Identification of a New Serotype and Antigenic Relationships Among Six Strains of Red Clover Necrotic Mosaic Virus

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ABSTRACT

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The antigenic relationships of three red clover necrotic mosaic virus (RCNMV) strains—Eng (England), Aus (Australia), and Can (Canada)—were compared with the three established serotypes of the virus—RCNMV-TpM 34 (serotype A), RCNMV-TpM 48 (serotype B), and RCNMV-Sw (serotype C)—by immunodiffusion tests. These tests differentiated RCNMV-Can from serotypes A and C but not from serotype B. It was demonstrated that both RCNMV-Eng and RCNMV-Aus constitute a new serotype, D, since they were serologically indistinguishable and differed from the three serotypes of the virus. By means of intragel cross-absorption experiments, antibodies specific for each serotype were demonstrated. These tests also showed that RCNMV-Aus antiserum contained heterospecific antibodies reacting specifically with serotypes B and C. Coat proteins from six RCNMV strains migrated as a single component in polyacrylamide gel corresponding to polypeptides with a molecular weight of about 40,000 daltons. Polyacrylamide gel electrophoretic analysis of coat proteins partially digested with V8 protease revealed significant differences between RCNMV-Sw (serotype C) and other strains of the virus. Similar analysis with chymotrypsin indicated that the coat proteins of RCNMV-Eng and RCNMV-Aus were identical and differed from the others. The antigenic and other properties of RCNMV serotypes are discussed in relation to taxonomic identity.

Additional key word: dianthoviruses

Red clover necrotic mosaic virus (RCNMV) is one of the three principal members of the dianthovirus group (10). Several strains of RCNMV from various geographic regions are serologically related, but considerable antigenic variation is known to occur (7). Recently, three serotypes of the virus—A (RCNMV-TpM 34), B (RCNMV-TpM 48), and C (RCNMV-Sw)—were identified (12). In the present study, we compared the antigenic relationships of three additional strains of the virus (RCNMV-Eng, RCNMV-Aus, and RCNMV-Can) with the three established serotypes, and we report the identification of a new serotype.

MATERIALS AND METHODS

Virus isolates. The following virus strains were used in this study: RCNMV-TpM 34 and RCNMV-TpM 48 (12), RCNMV-Sw (4), RCNMV-Eng (1), RCNMV-Aus (5), and RCNMV-Can (14).

Virus purification and preparation of antisera. All RCNMV strains were maintained and propagated in *Phaseolus vulgaris* L. 'Red Kidney'. The procedure used for the purification of all RCNMV strains was as described by Gould et al (5). Virus preparations used for immunization of rabbits were subjected to at least two cycles of sucrose density gradient centrifugation as a final purification step. Each adult rabbit received two intramuscular injections 1 wk apart containing 1 mg each of virus emulsified with an equal volume of Freund's complete adjuvant. A third injection containing 2 mg of virus was administered intravenously 2 wk later, and the animals were bled at least 1 wk after the final injection. The antisera to RCNMV-Can (14), RCNMV-Aus, RCNMV-Eng, and RCNMV-Sw were produced in our laboratory (Table 1) and none of these antisera reacted with healthy plant sap. Antisera to RCNMV-TpM 34 and RCNMV-TpM 48 were supplied by M. Musil, Institute of Virology, Bratislava, Czechoslovakia.

Serological methods. Immunodiffusion tests were done in agar (13), and intragel cross-absorption tests were as described by Van Regenmortel (16,17). In agar double-diffusion tests, antisera titers were obtained by testing twofold serial dilutions of an antiserum against a constant concentration (0.5 mg/ml) of the homologous or heterologous antigen. The titer of an antiserum was considered to be the highest dilution that produced visible precipitin lines when the plates were observed against a dark background.

Protein analysis. Protein samples prepared by boiling purified virus preparations in urea, SDS, and 2-mercaptoethanol were subjected to polyacrylamide gel electrophoresis (9). The gels were stained with Coomassie Blue.

