# Proteinase K-sensitive Factor Essential for the Infectivity of Southern Bean Mosaic Virus Ribonucleic Acid

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## ABSTRACT

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Treatment with Proteinase K reduced the infectivity of southern bean mosaic virus ribonucleic acid (SBMV-RNA) but not that of tobacco mosaic virus RNA. The structural integrity of the viral RNAs remainded unaffected after the Proteinase K treatment. The Proteinase K-sensitive moiety was associated intimately with the SBMV genome.

Southern bean mosaic virus (SBMV) is an isometric (diameter 30 nm), ribonucleic acid (RNA)-containing plant virus with a single stranded genome of $1.4 \times 10^6$ daltons (11). Purified SBMV loses infectivity when exposed to 60–65 C for 10 min with no detectable alterations in structural integrity or serologic reactivity (Veerisetty and Sehgal, *unpublished).* SBMV-RNA isolated from the heat-inactivated virions with the disruption medium (0.1 *M* tris-[hydroxymethyl]-amino methane [Tris]-HCl buffer, pH 7.5, 10 mM ethylenediamine tetraacetic acid [EDTA], and 1% sodium dodecyl sulfate [SDS]) at 25 C for 1 hr possessed low infectivity, but it was highly infectious when isolated with the disruption medium at 55 C for 5–10 min. The low specific infectivity of RNA isolated at 25 C from the heat-inactivated SBMV is probably due to the binding of some coat protein to the genome and its disengagement from the RNA may be essential for infectivity. Our attempts to enhance the infectivity of such RNA with Proteinase K were unsuccessful. We discovered, however, that the infectivity of the RNA isolated from the nonheated SBMV was abolished with Proteinase K. This report presents evidence that a Proteinase K-sensitive moiety is associated intimately with SBMV-RNA and is essential for its infectivity. The infectivity of tobacco mosaic virus (TMV) RNA, however, is not affected by Proteinase K.

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## MATERIALS AND METHODS

SBMV (bean strain) was purified according to Hsu et al (6) and TMV was isolated by the method of Francki (4). SBMV-RNA was isolated by treating virions with EDTA and SDS at pH 7.5 (6).

TABLE 1. Infectivity of southern bean mosaic virus (SBMV) virions, SBMV-ribonucleic acid (SBMV-RNA), and tobacco mosaic virus RNA (TMV-RNA) after Proteinase K treatment[a]

| Inocula | Inoculum concentration at assay (μg/ml) | Proteinase K concentration during incubation (μg/ml) | Infectivity[b] | | |
|---|---|---|---|---|---|
| | | | Primary leaves inoculated (no.) | Local lesions (no.) | Lesions/leaf |
| SBMV virions[c] | 1.0 | none | 8 | 673 | 84.1 |
| | | 1.0 | 10 | 892 | 89.2 |
| | | 10.0 | 14 | 1302 | 93.0 |
| SBMV + EDTA[d] | 50.0 | none | 12 | 244 | 20.3 |
| | | 1.0 | 12 | 53 | 4.5 |
| | | 10.0 | 12 | 12 | 1.0 |
| SBMV + EDTA + SDS[e] | 50.0 | none | 6 | 143 | 24.0 |
| | | 0.1 | 6 | 0 | 0 |
| | | 10.0 | 6 | 0 | 0 |
| Isolated SBMV-RNA[f] | 20.0 | none | 18 | 256 | 15.0 |
| | | 0.1 | 18 | 3 | 0.2 |
| | | 1.0 | 18 | 0 | 0 |
| Isolated TMV-RNA[g] | 50.0 | none | 13 | 716 | 55.1 |
| | | 5.0 | 13 | 643 | 49.0 |

[a]Proteinase K treatment was performed at 25 C for 1 hr.
[b]Infectivity assays were done on the primary leaves of *Phaseolus vulgaris* 'Pinto.' In each experiment the inocula were assayed on four to eight primary leaves.
[c]Virions were treated at a concentration of 50 μg/ml, diluted with 0.1 M Tris-HCL buffer pH 7.5, and then assayed.
[d]SBMV (500 μg/ml) in 0.1 M Tris-HCl buffer, pH 7.5, containing 10 mM EDTA, maintained at 25 C for 1 hr prior to the Proteinase K treatment.
[e]SBMV (500 μg/ml) in 0.1 M Tris-HCl buffer, pH 7.5, containing 10 mM EDTA and 0.1% sodium dodecyl sulfate, maintained at 25 C for 1 hr, prior to Proteinase K treatment.
[f]SBMV-RNA at 60 μg/ml was treated with Proteinase K and then diluted with 0.1 M Tris-HCl buffer, pH 7.5.
[g]TMV-RNA at a concentration of 150 μg/ml was treated with Proteinase K, diluted with 0.1 M Tris-HCl buffer, pH 7.5, and then assayed.

