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PURIFICATION OF BLACKGRAM MOTTLE VIRUS (BMoV) USING MAGNESIUM-BENTONITE

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Key Word Index—*Phaseolus vulgaris*; Fabaceae; blackgram; RNA virus; purification; coat protein; electron microscopy.

Abstract—Blackgram mottle virus was purified from French bean (*Phaseolus vulgaris*) cv. Contender tissue using two different methods involving differential centrifugation. The use of Mg-bentonite during purification produced a homogeneous and pure virus preparation. The M_r of RNA and coat protein were 1400 and 39k, respectively. The RNA was infectious at a concentration as low as 7 μg ml^{-1}. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Blackgram mottle virus (BMoV) has been reported to infect urad bean, mung bean, French bean and soybean in India and southeast Asia [1–5]. It has so far not been assigned to any virus group. Scott and Phatak [6] reported the purification of this virus by a differential centrifugation method. However, in spite of adequate precautions taken by us during purification, the same procedure resulted in a degraded virus preparation. In this report we describe an improved method using Mg-bentonite for purification of the virus and the molecular characterization of the purified virus.

RESULTS AND DISCUSSION

The major aim of any virus purification process is to eliminate the host cell constituents, especially proteases and nucleases, besides obtaining good yield [7–9]. In the present investigation, of the two methods employed to obtain intact and pure virus, Method I, involving two cycles of differential centrifugation, yielded a virus with A_{260}/A_{280} ratio 1.55, identical to the one reported by Honda *et al.* [2], but differed from the value given by Scott and Phatak [6]. The estimated yield of the virus ($E^{0.1}_{260nm}=0.5$) was *ca* 7.0 mg per 100 g tissue. The electron microscopy studies showed no variations in particle morphology. However, the purified virus showed two major polypeptides, with M_r 36 and 31k, respectively, on SDS–PAGE (Fig. 1, lanes 2 and 4) as against a single polypeptide subunit (M_r 38.2k) of coat protein suggested earlier by Scott and Phatak [6]. The reason could be either degradation of the virus during purification or contamination by host components. Incubation of intact virus particles with trypsin (1:25 ratio) for 4 hr resulted in complete disappearance of 31k polypeptide, accompanied by a few minor polypeptides (Fig. 1, lanes 3 and 5). This suggests the presence of trypsin susceptible sites on the surface of virus particles. Electroblot immunoassay (EBIA) of the virus protein using the BMoV-antiserum confirmed the origin of various polypeptides to virus protein itself (Fig. 2). Purification and repeated freezing and thawing of tobacco etch virus (poty virus) has also been reported to result in a doublet of 30 and 27k and a smaller band of 24k instead of a single 30k band [10].

In spite of all precautions taken, when analysed on 2.4% polyacrylamide–7 M urea gels the purified viral RNA did not produce any discrete RNA band, instead a smear was observed on every attempt. Scott and Phatak [6] also reported that the nucleic acid extracted with SDS–phenol from BMoV showed several components on polyacrylamide gels, but with Lane's dissociation buffer they reported the appearance of only one component. No explanation for this effect has been given. It could be suggested that the cleavage of coat protein during virus purification by Method I resulted in exposure of viral nucleic acid to the contaminating ribonucleases [11].

Dunn and Hitchborn [12] have reported that Mg-bentonite helps in reducing the contaminating ribonucleases and proteolytic enzymes. The Method II

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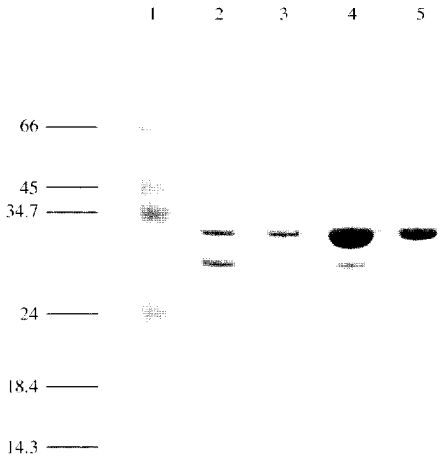

Fig. 1. SDS-PAGE of blackgram mottle virus (BMoV) protein. The virus purified by Method I was electrophoresed at 200 V for 4.5 hr. Lane 1: M_r markers (k) from top to bottom. Bovine serum albumin (66), egg albumin (45), pepsin (34.7), trypsinogen (24), β-lactoglobulin (18.4) and lysozyme (14.3); lane 2: 10 μg BMoV protein (preparation 1); lane 3: undissociated virus (10 μg) (preparation 1) incubated with trypsin (0.5 μg) for 4 hr before dissociation; lane 4: 10 μg of purified virus protein (preparation 2); lane 5: 10 μg purified virus (preparation 2) was incubated with trypsin (0.5 μg) for 4 hr before dissociation.

