

Etiology

Physicochemical Properties of Bean Rugose Mosaic Virus

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We wish to acknowledge the technical help of Reynaldo Pereira, Mayra L. Taylor, and Mark Coffey. We also express our appreciation to Rodrigo Gámez for advice and for critically reading the manuscript. This research was supported by the Vicerrectoría de Investigación of the Universidad de Costa Rica.

Accepted for publication 25 February 1987 (submitted for electronic processing).

ABSTRACT

Ramírez, P., Espinoza, A. M., Fuentes, A. L., and León, P. 1987. Physicochemical properties of bean rugose mosaic virus. *Phytopathology* 77:1317-1321.

Susceptible bean cultivars infected with bean rugose mosaic virus (BRMV) yield three sedimentation components in isopycnic and zonal gradients. The top component contains empty shells; the middle and bottom components, both required for infection, contain distinct RNA molecules of 1.4 and 2.1×10^6 , respectively. One large (41 K) and two small polypeptides (21 and 23 K) are found in all components. The 21 K

polypeptide appears to be derived by proteolytic cleavage from the 23 K polypeptide. In addition, each RNA contains a genome-bound peptide (VPg) on the 5' end and a poly-A tail on the 3' end of the molecule. All of these properties clearly support the inclusion of BRMV in the comovirus group, as previously proposed on the basis of immunoreactivity.

Bean rugose mosaic virus (BRMV) is a plant virus serologically related to several members of the comovirus group (2,11). A Costa Rican strain was the first to be described (11,13), and later, other related strains were found in Central and South America (3,10,20). BRMV is the only comovirus currently recognized in beans in Latin America (12). It is transmitted by chrysomelid beetles in the genera *Diabrotica* and *Cerotoma* and produces systemic infections in some cultivars of *Phaseolus vulgaris* L., localized lesions in others, and no visible symptoms or detectable virus in the so-called "immune cultivars." Resistance to virus infection is determined by

alleles that segregate in Mendelian fashion in crosses between susceptible and immune bean cultivars (21). BRMV is readily transmitted mechanically but is apparently not transmitted through seed (12). Some viral strains cause a severe mosaic with rugosities and deformation of leaves and form large, dense inclusions in the cytoplasm of susceptible cells; other strains induce milder symptoms and ultrastructural alterations in infected plants (16).

We report several of the physicochemical properties of BRMV particles, capsid polypeptides, and nucleic acids, using the Costa Rican strain BRMV-CR. In both genome and capsid properties, this strain strongly resembles other members of the comovirus group (2,28).

MATERIALS AND METHODS

Virus strains and maintenance. BRMV-CR, kindly provided by R. Gámez in our laboratory, was propagated in the México 80-R cultivar of *P. vulgaris*. Primary leaves were inoculated mechanically as described previously (12) and harvested 15 days later, soon after symptoms were first detected.

Isolation of virus. BRMV was isolated from 250-g batches of leaves washed with tap water and cut into small sections. An equal amount (w/v) of 0.01 M phosphate buffer, pH 7, was added to the cut material, which was then ground in a blender. The material was sieved through cheesecloth, clarified by centrifugation, and taken to 0.3 M NaCl and 8% polyethylene glycol (PEG 8000, Sigma). After 2 hr at 4 C, the virus was pelleted at $10,000 \times g$ for 15 min, resuspended, and clarified by centrifugation. This procedure was repeated three times. The last pellet was resuspended in 30 ml of 0.01 M phosphate buffer, pH 7, and centrifuged through a 40% sucrose cushion and then into a linear 10–40% sucrose gradient, using a Hitachi refrigerated ultracentrifuge (19). Virus concentration was determined by ultraviolet (UV) absorbance in a Hitachi 200-20 spectrophotometer, assuming an extinction coefficient of $7 \text{ (mg/ml)}^{-1} \text{ cm}^{-1}$ at 260 nm for unfractionated virus (12).

