

Glycosylation of the capsid proteins of cowpea mosaic virus: a reinvestigation shows the absence of sugar residues

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The previously reported (Partridge *et al.*, *Nature* 247, 391–392, 1974) glycosylation of the capsid proteins of cowpea mosaic virus (CPMV) has been reinvestigated. In initial studies, a preparation of purified CPMV particles was hydrolysed with HCl and amino acids and sugars were derivatized with o-phthalaldehyde (OPA). No glucosamine or galactosamine, amino sugars previously reported to occur in significant quantities in CPMV capsids, could be detected by reverse-phase high-performance liquid chromatography (RP-HPLC) of the derivatized hydrolysates. A complete analysis of all sugars potentially present was carried out by hydrolysing a sample of purified CPMV capsid proteins and derivatizing the sugars with 1-phenyl-3-methyl-5-pyrazolone. RP-HPLC analysis demonstrated that the capsids do not contain significant quantities of any sugar. The results show that, contrary to the previous report, the coat proteins of CPMV are not glycosylated.

Cowpea mosaic virus (CPMV) is a bipartite positive-strand RNA plant virus which is the type member of the genus *Comovirus*. Virus particles are composed of 60 copies each of a large (L) and small (S) coat protein arranged with icosahedral symmetry which encapsidate either of the two genomic RNAs (RNA-1 and RNA-2). As well as containing protein and RNA, particles have been reported as containing significant amounts of carbohydrate (Partridge *et al.*, 1974). The carbohydrate reported corresponded to 1.90 g/100 g of protein and consisted principally of glucosamine (0.70 g/100 g protein), glucose (0.51) and galactosamine (0.36) with more minor amounts of galactose (0.19) and mannose (0.14) being detected. These figures suggest that each of the 60 pairs of L and S subunits in the virus capsid contains, on average, 2 molecules

of glucosamine and 1 molecule each of glucose and galactosamine (Goldbach & Wellink, 1996). Potential sites for N-glycosylation can be found in both the L (three sites) and S (two sites) proteins (Goldbach & van Kammen, 1985). Partridge *et al.* (1974) suggested that the presence of sugar residues was associated with the seed transmissibility of CPMV since the related *Comovirus*, bean pod mottle virus (BPMV), was not seed-transmitted and no sugars could be detected.

The question of the extent and nature of any glycosylation on the capsids of CPMV has recently assumed increased significance owing to the potential use of such particles as a source of novel vaccines (for reviews see Lomonosoff & Johnson, 1995; Spall *et al.*, 1998; Lomonosoff & Hamilton, 1999). The possible presence of unusual glycan structures is an important regulatory consideration for the use of plant-expressed proteins as pharmaceuticals (Miele, 1997). Recent investigations of the structure of the capsid proteins of CPMV have cast some doubt on the presence of carbohydrate on the virus particles, at least at the levels reported by Partridge *et al.* (1974). Crystallographic analysis of virus particles revealed no electron density on either the L or S proteins which could be interpreted as sugar residues (Lomonosoff & Johnson, 1991; T. Lin, personal communication). This could, however, be due to the modification being partial at any one site. Electrospray mass spectrometry of the S protein gave only a single peak, the mass of which corresponded closely to that of an unmodified polypeptide chain (Taylor *et al.*, 1999). This suggests that if glycosylation does occur, it would have to be confined to the L subunits. To resolve the question of whether CPMV particles are glycosylated, we have reinvestigated the monosaccharide content of the proteins using modern analytical techniques.

CPMV was propagated in *Vigna unguiculata* var. California blackeye and purified by polyethylene glycol (PEG) precipitation and differential centrifugation essentially as described by van Kammen & de Jager (1978). However, a sucrose cushion was not used for the final ultracentrifugation step. The virus pellet was resuspended in 10 mM sodium phosphate pH 7.0. For an initial assessment of the glycosylation status of the viral coat proteins, we made use of a rapid method for the detection of amino sugars (Altmann, 1992). This method exploits the de-acetylation of N-acetyl amino sugars during