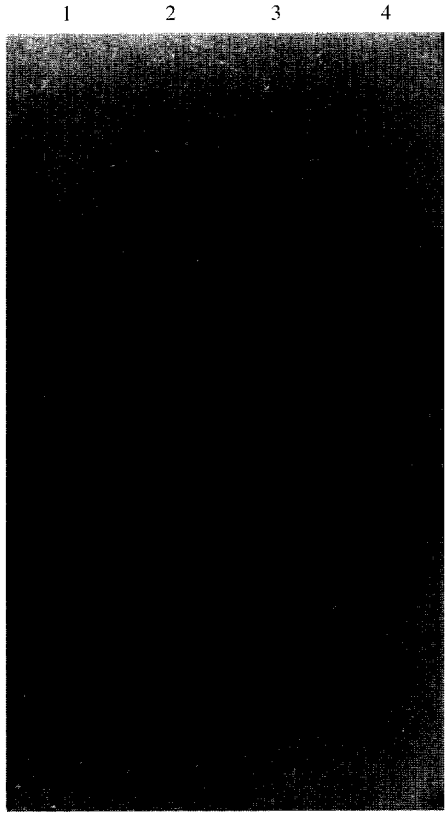

Fig. 2. Electroblot immunoassay of BMoV protein after SDS–PAGE. The protein after electrophoresis as in Fig. 1 was blotted on to a nitrocellulose membrane for 1.5 hr at 20 V. The blot was probed with anti-BMoV serum at 1:500 dilution (1 μl of antiserum for 500 μl of blocking solution) and developed by alkaline phosphatase mediated enzyme reaction. Lane 1: 10 μg of purified virus protein (preparation 1); lane 2: 10 μg of purified virus (preparation 1) digested with trypsin (25:1 ratio) before dissociation; lane 3: 10 μg of purified virus protein preparation 2; lane 4: 10 μg of purified virus from preparation 2 digested with trypsin before dissociation.

involving the use of Mg-bentonite before one-step differential centrifugation resulted in homogeneous, intact virus particles of uniform size (28 nm) and shape (Fig. 3). The UV absorption spectrum (210–300 nm) was typical of a nucleoprotein with absorption maximum at 260 nm and absorption minimum at 245 nm. The A_{260}/A_{280} ratio of 1.62 indicated that 20% of the virus particle was nucleic acid. The virus yield was around 6 mg per 100 g of leaf tissue. The lower yield as compared with Method I could possibly be due to the adsorption of virus on Mg-bentonite [12].

The purified preparation when analysed on SDS-PAGE produced a single polypeptide band of M_r 39k (Fig. 4, lane 2). Incubation of intact virus with trypsin (1:25 ratio) for 4 hr produced no effect (Fig. 4, lane 3). However, incubation of virus, after dissociation with Cleveland's buffer [13], with trypsin for 20 min resulted in complete digestion of 39k polypeptide to 34k polypeptide (Fig. 4, lane 4). Repeated freezing and thawing of the purified virus did not result in any degradation, indicating it to be a stable preparation.

Time course studies involving the limited proteolysis of dissociated BMoV by trypsin (25:1) showed that there are five or six preferred sites of tryptic cleavage which become available separately during proteolytic attack, resulting in a corresponding number of peptide products (Fig. 5).

BMoV nucleic acid isolated by Method II produced a typical A_{260}/A_{280} ratio of 2.3, and an average yield of 0.1 mg g^{-1} of virus. Various physicochemical studies such as the orcinol test and formaldehyde denaturation test indicate it to be an ssRNA virus.

The BMoV RNA was found to be infective at tested concentrations as low as 7 μg ml^{-1}. Purified RNA when analysed on 2.4% polyacrylamide–7M urea gel (Fig. 6, lane 2) produced a single RNA band of M_r 1400k, confirming the presence of an unsegmented single stranded RNA in the viral genome. Frequent freezing and thawing again did not affect the quality of the RNA. The present investigation shows that by using Mg-bentonite during the purification process, besides producing a homogeneous virus preparation, the purification time is also reduced.