Peptide analysis of protease-treated viral coat proteins. Viral coat proteins were partially digested with V8 protease or chymotrypsin and mapped by polyacrylamide gel electrophoresis (2). The gels were stained with silver (19).

RESULTS

Serological relationships. The homologous and heterologous titers of six RCNMV antisera are presented in Table 1. Antisera produced against RCNMV-Eng and RCNMV-TpM 34 reacted with the homologous as well as with one heterologous antigen, RCNMV-Aus. By contrast, antisera of RCNMV-Aus, RCNMV-TpM 48, RCNMV-Can, and RCNMV-Sw reacted with their homologous antigens as well as with all heterologous antigens. The relationships between RCNMV-Eng and RCNMV-Aus and between RCNMV-TpM 48 and RCNMV-Can were considered to be very close, since their heterologous titers were within twofold dilutions of each other. Similarly, RCNMV-TpM 34 was closely related to RCNMV-Eng, but not to RCNMV-Aus. On the other hand, RCNMV-Sw and RCNMV-TpM 48 were serologically distantly related to each other.

The antigenic relationships among the RCNMV strains were further examined by arranging the immunodiffusion patterns to determine the spur formation among all possible antigen pairs

(Fig. 1). Anti-RCNMV-Eng serum contained antibodies reacting with heterologous antigens RCNMV-TpM 34 and RCNMV-Aus (Fig. 1A). No definite serological relationship was observed between anti-RCNMV-Eng serum and the other three heterologous antigens, RCNMV-TpM 48, RCNMV-Can, and RCNMV-Sw (Table 1; Fig. 1A–C). In this respect, anti-RCNMV-Eng serum was analogous to anti-RCNMV-TpM 34 serum (Fig. 1J–L). However, the precipitin lines formed by anti-RCNMV-Eng serum with homologous antigen showed a distinct spur against RCNMV-TpM 34 (Fig. 1C). The complete fusion of the precipitin lines occurred between RCNMV-Eng and RCNMV-Aus when tested against either homologous or heterologous antisera (Figs. 1A and D). On the other hand, the antibody composition of RCNMV-Aus appeared to be different from that of RCNMV-Eng in that it contained antibodies reacting with all heterologous antigens (Table 1; Fig. 1D–F). Formation of definite spurs by anti-RCNMV-Aus serum occurred with homologous antigen and four heterologous antigens (Fig. 1D–F). The most interesting observation in these tests was the pattern of crossing or bilateral spurs between RCNMV-Sw and RCNMV-TpM 34 and between RCNMV-Sw and RCNMV-Eng (Fig. 1E).

Similar immunodiffusion tests also demonstrated that the anti-RCNMV-Can serum, like anti-RCNMV-Aus serum, reacted with all heterologous antigens (Fig. 1G–I). That RCNMV-Can has its own specific antigenic determinant was clearly illustrated when heterologous antigens were allowed to react with anti-RCNMV-Can serum (Fig. 1G–I). Formation of distinct spurs by the homologous antigen was observed for RCNMV-Can. Complete fusion of precipitin lines occurred with heterologous antigen RCNMV-TpM 48 (Fig. 1H and I). Reverse tests were also done with anti-RCNMV-TpM 34 serum (Fig. 1J–L), anti-RCNMV-TpM 48 serum (Fig. 1M–O), and anti-RCNMV-Sw serum (Fig. 1P–R). As reported previously (12), unilateral cross-reaction was observed among antisera of RCNMV-TpM 34, RCNMV-TpM 48, and RCNMV-Sw and their heterologous antigens (Table 1; Fig. 1J–R). The reactions between anti-RCNMV-TpM 48 serum and five other heterologous antigens (Fig. 1M–O) were analogous to anti-RCNMV-Can serum (Fig. 1G–I). Distinguishable patterns were also observed when anti-RCNMV-Sw serum reacted with heterologous antigens. As observed with anti-RCNMV-Aus serum (Fig. 1E), the precipitin lines formed by anti-RCNMV-Sw serum with the homologous antigens crossed with heterologous antigens, RCNMV-Eng and RCNMV-TpM 34 (Fig. 1Q). Distinct spurs between the homologous and heterologous antigens were observed for RCNMV-Sw (Fig. 1P–R).