Analytical ultracentrifugation. The buoyant densities of BRMV were determined by equilibrium centrifugation in CsCl of virus from purified sucrose gradient bands (19). Gradient bands were collected by upward displacement aided by an ISCO UA-5 monitor, and refractive indices were determined with a Bausch &

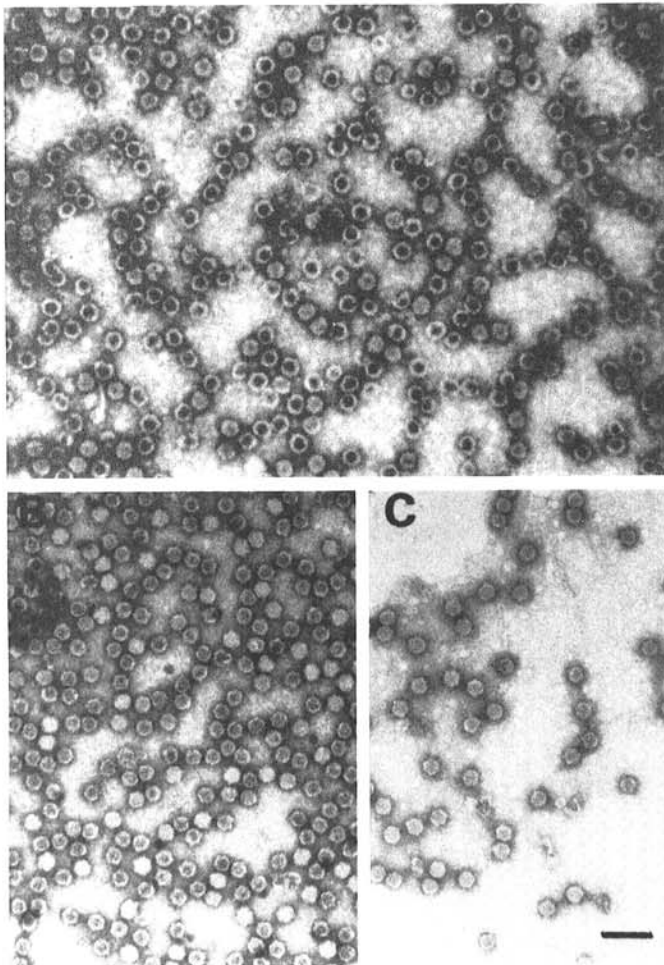


Fig. 1. Peaks of ultraviolet absorbance from a sucrose gradient were isolated and samples stained with 1% aqueous uranyl acetate. **A**, Top, **B**, middle, and **C**, bottom components photographed at the same magnification in a Hitachi HU12A electron microscope. Scale bar = $0.1 \mu\text{m}$.

Lomb refractometer. Percent RNA was estimated from the particle density according to Sehgal et al (25).

Electrophoresis of proteins. Viral proteins were analyzed by SDS-PAGE using 12.5 or 15% acrylamide in the separating gel, according to the procedure of Laemmli (17). Gels were stained with Coomassie brilliant blue, and molecular weights were estimated with several molecular weight markers (bovine serum albumin, 66 K; IgG heavy chain, 50 K; albumin tryptic digest, 46 K; ovalbumin, 45 K; myoglobin, 17 K; and lysozyme, 14 K [all from SIGMA]).

Analysis of nucleic acids. Viral nucleic acids were isolated as described by Ramírez (24), basically by two extractions with water-saturated phenol followed by treatment with ether and precipitation with 0.1 M NaCl and three volumes of cold 95% ethanol. Before RNA was analyzed, it was washed with 70% ethanol to remove excess NaCl. It was then loaded onto denaturing agarose gels containing formaldehyde and formamide (18,22). The unfixed gels were stained with toluidine blue (0.01 M sodium acetate, 0.001 M magnesium acetate, pH 5.5, and 0.05% toluidine blue).