Alternatively, an equal volume of 4.0 M LiCl (pH 8.0) was added to the EDTA-treated SBMV and the samples were frozen (−17 C) for 15 hr (3). After these samples were thawed at 25 C, the precipitated RNA was recovered by centrifugation (8,000 *g*, 20 min, 5 C), washed with 2.0 M LiCl and dissolved in 0.1 M Tris-HCl buffer, pH 7.5. For isolating TMV-RNA, virions (~1 mg/ml) in 0.1 M Tris-HCl buffer, pH 7.5, containing 10 mM EDTA and 1% SDS were emulsified with two volumes of neutralized phenol (90%) and centrifuged (5,000 *g*) to break the emulsion. The RNA in the aqueous phase was precipitated with three volumes of cold 95% ethanol and suspended in the Tris-HCl buffer. SBMV- and TMV-RNA preparations showed a maximum absorption at 260 nm and had a minimum absorption at 232 nm, with an $A_{260}/A_{232}$ ratio of 2.0–2.2. The infectivity of SBMV-RNA or TMV-RNA was lost completely with 0.1 μg/ml pancreatic ribonuclease (25 C, 1 hr) indicating the absence of any nuclease-resistant entities in these preparations. RNA concentration was determined spectrophotometrically ($E_{260}^{0.1\%} = 25.0$).

Proteinase K (EM Laboratories, Elmsford, NY 10523) was dissolved (1.0 mg/ml) in 0.1 M Tris-HCl buffer, pH 7.5. In preliminary studies, the Proteinase K-treated (25 C, 1 hr) SBMV-RNA was precipitated with ethanol, and after low speed centrifugation (8,000 *g*, 20 min, 5 C), the precipitate was suspended in 0.1 M Tris-HCl buffer and assayed for infectivity. Subsequent experiments showed that the ethanol precipitation step was unnecessary. Consequently, after appropriate dilutions the RNA was assayed directly for infectivity or analyzed by sucrose gradient centrifugation. Isolated SBMV-RNA exposed to the various reagents to denature its secondary structure was precipitated with ethanol, prior to subsequent characterization. The recovery of such treated viral RNA was ~95–100%. All bioassays were performed on the primary leaves of *Phaseolus vulgaris* L. 'Pinto' in the presence of 50 mg/ml Celite (10). The inocula were kept ice-cold during inoculations.

## RESULTS AND DISCUSSION

SBMV treated with Proteinase K in the presence of EDTA at pH 7.5 yielded an apparently intact(~25–30S) RNA (Fig. 1A). Further, the RNA released from the virions with EDTA and SDS was not fragmented in the presence of Proteinase K (Fig. 1B). Additionally, the sedimentation profiles of the isolated SBMV- and TMV-RNAs with and without Proteinase K treatment were identical (Fig. 1 C–F). These results show that structural integrity of the SBMV-and TMV-RNAs remained unaltered after the protease treatment. In the absence of EDTA, SBMV virions remained structurally intact when exposed to Proteinase K (25 μg/ml).

SBMV-RNA and TMV-RNA were treated (25 C, 1 hr) with Proteinase K (5 μg/ml), formamide was then added (final concentration, 50%), and the samples were heated at 55 C for 5 min.

TABLE 2. Infectivity of southern bean mosaic virus ribonucleic acid (SBMV-RNA) after selected treatments

| Treatments[a] | Temperature and duration | RNA concn at assay (μg/ml) | Infectivity[b] | |
|---|---|---|---|---|
| | | | Control | Treated |
| SBMV-RNA in buffer (control) | 60 C, 10 min | 20 | 233/5[c] | 205/5 |
| in 8.0 M urea | 60 C, 10 min | 20 | 309/11 | 288/11 |
| in 0.1% SDS | 60 C, 10 min | 20 | 138/4 | 127/4 |
| in 0.5 M urea, 1% SDS, and 1% β-mercaptoethanol | 60 C, 10 min | 20 | 289/13 | 306/13 |
| in 50% formamide and 25% dimethyl sulfoxide | 25 C, 1 hr | 20 | 509/17 | 524/17 |