In an effort to unravel the qualitative differences between heterologous reactivities of the sera (Table 1), a series of intragel cross-absorption tests was performed. The results (Table 2; Fig. 2) confirmed the existence of such differences very clearly and established the close relationships between RCNMV-Eng and RCNMV-Aus and between RCNMV-TpM 48 and RCNMV-Can. Furthermore, the results indicated that RCNMV-Eng and RCNMV-Aus constitute a new serotype, D, whereas RCNMV-Can is serologically identical with serotype B (TpM 48). These tests also revealed an interesting phenomenon associated with anti-RCNMV-Aus serum. Following the absorption of anti-RCNMV-

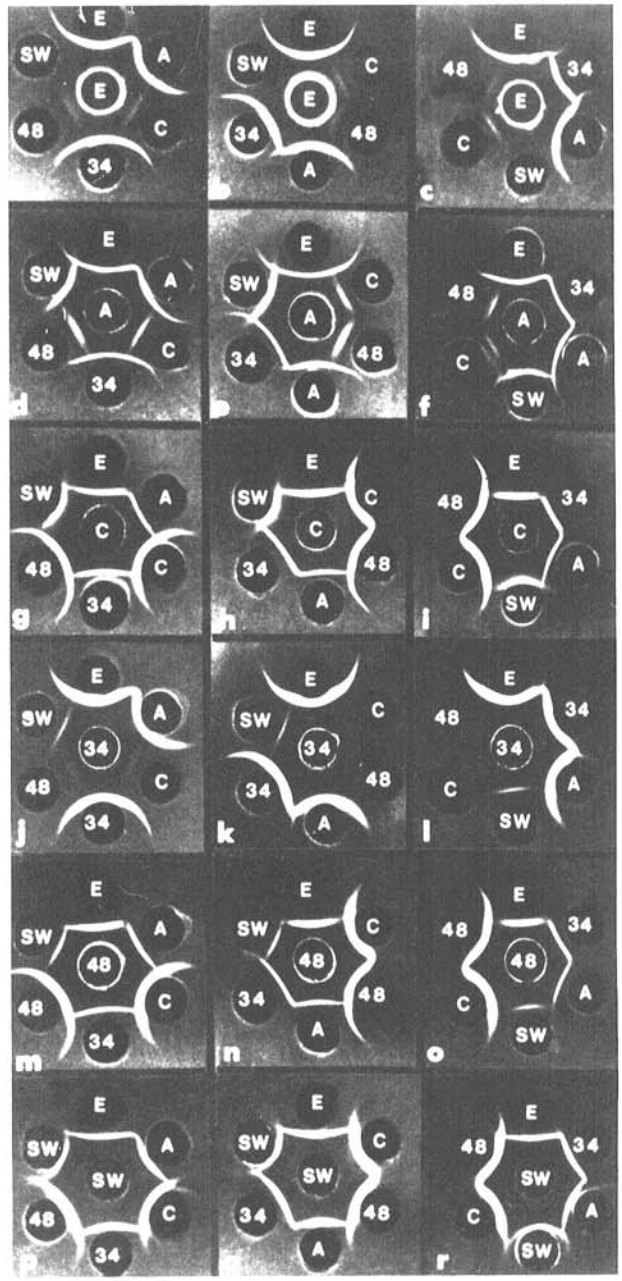

Fig. 1. Serological reaction in immunodiffusion plates. Center wells contained undiluted antiserum to red clover necrotic mosaic virus (RCNMV)-Eng (E) **(A–C),** RCNMV-Aus (A) **(D–F),** RCNMV-Can(C) **(G–I)**, RCNMV-TpM 34 (34) **(J–L)**, RCNMV-TpM 48 **(M–O)**, and RCNMV-Sw (Sw) **(P–R)**. Peripheral wells were charged with purified antigens (0.5 mg/ml) of RCNMV-Eng (E), RCNMV-Aus (A), RCNMV-Can (C), RCNMV-TpM 34 (34), RCNMV-TpM 48 (48), and RCNMV-Sw (Sw).

TABLE 1. Homologous and heterologous titers and precipitin patterns of antisera to six strains of red clover necrotic mosaic virus (RCNMV)

Antiserum	Antigen					
	RCNMV-TpM 34	RCNMV-TpM 48	RCNMV-Sw	RCNMV-Aus	RCNMV-Eng	RCNMV-Can
RCNMV-Eng	128[a]	−/n	−/−	512/c	*512*	−/n
RCNMV-Aus	8/s	128/n?	32/s	*256*	128/c	128/n?
RCNMV-Can	8/s?	256/c	8/s	64/s	8/s	*256*
RCNMV-TpM 34	*1,024*	−/n	−/−	4/s	256/s or c	−/n
RCNMV-TpM 48	16/s	*512*	4/s	16/s	16/n	512/c
RCNMV-Sw	16/s	128/s	*1,024*	8/s	32/?	16/?

[a] Titer when dilution series of each serum was reacted with each antigen (0.5 mg/ml)/ precipitin patterns of homologous with heterologous antigen. Pattern c = coalescence (complete fusion); n = faint, straight heterologous reaction not touching homologous reaction; s = partial fusion (spur of homologous over heterologous); and − = no reaction.