To determine the presence of genome-bound proteins, RNA purified from unfractionated middle and bottom components was labeled with ^{125}I in the presence of chloramine-T as described by Daubert et al (5,6). Aliquots of the labeled nucleic acid were digested with proteinase K at $0.45 \mu\text{g}/\mu\text{l}$ and pancreatic ribonuclease at $0.09 \mu\text{g}/\mu\text{l}$, then separated in agarose and acrylamide gels. TMV RNA was used as a negative control. Autoradiographs of the gels were obtained by applying Kodak X-omatic film overnight to the gels.

Purified RNA was fractionated in oligo(dT)-cellulose columns (Bethesda Research Labs, Bethesda, MD) to determine the presence of poly-A tails as described by Maniatis et al (22), then analyzed in the denaturing agarose gels.

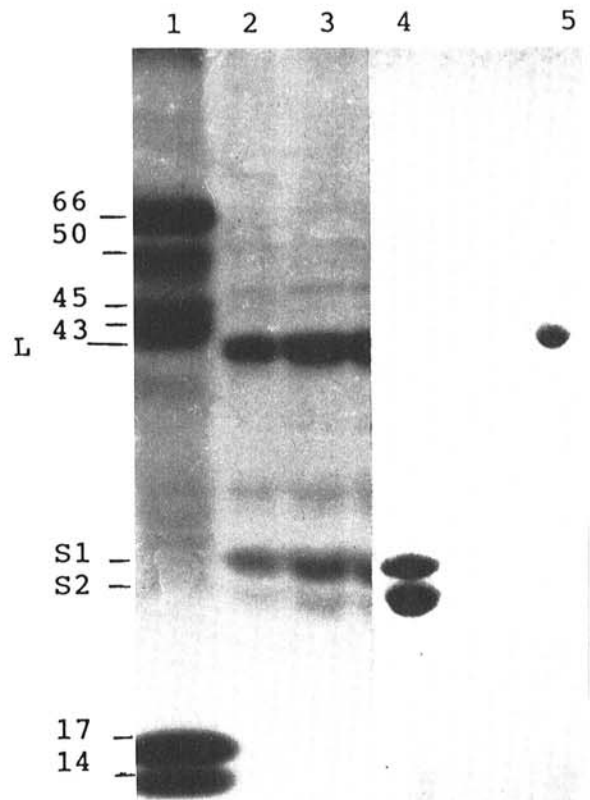


Fig. 2. Polyacrylamide gels (12.5%) of viral proteins stained with Coomassie blue. In lane 1, molecular weight markers were included. In lanes 2 and 3, 5 and 10 μg of purified capsids were added, respectively. In lanes 4 and 5, reruns of the S1 and L1 polypeptides were analyzed, respectively, after elution from the first gel and heating in boiling water bath for 2 min in sample buffer.

RESULTS

Properties of particles. Isolated virus separated into three components in zonal sucrose gradients, visible as light-scattering zones that coincided with peaks of UV absorbance. In CsCl gradients at pH 7.2, three bands were also seen, but the middle component was usually split into two overlapping peaks. The buoyant density and other properties of each component are provided in Table 1, and electron microscopy of each peak appears in Figure 1. From the buoyant density, we estimated a percentages of RNA of 0, 29, and 36 for top, middle, and bottom components, respectively.

Empty shells were usually present in different preparations but in varying amounts, depending on the age of infected leaf material. Preliminary observations indicated an increase in top component with time after infection. The proportion of the two heavier particles determined by UV absorbance of the gradients is also variable; the middle component is more abundant in most preparations and is slightly contaminated with particles that contain the larger RNA molecule, as observed in denaturing agarose gels.

The bean cultivar *Orguloso*, in which the virus produces local lesions, was used to test the infectivity of the three components obtained from CsCl gradients. The top component was not infectious, whereas an equimolar mixture of middle and bottom components produced the highest number of lesions. The number of local lesions scored in three separate trials were: top = 0, middle = 28 (standard deviation [SD] = 2), bottom = 1 (SD = 1), and middle + bottom = 98 (SD = 11).

