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Further structural studies of the carbohydrate moiety of the allergen Ag-54 (C1a h II) from the mould *Cladosporium herbarum*

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ABSTRACT

The carbohydrate moiety of the glycoprotein allergen Ag-54, isolated from the mould *Cladosporium herbarum*, has been characterised partly, using acetolysis, methylation analysis, and n.m.r. spectroscopy. Ag-54 contained a highly branched galactoglucomannan and two branched mannogluco-oligosaccharide chains. The oligosaccharides contained terminal, (1→4)-, and (1→4,6)-linked α-Glc residues and terminal, (1→2)-, and some (1→3)-linked α-Man residues. The n.m.r. data indicated the galactoglucomannan to have a main chain made up of (1→6)-linked α-Man and (1→4)-linked α-Glc residues, with the latter attached to position 6 of α-Man residues. Oligosaccharides with (1→6)-linked β-Gal*f* and (1→2)-linked α-Man were attached to the main chain. Acetolysis of the galactoglucomannan yielded linear and branched oligosaccharides. The presence of (1→2,3)-linked α-Man residues indicated either that other than (1→6) linkages were present in the main chain or that there was 2,3-branching in the side chains.

INTRODUCTION

Cladosporium herbarum is an airborne mould of the group Fungi Imperfecti. Its spores are found frequently in large numbers in both indoor and outdoor environments, and, in many countries, *C. herbarum* is a major source of inhalant allergy and allergic asthma in man[1]. Fungal material, including *C. herbarum*, contains numerous components with biological activity[2–4]. Extracts from *C. herbarum* contain at least 60 components with IgG-binding activity (antigens), of which >20 possess IgE-binding ability (allergens)[5]. One of the important allergens, Ag-54, which has been isolated and characterised immunologically[6], corresponds to the Cla h II according to the IUIS allergen nomenclature[7].

Ag-54 is a glycoprotein that contains 80% of carbohydrate, which is linked to the protein through one Man unit and two Glc units[8,9]. The main carbohydrate moiety has a molecular weight of 19000 (estimated by h.p.l.c.) and comprises mainly (1→2)- and

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(1→6)-linked D-Man, and some (1→4)-linked and (1→3,6)-linked D-Glc. (1→6)-Linked D-Gal*f* side-chains were proposed to be attached to positions 2 or 3 of (1→6)-linked Man units and position 3 of (1→2)-linked D-Man units[10]. The structures of the minor constituents of the carbohydrate moiety of Ag-54 were not determined, but were suggested to be small gluco-oligosaccharides.

We now report further details of the structure of the carbohydrate moiety of Ag-54.

RESULTS AND DISCUSSION

*Isolation and methylation analysis of the low-molecular-weight fractions (*B *and* C*).* — The carbohydrate material, released from the glycoprotein by treatment with alkaline borohydride, was fractionated on Bio-Gel P-4 (Fig. 1), to give fractions *A*–*C* that corresponded to the void volume and molecular weights of 2300–1600 and < 1600, respectively. The monosaccharide composition of each fraction was determined by g.l.c. of the trimethylsilylated methyl glycosides after methanolysis. The molecular weight of the fraction was estimated[10] to be 19000. The monosaccharide compositions of fractions *A*–*C* are given in Table I. Fractions *B* and *C* accounted for only 3.2% and 2.6%, respectively, of the original carbohydrate. No difference in the composition of the products was detected when the methanolysis was performed with methanolic M or 4M hydrogen chloride.

Methylation analysis of high-molecular-weight material that corresponded to fraction *A* has been reported[10]. The results of the methylation analysis of fractions *B* and *C* are summarised in Tables II and III, respectively. Two g.l.c. systems were used for identification of the partly methylated alditol acetates. The SP 2340 system was included

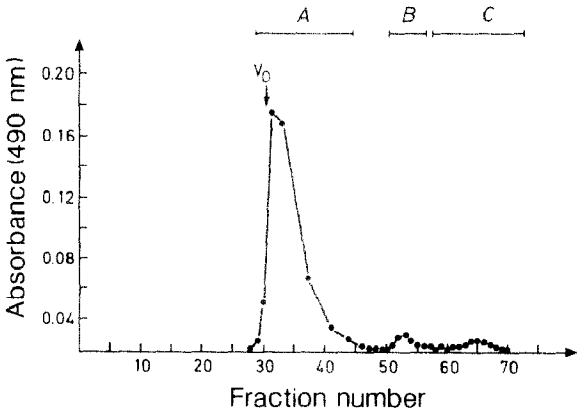

Fig. 1. Fractionation on Bio Gel P-4 (200–400 mesh) of the carbohydrate products obtained after treatment of Ag-54 with alkaline borohydride. The column was eluted with 0.075M ammonium hydrogencarbonate buffer (pH 7.9). Carbohydrates were detected by the phenol–sulfuric acid method: *A* corresponded to the void volume (V_0), *B* to a mol. wt. of 2300–1600, and *C* to a mol. wt. of < 1600.