Aus serum with either homologous or heterologous antigen, RCNMV-Eng, all the antibodies specific to the homologous antigens were completely removed (Table 2). However, anti-RCNMV-Aus serum still contained antibodies that specifically reacted with heterologous antigen, RCNMV-TpM 48, RCNMV-Sw, and RCNMV-Can (Table 2; Fig. 2B). Such antibody fractions, referred to as *heterospecific* (17), were not present in other antisera (Table 2).

Polyacrylamide gel electrophoresis of viral coat proteins. Proteins were dissociated from highly purified preparations of six RCNMV strains and analyzed by polyacrylamide gel electrophoresis (Fig. 3A). All preparations contained one major band with electrophoretic mobility corresponding to a polypeptide with a molecular weight of about 40,000 (±1,000) daltons. Similar estimates were obtained from four different electrophoresis experiments. In addition to the major polypeptide band, at least one minor band, migrating faster than the major band, was detected in all preparations. To determine the nature of this minor band, each RCNMV strain was centrifuged in sucrose density gradients and each gradient was divided into 15 fractions. When each fraction was analyzed by polyacrylamide gel electrophoresis, the minor bands were associated only with fractions containing the virus (*data not shown*), indicating that none of these minor polypeptides are of host origin. Furthermore, peptide mapping showed that the minor polypeptide is a degradation product of the major polypeptide (see below).

Comparison of viral proteins by peptide mapping. Polyacrylamide gel electrophoresis was performed following partial digestion of coat proteins from six RCNMV strains with V8 protease and chymotrypsin. The protein from each RCNMV strain yielded several peptides that migrated at different rates in the gel (Fig. 3B and C). RCNMV-Sw had a distinctive peptide pattern when it was digested with V8 protease (Fig. 3B, lane 3), whereas others were indistinguishable. Similar experiments with chymotrypsin showed that the proteins of RCNMV-Eng and RCNMV-Aus have identical peptide patterns that are readily differentiated from others (Fig. 3C, lanes 4 and 6). When the minor polypeptide band from RCNMV-Can was digested with chymotrypsin, its peptide map appeared to be very similar to that of the major polypeptide (Fig. 3C, lane 8). Similar results were obtained with other strains of the virus.