Properties of capsid proteins. Empty and full capsids were analyzed by electrophoresis in denaturing acrylamide gels. In SDS-PAGE, all centrifugal components contained three polypeptide bands that stained with Coomassie blue (Fig. 2) and with periodic acid-Schiff (data not shown, indicates that the proteins are glycosylated). The slowest band has an apparent molecular weight of 41,000, and the two smaller ones, molecular weights of 23,000 and 21,000 (Fig. 2). These are known respectively as L, S1, and S2. The S1 band, obtained from acrylamide gels and treated with proteases according to Cleveland et al (4) or heated (2 min in boiling water), generated the S2 polypeptide band (Fig. 2).

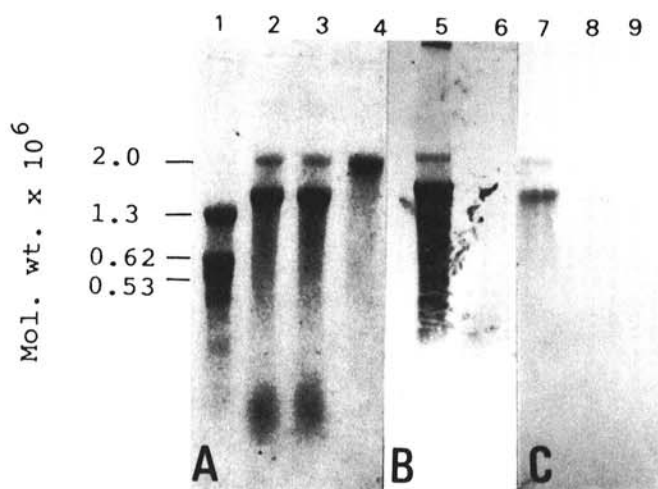


Fig. 3. Denaturing agarose gels of **A**, purified BRMV RNA (lanes 2 and 3) with maize nuclear and chloroplast ribosomal RNA (1.3×10^6 , 0.6×10^6 , and 0.5×10^6 , lane 1) and TMV RNA (2×10^6 , lane 4) as molecular weight markers, stained with toluidine blue. The diffuse, high-mobility bands in lanes 2 and 3 are degradation products often detected. **B**, Autoradiograph of viral nucleic acids labeled with ^{125}I using chloramine T. RNA from BRMV (lane 5) and from TMV, which lacks a genome-associated peptide (lane 6), are compared. **C**, RNAs recovered from an oligo(dT)-cellulose column. Lane 9 contains the flow-through fraction, and lanes 7 and 8, the first two eluted fractions with the RNA that was retained in the column.

Also, the relative quantity of the S2 polypeptide changed with storage.

Properties of nucleic acids. Nucleic acids isolated from the middle and bottom component showed a UV hyperchromicity after treatment with pancreatic RNase, but not with DNase I (data not shown). Absorbance ratios between 1.9 and 2.1 at 260/280 nm were obtained for total, purified RNA. In denaturing formaldehyde-formamide agarose gels, two RNA bands appeared with estimated molecular weights of 2.1 and 1.4×10^6 (Fig. 3A). After the unfractionated RNA were labeled with ^{125}I , using conditions that iodinate only polypeptides (6), both molecules were strongly labeled, whereas TMV RNA treated similarly was not (Fig. 3B). To test the possibility that the labeled polypeptide was a contaminating capsid protein, we used a 15% acrylamide gel to compare the sizes of the capsid and labeled bands. With this system, the large RNA molecules barely entered the gel (Fig. 4A). However, RNase digestion destroyed the RNA band and the radioactivity, then migrated slightly behind the buffer front but ahead of the capsid polypeptides (Fig. 4B). Proteinase K caused the radioactivity to migrate with the buffer front. Again, only trace amounts of label were associated with the TMV RNA (Figs. 3B and 4B). These experiments support the notion that the two RNA molecules in BRMV contain a genome-bound peptide.

