Acta virol. **37:** 47-53, 1993

SOME PROPERTIES OF COAT PROTEINS OF TWO COMOVIRUSES

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Received May 14, 1992; revised June 9, 1992

Summary. - Properties of coat proteins of red clover mottle virus (RCMV) and broad bean stain virus (BBSV) belonging to comoviruses were studied using polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), proteolytic cleavage, Western blot analysis and monoclonal antibodies (MoAbs). Boiling in the absence of detergent did not cause disintegration of virus particles, but the latter occurred in the presence of 0.2 % SDS. With 1 % SDS the disintegration began at 50 °C and above 60 °C the virus particles were completely disintegrated. The relative molecular weights of the coat proteins as determinated by SDS-PAGE method were 37.5 K and 20.5 K for RCMV, and 36.5 K and 22 K for BBCV, respectively. A spontaneous shortening of both coat proteins by proteolytic cleavage occurred *in vitro*. After cleavage with V8-protease the larger proteins gave 5 and 6 products, respectively (2 and 3 of them being the products of incomplete or nonspecific cleavage), the smaller proteins 4 products. The epitopes distinguished by 7 MoAbs were localized on only two V8-digest products of the larger coat proteins, but no MoAb binding to the smaller coat protein was observed.

Key words: red clover mottle virus; broad bean stain virus; coat protein; Western blot analysis

Introduction

The comovirus group includes plant viruses with genome divided in two single stranded (+) RNA chains packed separately into two identical icosahedral capside (Crowther *et al.*, 1974). The coat of every particle consists of two proteins called L (40 K) and S (20 K), respectively.

Though the cowpea mosaic virus, a typical representative of comovirus group, was well studied, much less is known about other comoviruses. Since the description of RCMV (Sinha, 1960) and determination of its basic physical and chemical properties (Oxelfelt, 1976), the attention has been paid especially to the problems of its infectivity (Valenta and Marcinka, 1968; Laphic *et al.*, 1976;

Oxelfelt and Abdelmoeti, 1978) and serology (Devergne and Cardin, 1968; Gallo and Musil, 1988).

Similar situation is with BBSV, because after description and basic characterization of this virus (Gibbs *et al.*, 1968) only few workers were interested in its further study (Govier, 1975; Gallo and Musil, 1988).

In this paper we tried to extend the knowledge on the properties of RCMV and BBSV coat proteins using SDS-PAGE, proteolytic cleavage, Western blot analysis and MoAbs.

Materials and Methods

Viruses. TpM36 isolate of RCMV (Musil and Gallo, 1984) and VsM isolate of BBSV (Musil *et al.*, 1978) propagated on pea were purified as described before (Musil *et al.*, 1983). Purified viruses were diluted to the concentration 1 mg/ml and stored at +4 °C. The concentration was determined spectrophotometricaly (Boswell and Gibbs, 1983).

Preparation of virus proteins. The coat proteins were prepared by precipiting the virus nucleic acid in the mixture of guanidine hydrochloride and lithium chloride (Wu and Bruening, 1971). To the prepared protein in 25 mmol/l Tris-HCl pH 7.0, both SDS and 2-mercaptoethanol (2-ME) to a 1 % concentration were added, followed by 5 min incubation in boiling water. After cooling the protein was loaded on a Sephadex G-150 column (1.5 × 45 cm) equilibrated with 25 mmol/l Tris-HCl pH 7.0, 0.1 % SDS, 0.1 % 2-ME and eluted with the same buffer by flow rate 3 ml/hr at 15 °C. Collected fractions were electrophoretically analyzed.

Proteolytic cleavage. The endoproteinase Glu-C from *Staphylococcus aureus*, strain V8 (V8-protease, Sigma) was used. After boiling for 5 min with 1 % SDS and 1 % 2-ME the proteins were cleaved in 125 mmol/l Tris-HCl pH 7.6. We used 1 μg of protease for cleavage of 20–50 μg protein. The cleavage was carried out at 37 °C for 1 hr and stopped by boiling for 1 min.

Electrophoresis. The SDS-PAGE of protein was run according to Laemmli (1970). The gels were silver stained as described by Blum *et al.* (1987), stored in distilled water at +4 °C or dried between two cellophane sheets. Low molecular weight standards (Pharmacia) were used for determination of M_r.

MoAbs against RCMV (MoAbs 2-5) and BBSV (MoAbs 1, 6, 7) were prepared by immunization of Balb/c mice with native viruses as described before (Gallo and Matisová, 1993).

Western blot analysis. The cellulose nitrate or nylon membranes (Schleicher and Schuell, 0.45 μm) were employed. This procedure was carried out in a semidry system according to Hirano and Watanabe (1990). The membranes were dried at 80 °C for 15 min and stained immunospecifically after blotting. The MoAbs were diluted 1000-fold, 10 % non fat milk was used for blocking in all steps. The membranes were usually soaked overnight in the solution of primary and for 2–4 hr in that of secondary antibody. We used swine anti-mouse IgG conjugated with horseradish peroxidase (ÚSOL, Prague) and diaminobenzidine hydrochloride (Sigma) as substrate.