[a]SBMV-RNA at a concentration of 350 μg/ml was treated as indicated. The treated, as well as the control RNAs, were precipitated with cold ethanol and suspended in 0.1 M Tris-HCl buffer, pH 7.5
[b]Infectivity assays were performed on the primary leaves of *Phaseolus vulgaris* 'Pinto.' In each assay the control and the treated RNAs were inoculated on the opposite primary leaves of the same plant.
[c]Numerator = no. of local lesions induced; denominator = no. of the primary leaves inoculated. In each test four to six primary leaves were employed.

These samples were analyzed in formamide-containing sucrose gradients. The enzyme-treated SBMV- and TMV-RNAs sedimented as homogenous entities with no evidence of fragmentation. These experiments were performed to detect any hidden "nicks" for the protease-treated RNAs, but none were found.

No decline was detected in the infectivity of SBMV virions after treatment with Proteinase K (Table 1). The infectivity of SBMV-RNA was abolished if treated with Proteinase K in the presence or absence of SDS in the incubation mixture. When the effect of Proteinase K on the isolated SBMV- and TMV-RNAs was compared, the infectivity of SBMV-RNA was lost with 0.1 μg/ml Proteinase K while the infectivity of TMV-RNA remained largely unaffected, even by a 50-fold greater enzyme concentration. Other studies showed that SBMV-RNA (70 μg/ml) was rendered noninfectious when treated with 1.0 μg/ml Proteinase K at 25 C for 10 min.

SBMV-RNA heated at 60 C for 10 min in the presence of 0.1% SDS or 8 M urea or 0.5 M urea plus 1% SDS plus 1% β-mercaptoethanol possessed the same specific infectivity as the control RNA (Table 2). Similarly, no loss of SBMV-RNA infectivity occcurred when treated (25 C, 1 hr) with 50% formamide plus 25% dimethyl sulfoxide. These results suggest that the Proteinase K-sensitive moiety is associated intimately with the SBMV-RNA.

Covalently-linked genome proteins have been identified in many animal and bacterial viruses and in some, specific sites on the genome for protein interaction have been established (eg, 1,7,9,12). Several roles have been ascribed to such proteins, including as initiators of the genomic infection and/or replication processes as well as of a direct involvement in virion morphogenesis. Among plant viruses, the genomes of cowpea mosaic virus and the nepoviruses possess covalently-linked proteins (2,5,8). The role of protein complexed with cowpea mosaic virus RNAs is unknown; however, it is neither essential for infectivity nor for in vitro RNA translation (12). For nepoviruses, the protein linked to the RNAs is needed for infectivity (5). Our results demonstrate that in SBMV, a single-RNA-component virus, the genomic infectivity is dependent also upon the presence of a protease-sensitive moiety. We have learned (P. Kaesberg, *personal communication*) that a protein covalently linked to the 5′ terminus of SBMV-RNA has recently been recognized. The precise function of the genome-linked protein in the biology or structure of SBMV, however, remains to be established.