TABLE I

Composition of the carbohydrate components released from Ag-54 after treatment with alkaline borohydride (see Fig. 1)

Fraction	*Mol. wt.*	*Man:Gal:Glc*	*Relative proportion (%)*
A	19 000	1:0.7:0.4	94.2
B	2300–1600	1:0:1.6	3.2
C	<1600	1:0:2	2.6

TABLE II

Methylation analysis of fraction B (Fig. 1)

Alditol acetates corresponding to	*Type of linkage*	T[a]	*Relative peak area*	
		DB-5	*DB-5*	*SP2340*
2,3-Di-*O*-methylglucitol	(1→4,6)	1.99	2.2	
2,4-Di-*O*-methylglucitol	(1→3,6)	2.06	0.3	
2,3,6-Tri-*O*-methylglucitol	(1→4)	1.34	2.2	
2,4,6-Tri-*O*-methylmannitol	(1→3)	1.37	1.0	
3,4,6-Tri-*O*-methylmannitol	(1→2)	1.28	1.7	
2,3,4,6-Tetra-*O*-methylmannitol/glucitol		1.0	2.5	1.5–1.0

[a] Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

TABLE III

Methylation analysis of fraction C (Fig. 1)

Alditol acetates corresponding to	*Type of linkage*	T[a]	*Relative peak area*	
		DB-5	*DB-5*	*SP2340*
2,3-Di-*O*-methylglucitol	(1→4,6)	1.99	1.0	
2,3,6-Tri-*O*-methylglucitol	(1→4)	1.34	1.1	
3,4,6-Tri-*O*-methylmannitol	(1→2)	1.29	1.0	
2,3,4,6-Tetra-*O*-methylmannitol/glucitol		0.99	2.5	1.0–2.0

[a] See Table II.

primarily for its ability to separate 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol from the corresponding D-mannitol derivative.

Fraction *B* possessed 10 units as judged from the results of methylation analysis (Table II). Glc was the main monosaccharide and was involved in the branch points with (1→4) and (1→4,6) linkages. The Man was mainly (1→2)-linked, but a small proportion of (1→3) linkages was detected.

The results obtained with the DB-5 system indicated that fractions *B* and *C* were similar, but the latter was smaller and had equal proportions of (1→2)-linked Man, (1→4)-linked Glc, and (1→4,6)-linked Glc, together with terminal Man and Glc. Use of the SP 2340 system showed that there was twice as much terminal Glc as terminal Man, and indicated microheterogeneity in the oligosaccharide, which may have had either Man or Glc as the end unit in the branch, or have been a mixture of oligosaccharides.

The end-group alditol of fraction *C* was detected by g.l.c.–m.s. in the DB-5 system (*T* 0.62 relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol) and gave primary fragments with *m/z* 45, 89, 133, 161, and 205, which demonstrated that it was a 2-*O*-acetyl-1,3,4,5,6-penta-*O*-methylhexitol. Man was the only sugar in Ag-54 that was (1→2)-linked. Because fraction *C* was linked to the protein through Man, it is concluded that fractions *A* and *B* must have been linked through Glc, since the three carbohydrate components of Ag-54 are linked to protein through one Man and two Glc units[9]. The end-group alditol of fraction *B* could not be detected.

Acetolysis of fraction A. — Acetolysis (40°, 12 h) of fraction *A* was carried out with the aim of cleaving the Man (1→6) linkages and leaving the Man (1→2) linkages intact. The products were fractionated on Bio Gel P-2 (Fig. 2), and t.l.c. of the fractions indicated that they were homogeneous and confirmed the sizes. The monosaccharide fraction (M_1) accounted for ~40% of the mixture and, of the higher saccharides (M_2–M_5), the pentasaccharide (M_5) preponderated. Fraction M_1 contained Man, Gal, and Glc in the proportions 1:0.8:1.3 as determined by g.l.c. of the trimethylsilylated methyl glycosides. Methylation analysis demonstrated that Man was the only monosaccharide present in the fractions M_2–M_5. As indicated in Table IV, these fractions consisted of blocks of (1→2)-linked Man residues; no (1→6)-linked Man was detected.

In order to determine the origin of the fractions of low molecular weight, a pentasaccharide fraction (M_5) obtained after acetolysis of fraction *A* for 2 h at 40° (Fig. 3) was isolated and acetolysed for 5 h at 40°. The elution profile of the products on Bio Gel P-2 (Fig. 4) showed that small proportions of monosaccharides were split off, which indicated that M_5 was a mixture of a (1→2)-linked mannopentaose and another penta-saccharide. Acetolysis for another 12 h at 40° of the new fraction M_5 did not release further oligosaccharide fractions (Fig. 4). Thus, the ratios of the higher saccha-

TABLE IV

Methylation analysis of the oligosaccharide fractions M_2–M_5 (Fig. 2)

Alditol acetates corresponding to	*Type of linkage*	T[a]	*Relative peak area*			
			M_2	M_3	M_4	M_5
4,6-Di-*O*-methylmannitol	(1→2,3)	1.88	0	0	0	0.1
3,4,6-Tri-*O*-methylmannitol	(1→2)	1.30	0.7	2	2.8	4.1
2,3,4,6-Tetra-*O*-methylmannitol/glucitol		1.0	1	1	1	1

[a] See Table II.