Results and Discussion

Both RCMV and BBSV preparations gave a SDS-PAGE pattern characteristic for all comoviruses: two groups of protein bands, L and S. It was not substantially influenced by the 2-ME concentration in the sample. On the other hand 0.2 % SDS at 100 °C for 3 min was sufficient for full disintegration of virus particles. In further experiments we usually used 1 % SDS. Strong reciprocal affinity of proteins (protein - protein binding) caused their reassociation after

SDS removal. This fact resulted, e.g. in the impossibility to separate the coat proteins by "native" electrophoresis. The presence of only 0.1 % SDS either in the electrophoretic or chromatographic system was sufficient to keep the proteins solubilized and mutually separable (data not shown).

Oxelfelt (1976) described the absence of the S protein band in some RCMV electrophoretic runs, when the virus was warmed to 50 °C and the gel was stained with Coomassie Brilliant Blue (CBB). When investigating the degree of RCMV disintegration in the presence of 1 % SDS at various temperatures we ascertained that the disintegration of virus particles began just at 50 °C and that the L protein band was really markedly more distinct than the S protein band (Fig. 1). In contrast, the S protein but not the L protein band was well visible in BBSV preparation at this temperature (Fig. 2). This could be caused by greater mutual affinity of L-L than S-S, and S-L proteins, respectively. In this connection the S protein solubilization occurs earlier due the conditions at the limits of denaturation. On the other hand, for RCMV from this consideration follows the greater association ability of the S rather than the L proteins. We do not assume that it was caused by an artefact during silver staining, because the proteins of either virus stained equally well under usual conditions of the sample preparation (100 °C). The existence of affinity between the same S or L molecules was also documented by the fact, that the association occurred with the isolated coat

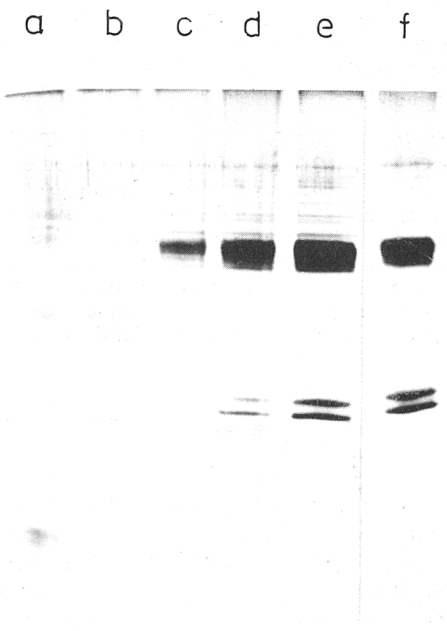

Fig. 1
Temperature dependence of RCMV disintegration degree in the presence of 1 % SDS
SDS electrophoresis in 14 % PAG. Lanes a–e: temperatures of 40 °C, 45 °C, 50 °C, 55 °C, and 60 °C, respectively. Lane f: control sample (100 °C).

proteins preparations after their dialysis against the buffer solution without SDS, and that the warming in the presence of detergent was required for their resolubilization.

Above 50 °C, the degree of dependence of protein disintegration on an increased temperature was very steep, eg. at 60 °C for 5 min both viruses were fully disintegrated as well as by boiling (Fig. 1 and 2). This observation corresponds to the results of RCMV thermal inactivation studies (Musil and Lešková, 1969), when the infectivity of the sap from infected plants decreased 1000-times between 50 °C and 60 °C, and about 10 000-times between 50 °C and 65 °C, respectively.

Using the SDS-PAGE method we determined the relative molecular weights of coat proteins as 37.5 K (35.5 K) and 20.5 K (19.5 K) for RCMV, and 36.5 K (34 K) and 22 K (20.5 K) for BBSV, respectively. When the virus preparations were allowed to stand *in vitro* (in Tris or phosphate buffer pH 7.0 at +4 °C), the proteolytic shortening of protein chains to the values showed in parentheses was observed and the concentration of shortened chains in the samples was gradually growing. In the case of S proteins the chains with original molecular weights even fully disappeared, but with L proteins this process was less distinct. Such an *in vitro* and also *in vivo* (in the host plants) proteolysis - not

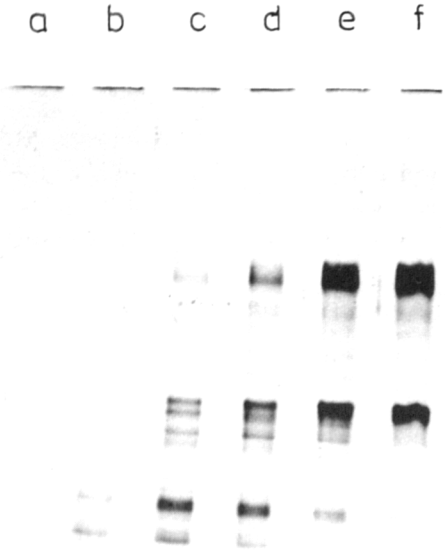

Fig. 2
Temperature dependence of BBSV disintegration degree in the presence 1 % SDS Electrophoretic conditions and lanes a–f as in Fig. 1.