EXPERIMENTAL

BMoV was cultured on French bean cv. Contender under glasshouse conditions. Infected leaves (16 days

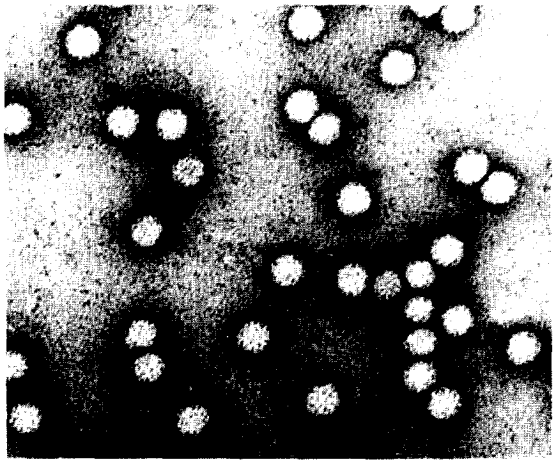

Fig. 3. Transmission electron micrograph of BMoV (× 140 000) purified by Method II. About 2 μl of sample containing purified virus (3 μg) was spotted on the grid, stained with 2% uranyl acetate and observed under a Jeol-100 CX-11 transmission electron microscope.

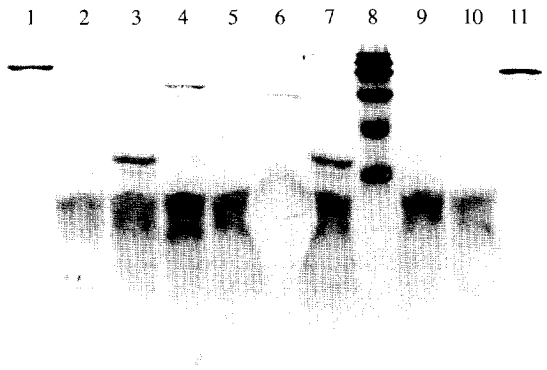

Fig. 5. Time course study of trypsin digestion of dissociated BMoV protein. The virus (42 μg), purified by Method II, was dissociated with an equal volume of 2 × Cleveland buffer at 100° for 3 min and digested with trypsin at 37°. At specified time intervals, samples (6 μg) were withdrawn and made 10% in 2-mercaptoethanol and 2% in SDS before denaturing and loading on to the gel. Lane 1: control; lane 2: 15 min; lane 3: 10 min; lane 4: 5 min; lane 5: 20 min; lane 6: trypsin; lane 7: 10 min; lane 8: marker proteins; lane 9: 30 min; lane 10: 40 min; lane 11: undissociated BMoV (6 μg) incubated with trypsin (0.24 μg) for 24 hr.

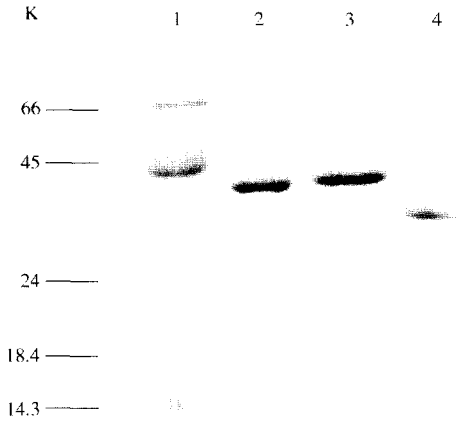

Fig. 4. SDS–PAGE of BMoV. The virus purified by Method II was electrophoresed at 100 V for 3 hr at room temperature after dissociation. Lane 1: M_r markers as described in Fig. 1; lane 2: 5 μg of blackgram mottle virus protein; lane 3: 6 μg undissociated BMoV was incubated with trypsin (0.24 μg) for 4 hr before dissociation; lane 4: 6 μg of BMoV dissociated in Cleveland buffer and digested with trypsin (0.24 μg) for 20 min.