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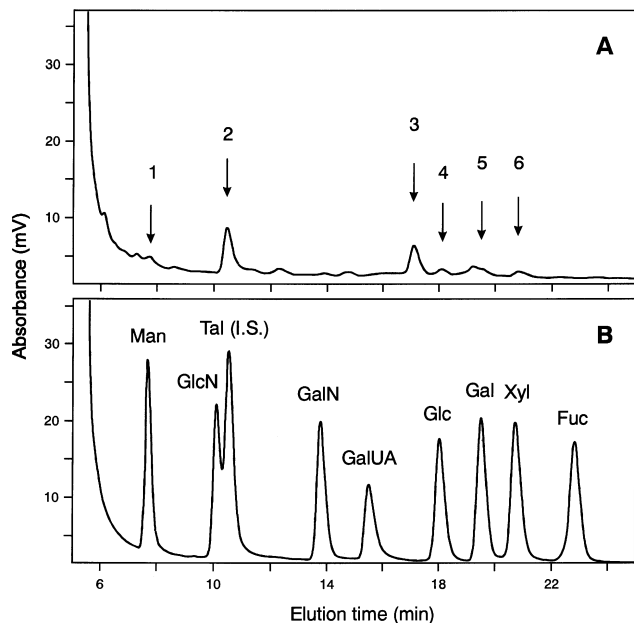


Fig. 1. Analysis of monosaccharides present in a preparation of CPMV capsid proteins. Sugars were analysed as PMP-derivatives by RP-HPLC. (A) Analysis of a 0.5 mg aliquot of purified CPMV capsid proteins. (B) Analysis of a standard mixture containing 5 nmol each of mannose (Man), glucosamine (GlcN), galactosamine (GalN), galacturonic acid (GalUA), glucose (Glc), galactose (Gal), xylose (Xyl) and fucose (Fuc) plus 6.5 nmol of talose (Tal), which was used as internal standard (I.S.) and was not expected to be present in CPMV.

acid hydrolysis and the subsequent derivatization of the resultant primary amines with *o*-phthalaldehyde (OPA). Thus *N*-acetyl amino sugars are identified as their de-acetylated OPA derivatives. Using this method it is possible to analyse both the amino sugar and amino acid content of a protein simultaneously, providing an internal control for the level of amino sugars. A 50 µg sample of purified virus was hydrolysed in 4 M HCl for 4 h at 100 °C. Prior to derivatization, the amino sugars were reduced to their corresponding sugar alcohols using sodium borohydride, as this improves the accuracy of the subsequent HPLC analysis. Derivatization and analysis of amino acids and amino sugars as OPA derivatives by reverse-phase high-performance liquid chromatography (RP-HPLC) was as previously described (Altmann, 1992). In the resulting chromatogram of the mixture of derivatized amino acid and amino sugar standards, peaks due to (*N*-acetyl)glucosamine and (*N*-acetyl)galactosamine were well separated. No corresponding peaks were observed in the hydrolysate of CPMV particles. By comparison with the peak heights for the amino acids in the hydrolysate, it was estimated that levels of (*N*-acetyl)glucosamine and (*N*-acetyl)galactosamine of approximately 0.02 g/100 g and 0.05 g/100 g of protein, respectively, would have been easily detected. These levels are significantly below the figures of 0.70 g/100 g and 0.36 g/100 g of protein reported by Partridge *et al.* (1974). These results raised serious doubts about the previous

conclusions regarding glycosylation, at least as far as amino sugars were concerned.

To confirm the apparent lack of glycosylation of CPMV capsids, a total sugar analysis was undertaken. It was necessary to perform this analysis on the isolated capsid proteins as the vigorous hydrolysis conditions required released large quantities of ribose from the viral RNA when whole capsids were analysed. The capsid proteins were separated from RNA by the guanidinium–LiCl method of Wu & Bruening (1971) and the resulting protein pellet was washed twice with 0.1 M ammonium acetate in 95% (v/v) ethanol to minimize the level of contaminating nucleotides. This was the same method of protein purification as used by Partridge *et al.* (1974). Following hydrolysis of a 500 µg sample of the coat proteins by 4 M trifluoroacetic acid at 100 °C for 4 h, monosaccharides were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP). The resulting mixture was analysed by RP-HPLC as previously described by Fu & O'Neill (1995) except that a 5.5 µm ODS Hypersil column (250 × 3 mm) was run at a flow rate of 1.2 ml/min. Eluents A and B contained 15% (v/v) and 30% (v/v) acetonitrile, respectively, in 0.1 M ammonium acetate, pH 5.5. PMP-sugars were eluted by a gradient from 12–18% B for 8 min followed by an increase to 55% B for 22 min.