Table 1. Molecular weights of V8-digest products of RCMV and BBSV L coat proteins

Virus	M_r of fragment (in thousands)					
	A1	A2	A3	B	C	D
RCMV	29.0	27.0	-	15.5	14.0	12.0
BBSV	27.0	25.0	23.5	14.0	13.5	12.0

accompanied by a loss of infectivity - is well known with comoviruses and it is causally connected with the existence of so-called electrophoretic forms of virus particles (Niblett and Semancik, 1969; Musil and Gallo, 1986).

Molecular weights of RCMV coat proteins determined in our experiments differed from 40 K and 22.2K (18.3 K) values described before (Oxelfelt, 1976). It is possible that different virus isolates or strains were analyzed or - what is more probable - that we used a more sensitive system and detection (slab gels stained with silver instead of rod gels stained with CBB employed in Oxelfelt's experiments (Oxelfelt, 1976)).

By the use of V8-protease for the RCMV and BBSV coat proteins digestion, we obtained exactly defined polypeptide chains (Tables 1 and 2), irrespective of whether disintegrated complete viruses or isolated coat proteins were digested (Fig. 3). Both viruses gave analogous digest products. The 23.5 K and 25 K BBSV polypeptides arose from the 27 K precursor either as a result of an incomplete proteolysis at specific sites or due to a partial protease nonspecificity as follows from the same reactivity of these bands in the Western blot analysis. Similarly, 27 K polypeptide appeared in some cases of RCMV digestion.

Of seven MoAbs available, the MoAbs 1-4 were group-specific, the MoAb 5 RCMV-specific and the MoAbs 6 and 7 BBSV-specific (which applies to RCMV and BBSV, but not to other comoviruses). None of these MoAbs bound to the S proteins. Except for epitopes 2 and 3 localized on the C fragments, all other epitopes were on the A fragments (Fig. 4).

The most immunogenic sites of both studied comoviruses are evidently

Table 2. Molecular weights of V8-digest products of RCMV and BBSV S coat proteins

Virus	M_r of fragment (in thousands)			
	a	b	c	d
RCMV	17.5	16.0	12.5	11.5
BBSV	18.0	16.5	12.5	11.5

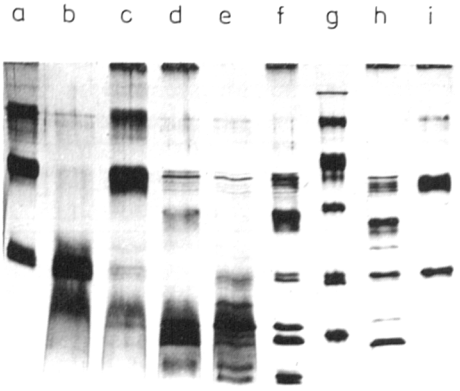

Fig. 3
SDS-PAGE electrophoresis of RCMV and BBSV coat proteins cleavage by V8-protease
SDS-electrophoresis in 14 % PAG. Nondigested controls: (a) RCMV, (b) RCMV-S, (c) RCMV-L, (i) BBSV. Digested samples: (d) RCMV-L/V8, (e) RCMV-S/V8, (f) RCMV/V8, (h) BBSV/V8, (g) M_r standards: phosphorylase b (94 K), albumin (67 K), ovalbumin (43 K), carbonic anhydrase (30 K), trypsin inhibitor (20.1 K) and α-lactalbumin (14.4 K).

localized on these two digest products of the major coat proteins. The number of epitopes statistically agrees with the fragment length. The A fragment is about twice longer than C fragment and contains also approximately twice more epitopes.

Because the MoAbs were obtained from mice immunized with native viruses, the localization of epitopes indicates that two A and C fragments originated from the L protein part, which on the native virus particles is directed toward the exterior so that it is more easily accessible to the immune system response. On the contrary, the S protein appeared to be a very poor immunogen by our immunization method.

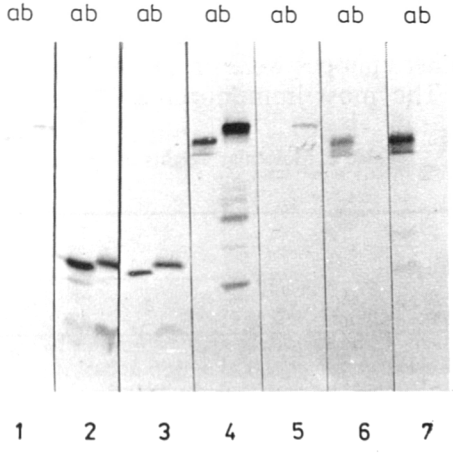

Fig. 4
Western blot analysis of RCMV and BBSV coat proteins after V8-proteolysis
BBSV (lane a), RCMV (lane b), MoAbs 1–7 (lanes 1–7).

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