TABLE 2. Intragel cross-absorption tests with six red clover necrotic mosaic virus (RCNMV) antisera cross-absorbed with homologous and heterologous antigens

Antiserum absorbed	Antigen with which absorbed	Antigen tested					
		Eng	Aus	Can	TpM 34	TpM 48	Sw
RCNMV-Eng	Eng	−	−	−	−	−	−
	Aus	+	−	−	+	−	−
	Can	+	+	−	+	−	−
	TpM 34	+	+	−	−	−	−
	TpM 48	+	+	−	+	−	−
	Sw	+	+	−	+	−	−
RCNMV-Aus	Eng	−	−	+	−	+	+
	Aus	−	−	+	−	+	+
	Can	+	+	−	+	−	+
	TpM 34	+	+	+	−	+	+
	TpM 48	+	+	−	+	−	+
	Sw	+	+	+	+	+	−
RCNMV-Can	Eng	−	−	+	−	+	+
	Aus	−	−	+	−	+	+
	Can	−	−	−	−	−	−
	TpM 34	−	−	+	−	+	+
	TpM 48	−	−	−	−	−	−
	Sw	+	+	+	+	+	−
RCNMV-TpM 34	Eng	−	−	−	+	−	−
	Aus	−	−	−	+	−	−
	Can	+	+	−	+	−	−
	TpM 34	−	−	−	−	−	−
	TpM 48	+	+	−	+	−	−
	Sw	+	+	−	+	−	−
RCNMV-TpM 48	Eng	−	−	+	+	+	+
	Aus	−	−	+	+	+	+
	Can	−	−	−	−	−	−
	TpM 34	−	−	+	−	+	+
	TpM 48	−	−	−	−	−	−
	Sw	+	+	+	+	+	−
RCNMV-Sw	Eng	−	−	+	+	+	+
	Aus	−	−	+	+	+	+
	Can	+	+	−	+	−	+
	TpM 34	−	−	+	−	+	+
	TpM 48	+	−	−	−	−	+
	Sw	−	−	−	−	−	−

DISCUSSION

Data presented in this paper on the serological relationships of six RCNMV strains indicate that they represent four distinct serotypes. In addition to the three previously established serotypes—A (typified by RCNMV-TpM 34), B (RCNMV-TpM 48), and C (RCNMV-Sw) (12)—this study has shown that virus strains from Australia (Aus) and England (Eng) constitute a new serotype, D, whereas the Canadian strain (Can) is serologically identical with serotype B. Our results also indicate the extent of serological differences among the six RCNMV strains. Serological relationships among certain RCNMV strains are definitely closer than others. For example, RCNMV-Eng and RCNMV-Aus were so closely related that a distinction between them was almost impossible when their homologous antisera were used. The same was true for RCNMV-TpM 48 and RCNMV-Can. RCNMV-Eng and RCNMV-Aus were better distinguished with antisera to RCNMV-TpM 48, RCNMV-Can, and RCNMV-Sw than with their homologous antisera (Table 1). However, as illustrated by data presented in Table 2, the most definitive separation of RCNMV-Eng and RCNMV-Aus was obtained by sera cross-

