) typical of background staining may be observed, but cylindrical inclusions (CI) are not stained more heavily than background. Bar marker represents 500 nm.

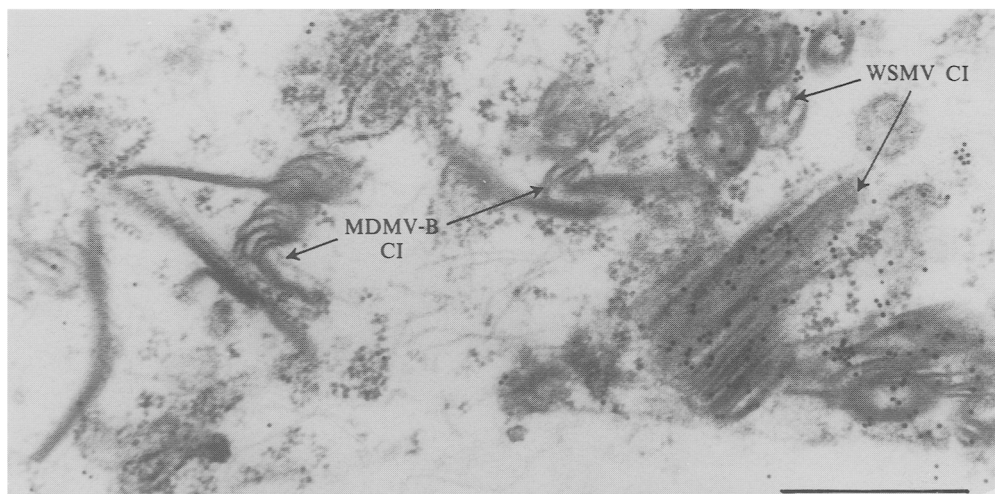


Fig. 5. Thin section of a maize cell from a leaf infected with both WSMV and MDMV-B. The cell was stained with gamma globulin from antiserum to WSMV M_r 66000 protein as in Fig. 3. The numbers of gold particles over the inclusions at the left and in the centre (MDMV-B CI) are no higher than background, whereas a large number of gold particles covers the inclusion bodies at the right (WSMV-CI). Bar marker represents 500 nm.

reacted with both antisera, i.e. to virions and to M_r 66000 protein, showing that both capsid protein and M_r 66000 protein were present in both pellets although in different proportions.

In Western blots, the antibodies to M_r 66000 protein reacted with the M_r 66000 protein but not with capsid protein. Conversely, antibodies raised to virions reacted with capsid protein but not with M_r 66000 protein (Fig. 1). The unadsorbed M_r 66000 antiserum reacted slightly with the large subunit of ribulose biphosphate carboxylase and also to a M_r 66000 protein of uninfected plants. This latter protein is apparently a cell wall protein since unadsorbed antiserum stained cell walls in thin sections. This reaction disappeared after adsorption with a cell wall preparation from uninfected plants. The antibodies to the M_r 66000 protein specifically stained pinwheel inclusions and laminated aggregates in thin sections (Fig. 3). Sections of uninfected cells were not stained (data not shown). The specificity of the staining is also shown by the lack of staining of other parts of the cell by the antibody to the M_r 66000 protein (Fig. 3), and lack of staining of wheat spindle streak mosaic virus-induced cylindrical inclusions by antiserum to the WSMV M_r 66000 protein (Fig. 4). Furthermore, these antibodies did not stain inclusions in maize cells infected with MDMV-B (data not shown). The antibodies did stain some inclusions, but not others, in maize cells infected with both WSMV and MDMV-B (Fig. 5). The unstained inclusions are morphologically similar to those in cells infected with MDMV-B only.

DISCUSSION

The fact that antibodies to the M_r 66000 protein specifically stain pinwheel inclusions and laminated aggregates shows that this protein is a component of virus-induced inclusion bodies. The relatively high concentration of the M_r 66000 protein suggests it is a major component of the inclusion bodies, but a definitive answer to this question will depend on chemical analysis of highly purified inclusion bodies. The inclusion bodies also stain with antibody to virions, though to a lesser extent than with antibody to M_r 66000 protein, showing that capsid protein or virions are also components of, or adsorbed to, inclusion bodies (Langenberg, 1986). The absence of a reaction between the antisera to M_r 66000 protein and capsid protein, and between antisera to virions and the M_r 66000 protein (Fig. 1), shows that the two proteins are not serologically related and that staining of inclusion bodies by both antisera does indeed indicate presence of both proteins.

The antibodies to the M_r 66000 protein precipitated six times as much of the *in vitro* translation products of WSMV RNA as did normal serum (J. Joshi & M. K. Brakke, unpublished). This result, together with the lack of staining of cylindrical inclusions found in wheat infected with wheat spindle streak mosaic virus and in maize infected with MDMV-B, suggests that the M_r 66000 protein is virus-coded. The function of the inclusion bodies and the M_r 66000 protein is unknown, but the fact that it is present in higher concentration than virions (approx. 100 $\mu\text{g/g}$ for M_r 66000 compared to 10 to 40 $\mu\text{g/g}$ for virions) suggests that the protein does not function as an enzyme for synthesis of virion components. It could serve a structural function within the inclusion body, possibly to modify the cell cytoskeleton to adapt it for virus synthesis and/or translocation. A role for the cytoskeleton in replication of animal viruses has been postulated (e.g. Weed *et al.*, 1985). The heavy staining of some inclusions, and lack of staining of others (Fig. 5), suggests a considerable specificity in the assembly of these inclusions from their protein subunits. The stained inclusions must contain the WSMV-coded M_r 66000 protein, whereas the unstained inclusions presumably are composed of a MDMV-B-coded protein.

The M_r 66000 protein could be purified from fully expanded green leaves regardless of their age. In contrast, WSMV virions are readily purified only from the two youngest, fully expanded wheat leaves (Brakke & Ball, 1968). The difficulty in purifying virions from older leaves lies partly in the stability of plant proteins and other components in these leaves to the denaturing conditions of the purification procedure, and partly to changes in virions with leaf age. The capsid protein from purified WSMV is usually partly degraded (e.g. see lane 3, Fig. 1*a*). The extent of degradation increases with leaf age, and sometimes none of the intact (apparent M_r 46000 in 12% gels) capsid protein remains (M. K. Brakke & L. C. Lane, unpublished). The instability of the virions and of the capsid protein together with their low concentration makes accurate assay of virions difficult. The assays of virions reported earlier (Brakke & Ball, 1968) and of the M_r 66000 protein reported here indicate the amounts that can be extracted, not necessarily the amounts synthesized or present in cells. The accuracy of the estimation is further limited by the variability in dye binding from protein to protein (e.g. see Sedmak & Grossberg, 1977).

The M_r 66000 protein of WSMV is similar in size to the cylindrical inclusion body proteins of potyviruses (Hollings & Brunt, 1981). It differs in size from rapidly pelleting, presumed inclusion body proteins of wheat spindle streak mosaic virus (protein subunit M_r 70000; unpublished data) and soil-borne wheat mosaic virus (M_r 90000; Hsu & Brakke, 1985). These proteins are potentially useful for virus identification, either by serology or by SDS-PAGE of partially purified preparations. Their relative abundance makes them readily detectable by SDS-PAGE of material pelleted by low speed centrifugation of extracts treated with non-ionic detergents.

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