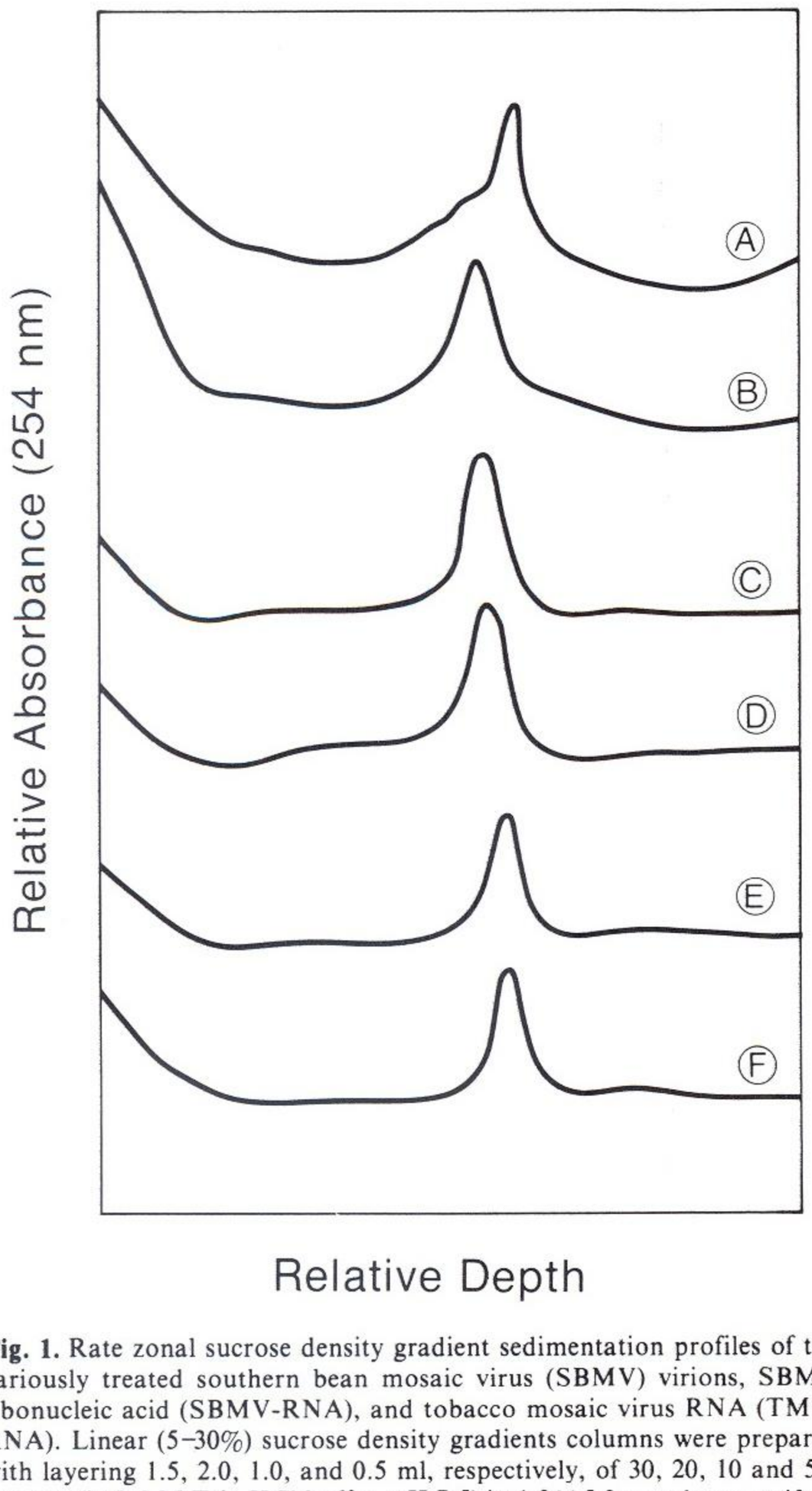


**Fig. 1.** Rate zonal sucrose density gradient sedimentation profiles of the variously treated southern bean mosaic virus (SBMV) virions, SBMV ribonucleic acid (SBMV-RNA), and tobacco mosaic virus RNA (TMV-RNA). Linear (5–30%) sucrose density gradients columns were prepared with layering 1.5, 2.0, 1.0, and 0.5 ml, respectively, of 30, 20, 10 and 5% sucrose (in 0.1 M Tris-HCl buffer, pH 7.5) in 1.3 × 5.0-cm ultracentrifuge tubes and maintained at 5 C for 18 hr. A 0.2-ml sample containing 4.5 μg RNA was layered on the gradient and centrifuged at 45,000 rpm (Beckman SW 50.1 rotor) for 2.5 hr at 10 C in a Beckman L3-50 ultracentrifuge. The gradients were fractionated and monitored at 254 nm with an ISCO UA-5 analyzer. **A,** SBMV (100 μg/ml) in 0.1 M Tris-HCl buffer, pH 7.5, plus 10 mM ethylenediamine tetraacetic acid (EDTA) maintained at 25 C for 1 hr and then treated with 10 μg/ml Proteinase K. **B,** As in (A), but 0.1% sodium dodecyl sulfate (SDS) was included in the Tris-EDTA buffer. **C,** SBMV-RNA isolated with the EDTA: LiCl:freezing method, untreated. **D,** As in (C), but treated with 10 μg/ml Proteinase K. **E,** TMV-RNA isolated with phenol plus SDS, untreated. **F,** As in (E), but treated with 10 μg/ml Proteinase K. Proteinase K treatment was performed at 25 C for 1 hr.

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