A large fraction of BRMV RNA (>60%) bound to oligo(dT)-cellulose columns. Analysis of the bound fraction revealed that both viral RNA molecules were retained by the column, whereas the flow-through fraction did not contain intact RNA molecules (Fig. 3C).

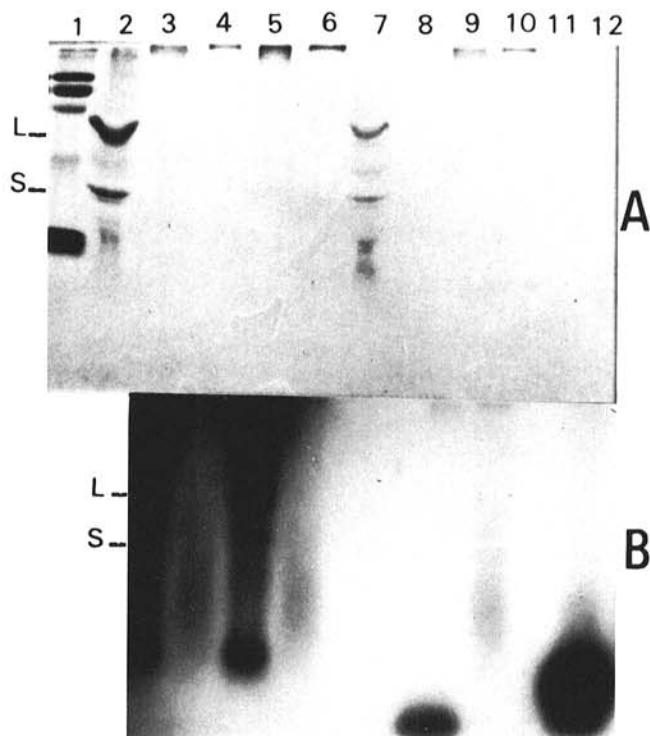


Fig. 4. Iodinated RNA was analyzed in a 15% SDS-PAGE to determine if the labeled polypeptides comigrated with the capsid proteins L and S. **A**, Coomassie blue-toluidine blue stained gel with molecular weight markers in lane 1 and BRMV capsid proteins in lanes 2 and 7. As expected for these gels, most of the RNA remains near the origin of the separating gel (see lanes 3-6). **B**, Autoradiograph of lanes 3-12 from the same gel. In lanes 3 and 5, unheated and heated-labeled BRMV RNAs were respectively included, beside TMV RNA treated in the same way (lane 4, unheated sample, and lane 6, heated sample). All the radioactive label migrated with the buffer front after digestion with proteinase K (lane 9). Treating the BRMV with pancreatic RNase (lane 12) also allows the label to migrate into the gel, much farther than the capsid proteins, but behind the buffer front. In lanes 10 and 11, iodinated TMV RNA was treated in the same way as the BRMV RNA, with proteinase K and ribonuclease, respectively. Lane 8 was loaded with proteinase K alone.

DISCUSSION

The main physicochemical characteristics of BRMV-CR are similar to those described for other members of the comovirus group (Table 1), including the type member, cowpea mosaic virus (CPMV) (2,29), and two serologically related viruses, cowpea severe mosaic virus (CPSMV) and bean pod mottle virus (BPMV) (7,26). A closely related Colombian strain of BRMV, BRMV-CO (1), was also compared. The Costa Rican and Colombian strains of BRMV are similar in buoyant density properties and in the size of the proteins and RNAs, but these properties alone are not sufficient to discriminate between members of different serogroups (9).

The two glycosylated polypeptides in the capsid, L and S, are found in approximately equimolar amounts. These are presumably assembled as 60 dimers in a T = 1 icosahedral shell, as in other comoviruses (14). A third protein band (S2 polypeptide) with an estimated molecular weight of 21,000 is apparently produced by proteolysis or by conformational alteration of S1. This is based on the detection of S2 polypeptide in gels loaded with S1 polypeptide extracts from gels (Fig. 2) and on similar peptide maps generated by both polypeptides (L. Taylor, *unpublished*).