Chromatographs of the hydrolysate of CPMV capsid proteins (panel A) or a mixture of monosaccharide standards (panel B) are shown in Fig. 1. In (A), peak 2 was assigned as ribose rather than talose on the basis of its retention time. Peaks 1, 4, 5 and 6 represent traces of mannose, glucose, galactose and xylose/arabinose (not separable under the conditions employed) while peak 3 could not be identified. The weight of protein hydrolysed corresponded to approximately 8 nmol of each viral capsid protein. The chromatograph of the mixture of standard monosaccharides (panel B) contained 5 nmol of each sugar. Thus, if each coat protein molecule contained a single molecule of a given sugar (as suggested for glucosamine by Partridge *et al.*, 1974), the peak obtained in panel (A) should be at least as high as that found for the corresponding standard sugar in panel (B). This is clearly not the case. The two most prominent peaks (2 and 3) in panel (A) represent ribose (presumably derived from hydrolysis of residual RNA in the protein preparation) and an unidentifiable contaminant, neither of which was reported by Partridge *et al.* (1974). The deduced level of each monosaccharide in the hydrolysate of the CPMV capsid proteins is given in Table 1. From the data, it is clear that CPMV capsid contains a far lower level of monosaccharides than previously reported. The fact that ribose was the most abundant sugar detected, despite attempts to minimize the level of RNA in the sample, confirms the sensitivity of the method used for the analysis. The extremely low levels which are found are most probably due to low-level contamination with other plant material. Even if all the monosaccharides detected were genuinely derived from the CPMV capsid proteins they would represent on average less than one molecule each of mannose, glucose, galactose and

Table 1. Amounts of monosaccharides found after PMP analysis of hydrolysed CPMV protein samples

The level of each sugar was calculated from the areas under each peak in Fig. 1.

Monosaccharide	Amount (g/100 g protein)	
	This report	Partridge <i>et al.</i> (1974)
Glucose	0.0064	0.51
Mannose	0.0054	0.14
Ribose	0.0496	Not reported
Xylose/arabinose*	0.0056	None found
Galactose†	<0.010	0.19
Glucosamine	None found	0.70
Galactosamine	None found	0.36

* Xylose and arabinose are not resolved by the HPLC method used in this paper.

† Galactose co-migrated with an unknown contaminant making more accurate quantification impossible.

xylose/arabinose per virus particle. As with the OPA analysis, there was no evidence at all for modification with glucosamine or galactosamine, two of the sugars reported as occurring in the largest quantities by Partridge *et al.* (1974). We regard our findings of only low levels of sugars as reliable since the hydrolysis conditions used for the PMP-monosaccharide analysis had previously been shown to allow quantitative recovery of both hexoses and aminohexoses and at least 90% recovery of pentoses and deoxypentoses. Furthermore, when similar analyses were performed on a variety of other glycoproteins using the identical method and apparatus, the expected levels of sugars were always detected.

The results presented in this paper refute the conclusions previously reported by Partridge *et al.* (1974). It is unclear why the previous authors obtained such apparently high levels of monosaccharides in their analysis. One possible explanation lies in the methods of virus purification used. Though Partridge *et al.* (1974) give few details, they state that they used 'sucrose gradient electrophoresis' to purify both CPMV and BPMV. The use of sucrose during purification might have led to introduction of extraneous sugars into their samples though why the levels should have been greater for CPMV than BPMV is unclear. Whatever the cause of the discrepancy, we are convinced that our current findings are correct, especially since the levels of amino sugars were assessed by two independent methods.

The results presented in this paper indicate that administration of CPMV-based chimaeras to experimental animals should not cause unwanted immunological reactions due to the presence of plant-specific glycan. In addition, the results undermine the hypothesis that glycosylation determines seed-

transmissibility. Indeed, the significance of glycosylation of plant viral coat proteins is open to question. The only other report of glycosylation on a plant virus capsid concerns potato virus X (PVX; Tozzini *et al.*, 1994). In this case, evidence based on enzymatic and chemical digestion was presented to suggest that the PVX coat protein contains O-linked sugars. O-linked N-acetylglucosamine has been found in mammalian cytosolic proteins, so such a modification is, in principle, possible. However, the precise nature of glycan in the PVX coat protein was not investigated and its possible significance is unclear. A more thorough analysis of this single case of glycosylation is undoubtedly warranted as is a more general survey of the occurrence of glycosylation in plant viruses.

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