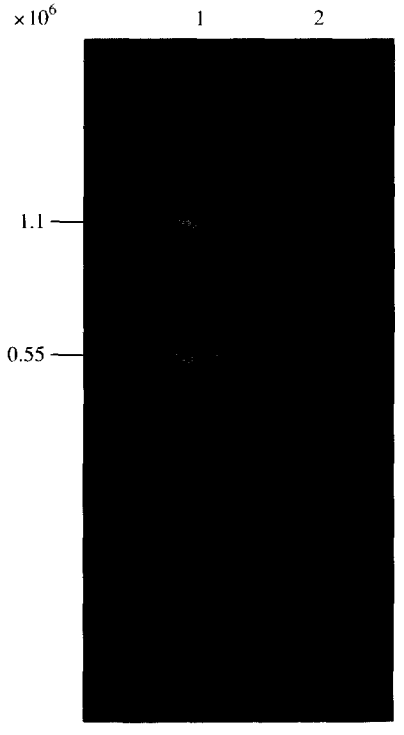

Fig. 6. Polyacrylamide–urea gel electrophoresis of purified BMoV RNA. The RNA samples, after denaturing at 5° for 5 min in loading buffer, were loaded on 2.4% polyacrylamide–7 M urea gel and electrophoresed at 40 V for 10 hr and stained with ethidium bromide. Lane 1: 3 μg of *E. coli* RNA composed of 23S, 1.1×10^6 and 16S, 0.55×10^6 γRNA purified as described in ref. [23]; lane 2: 1.5 μg of purified BMoV RNA.

after inoculation) were harvested and used for virus purification.

Purification. Infected leaves were homogenized in 0.2 M Na-Pi buffer (pH 7.2)–0.1 M ascorbic acid (1:1) followed by clarification with an equal vol. of *n*-BuOH–$CHCl_3$ (1:1) and centrifugation at 5000 *g* for 10 min. The aq. phase was collected and processed by 2 slightly different methods. Method I involved 2 cycles of differential centrifugation (9000 *g*/10 min and

80 000 *g*/1 hr) [6]. Method II was as follows. The aq. phase, after storing overnight at room temp., was treated with Mg-bentonite (100:1) [12]. The contents, after thorough mixing, were centrifuged at 3500 *g* for 10 min. The resulting supernatant was subjected to centrifugation (80 000 *g* for 1 hr). The pellet was subjected to rate zonal centrifugation on a sucrose density gradient (10–40%) at 93 000 *g* for 75 min. The light scattering band was collected, dialysed and the virus pelleted at 80 000 *g* for 1 hr. The virus pellet was dissolved in 0.01M Na-Pi buffer (pH 7.2). Samples were examined under Jeol-100 CX-11 transmission electron microscope after staining with 2% uranyl acetate. Spectrophotometric studies were conducted by determining the absorption spectrum of purified virus between 230 and 300 nm followed by protein [14] and nucleic acid estimation [15].

Nucleic acids. Viral nucleic acid was purified by SDS–EDTA disruption of the virus followed by proteinase-K treatment and phenol–$CHCl_3$ extraction [16]. The type of nucleic acid was determined by employing the orcinol test for RNA [17] and diphenylamine test for DNA [18].

Polyacrylamide–urea gel electrophoresis of RNA. Nucleic acid was electrophoresed on 2.4% polyacrylamide–7 M urea gels buffered with 1 × TBE [19] (5 × TBE stock, pH 8.3, containing 0.445 M Tris, 0.445 M boric acid and 0.01 M EDTA). RNA samples were mixed with loading buffer (1 × TBE, 10 M urea, 20% glycerol and 0.001% bromophenol blue) in the ratio 1:2 (v/v) and heated at 55° for 5 min. After electrophoresis for 7 hr at 60 V, the gel was stained with ethidium bromide (0.5 $\mu g\ ml^{-1}$).

SDS–PAGE. Virus particles were dissociated and denatured with sample buffer (60 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS and 5% 2-mercaptoethanol) and subjected to 1D SDS–PAGE [20].

EBIA. EBIA was performed after SDS–PAGE as in refs [21, 22] using nitrocellulose membrane and alkaline phosphatase conjugated antibodies (GAR^{XAP}). Antiserum against BMoV was prepd by immunizing rats with purified virus. The polyclonal antiserum so obtained was used at 1:500 ratio (1 μl antigen/500 μl blocking soln).

Trypsin digestion. The procedure in ref. [13] was followed. Dissociated and undissociated virus prepns were digested at 37° with trypsin at a virus–trypsin ratio of 25:1 and subjected to SDS–PAGE.

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