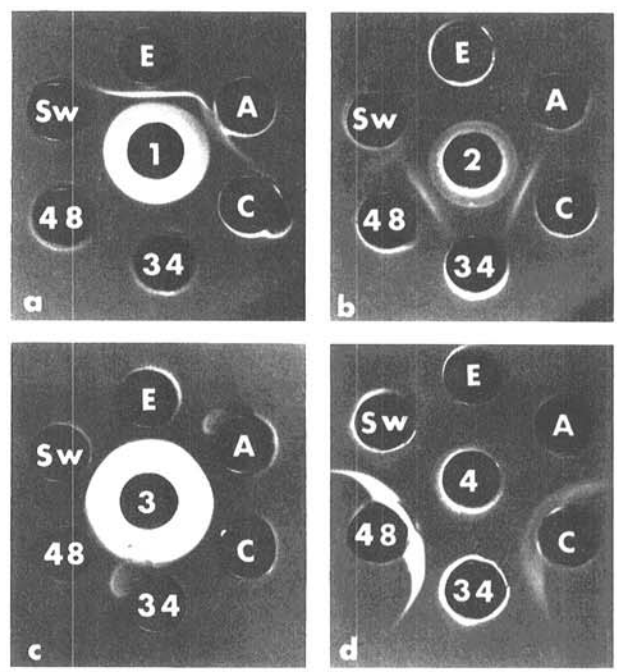

Fig. 2. Intragel cross-absorption tests: **A,** Central well filled initially with red clover necrotic mosaic virus (RCNMV)-TpM 34 antigen, then 24 hr later with anti-RCNMV-Eng serum. Peripheral wells contained antigens of RCNMV-Eng (E), RCNMV-Aus (A), RCNMV-Can (C), RCNMV-TpM 34 (34), RCNMV-TpM 48 (48), and RCNMV-Sw (Sw). **B,** Central well filled initially with RCNMV-Aus antigen, then 24 hr later with anti-RCNMV-Aus serum. Peripheral wells as in A. **C,** Central well filled initially with RCNMV-TpM 48 antigen, then 24 hr later with anti-RCNMV-Can serum. Peripheral wells as in A. **D,** Central well filled initially with RCNMV-Aus antigen, then 24 hr later with anti-RCNMV-Can serum. Peripheral wells as in A.

absorbed with these two antigens. The existence of heterospecific antibodies in anti-RCNMV-Aus serum is a unique feature. Until now, such antibodies are only known to occur in sera obtained from animals immunized with tobacco mosaic virus (17). It was suggested (17) that heterospecific antibodies appear as a result of the unfolding of otherwise hidden epitope(s) of an antigen in the immunized animal and would only react with heterologous antigen when the particular epitope is rendered more accessible than in the homologous antigen.

As reported earlier (12), the serological relationship between RCNMV-TpM 34 and RCNMV-TpM 48 was distinct as indicated by their unilateral reactions (Table 1). The weak reaction observed between anti-RCNMV-TpM 34 serum and the heterologous antigens RCNMV-TpM 48, RCNMV-Sw, and RCNMV-Can (Fig. 1J–L) is probably caused by degradation of viral particles into coat protein, and these reactions were not seen beyond one-half dilution of the serum (Table 1). There could be many reasons for the unilateral cross-reactivity observed in this study, and also by previous workers (12). First, variations in the degree of apparent cross-reactivity exhibited by different antisera may arise from differences between individual animals (18). However, variations among individual animals are not a major reason in our experiments, as indicated by similar results obtained in independent tests with RCNMV-TpM 34 and RCNMV-TpM 48 antisera prepared several years ago (11), as well as the ones prepared in our laboratory (*data not shown*) using different rabbits at different times. Second, some differences may arise in homologous and heterologous antisera because of the different timing of bleedings of the same animal (8). Since most of our tests were based almost entirely on antisera from one or two animals for each of the virus strains, the use of antisera from several bleedings over an extended period of immunization would shed further light on detailed serological relationships among dianthoviruses.

In spite of distinct serological differences among certain RCNMV strains, no differences were observed in coat protein size. However, peptide analysis with V8 protease differentiated serotype C, and chymotrypsin differentiated serotype D from other serotypes. Peptide mapping with several other site-specific proteases might be able to resolve further differences of coat proteins among serological groups.