A top component that lacks RNA is occasionally recovered from infected leaves, indicating that the shell is stabilized primarily through protein-protein interactions, although empty capsids more easily dissociated during isolation and, in many preparations, are not obtained. Estimated total molecular weights for the top, middle, and bottom components are 3.8, 5.2, and 5.9×10^6 , respectively, based on the molecular weights of the proteins and the RNAs. These values correlate well with the sedimentation coefficients of the components (12). The percentage of RNA in the middle and bottom components, estimated from the buoyant densities, also agrees closely with the expected values based on the mass of the individual components, assuming the capsid structure mentioned above.

TABLE 1. Physicochemical properties of BRMV and related comoviruses^a

Property	BRMV-CR	BRMV-CO	CPMV	CPSMV	BPMV
Density in CsCl					
Top ^b	1.293	1.305	1.288	nr ^b	nr
Middle					
1	1.401	1.395	1.395	(1.386) ^c	nr
2	1.414	1.412	nob ^d		nr
3	nob	1.428	nob		nr
Bottom					
1	1.450	1.470	1.406	1.44 ^e	nr
2	nob	nob	1.447		nr
S _w , 20					
Top	59 ^f	nr	58	58	54
Middle	97	nr	95	95	91
Bottom	113	nr	115	115	112
%RNA					
Middle					
1	29	29	25	nr	30
2	31	31	nr	nr	nr
Bottom	36	38	36	nr	nr
Mol wt of proteins (K)					
L	41	40	37	nr	nr
S1	23	25	22	nr	nr
S2	21	22	nr	nr	nr
Mol wt of RNAs ($\times 10^6$)					
RNA 1	2.1	nr	2.02	nr	2.5
RNA 2	1.4	nr	1.37	nr	1.5

^aOur data are averages of five repetitions. References: BRMV (1), CPMV (29), CPSMV (7), and BPMV (26).

^bTop, middle, and bottom components; nr = not reported.

^cDensity in parentheses indicates that a range of densities is reported from 1.386 to 1.404 as the pH is raised from 6.5 to 8.5.

^dnob = Not observed.

^eBottom component is reported to band at two densities (1.403 and 1.443) at pH 6.5 but only at 1.443 at pH 8.5.

^fFrom Gámez (12).

In isopycnic buoyant density gradients of comoviruses, overlapping double peaks are usually reported for the middle and bottom components (Table 1). BRMV-CR, under the conditions tested, showed a bimodal middle component, whereas in BRMV-CO, both top and middle components are bimodal. Buoyant density studies indicate that the appearance of multiple peaks depends on chelating agents, ion types, and pH of the gradient solution (1,15). The absence of multiple peaks in sedimentation studies and the unimodal distribution of each viral RNA in electrophoretic separations support the conclusion that the multiple buoyant density components result from differences in hydration and conformation of the capsid proteins.

Two different RNAs are isolated from the middle and bottom components. In CPMV and other comoviruses, each RNA has an associated peptide at the 5' end and a polyadenylated sequence at the 3' end of the molecule (5,6,8,23,27). We were therefore interested in determining if BRMV shared these properties. The denaturing formaldehyde-formamide gel used showed no changes in mobilities of BRMV RNAs after protease treatment. However, by iodinating the isolated nucleic acids under conditions where only amino acids react, clear evidence for genome-bound peptides (VPg) was obtained (Fig. 3B). In acrylamide gels (Fig. 4B), we determined that the labeled peptide is not a contaminating capsid protein. Our results also indicate that both of the RNAs from BRMV contain a poly-A tail, because they bind to oligo(dT)-cellulose columns.

Physicochemical properties of plant viruses are very useful for establishing the major viral phylogenies, but they discriminate little at lower taxonomic levels when related strains are compared. Sequence studies will be needed to discover virus evolutionary relationships at the lower taxonomic levels.

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