In taxonomy of plant viruses, there are still no generally accepted criteria that clearly distinguish whether two viruses are distinct or are strains of the same virus (3). It has been suggested that, at present, antigenic properties and host ranges of the isolates are the most acceptable criteria upon which to base these decisions (6). RCNMV-TpM 34 and RCNMV-TpM 48, currently considered to be strains of the same virus, differ (i) antigenically by a serological differentiation index of more than 5 (Table 1); (ii) in their host range and symptomatology; and (iii) in genetic compatibility (the ability to form pseudorecombinants) and nucleotide homology (15). Further studies are required to resolve this taxonomic dilemma.

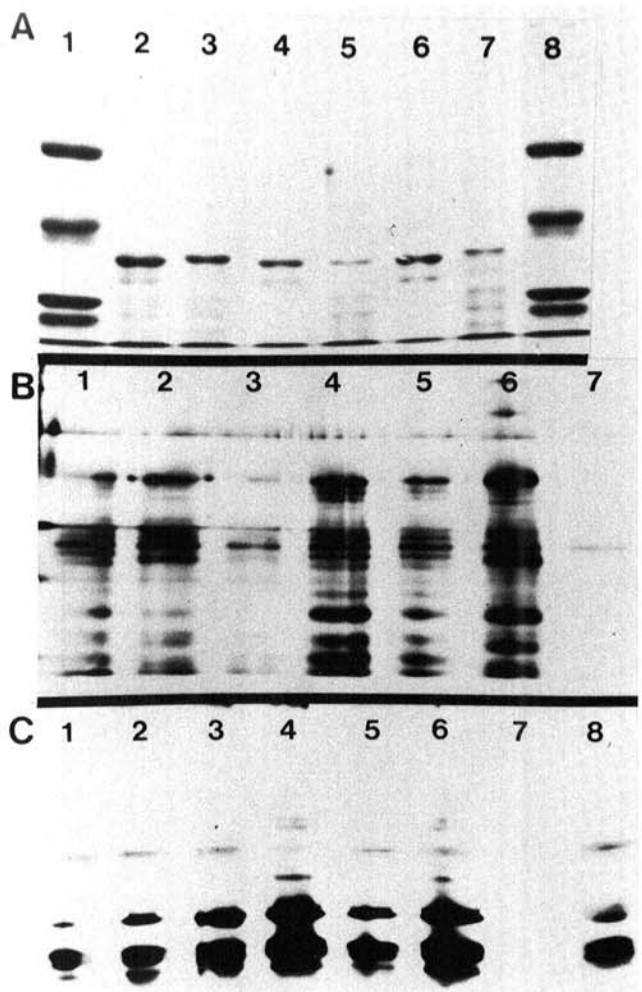

Fig. 3. A, Discontinuous SDS polyacrylamide gel electrophoresis (9%) of dissociated coat proteins from red clover necrotic mosaic virus (RCNMV)-TpM 34 (2), RCNMV-TpM 48 (3), RCNMV-Sw (4), RCNMV-Eng (5), RCNMV-Can (6), and RCNMV-Aus (7). Lanes 1 and 8 were molecular weight markers containing (from top) bovine plasma albumin (66,000 daltons), ovalbumin (45,000 daltons), trypsinogen (24,000 daltons), β-lactoglobulin (18,400 daltons), and lysozyme (14,300 daltons). **B,** Discontinuous SDS polyacrylamide gel electrophoresis (15%) of coat proteins of RCNMV strains following partial proteolysis with V8 protease. Lanes 1–7: RCNMV-TpM 34 (1), RCNMV-TpM 48 (2), RCNMV-Sw (3), RCNMV-Eng (4), RCNMV-Can (5), RCNMV-Aus (6), and V8 protease (7). **C,** Discontinuous SDS polyacrylamide gel electrophoresis (15%) of coat proteins of RCNMV strains following partial proteolysis with chymotrypsin. Samples in lanes 1–6 are as in B; lane 7 contained chymotrypsin enzyme, and lane 8 was the digestion product of protein band migrating faster than the coat protein of RCNMV-Can in A.

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