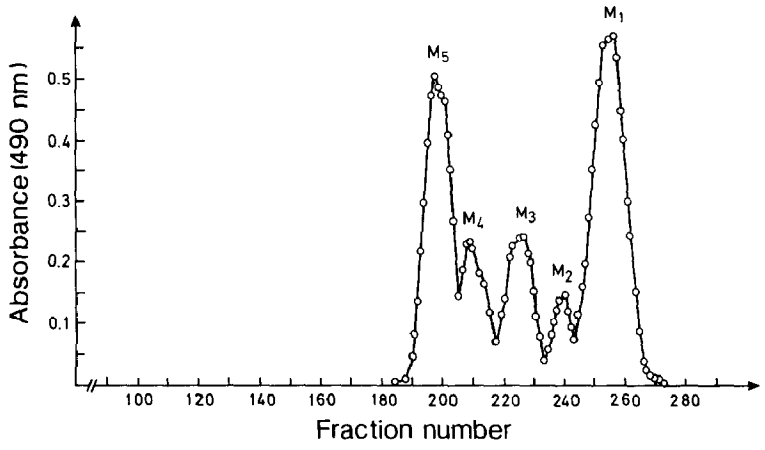

Fig. 2. Elution of products of acetolysis of fraction *A* (Fig. 1) with 10:10:1 acetic anhydride–acetic acid–sulfuric acid at 40° for 12 h from a column (2.5 × 95 cm) of Bio Gel P-2 (400 mesh); 1.5-mL fractions were collected. M_1–M_5 connote mono- to penta-saccharide, respectively. Molar ratios: $M_1 = 16$, $M_2 = 1.2$, $M_3 = 1.8$, $M_4 = 1.3$, and $M_5 = 2.1$.

rides formed after acetolysis of the Ag-54 carbohydrate moiety for 12 h are considered to be similar to that present in the carbohydrate moiety itself.

The results of methylation analysis of the fractions I–IV (Fig. 3) of molecular weight greater than that of M_5 are summarised in Table V. Fraction I, which contained (1→4), (1→2), and (1→6) linkages, and also branch points, had most of the character-

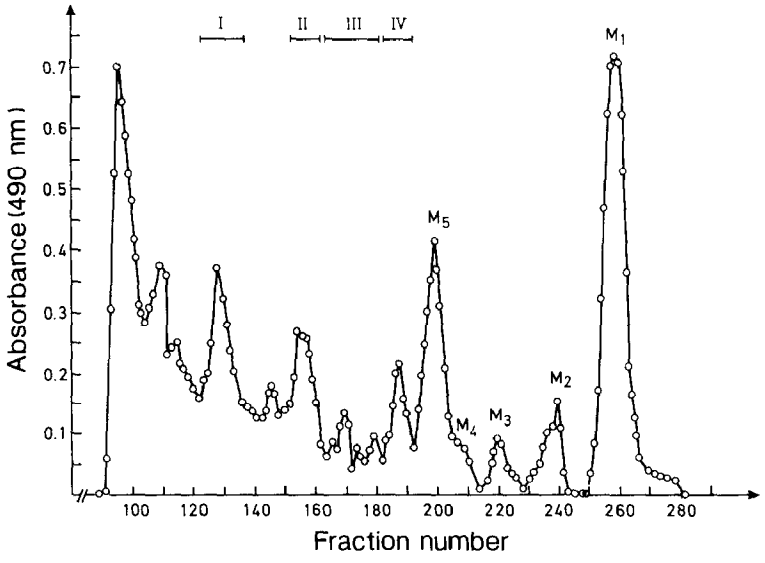

Fig. 3. Fractionation of the products obtained by acetolysis (2 h, 40°) of *A* (Fig. 1) on a column (2.5 × 95 cm) of Bio Gel P-2 (400 mesh).

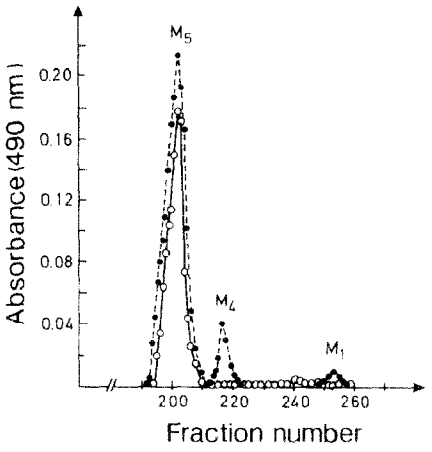

Fig. 4. Fractionation (---●---) of the products obtained by acetolysis (5 h, 40°) of fraction M_5 (Fig. 3) on a column (2.5 × 95 cm) of Bio Gel P-2 (400 mesh) (molar ratios: $M_1 = 2.0$, $M_4 = 1.8$, and $M_5 = 16.2$). When the new fraction M_5 was isolated and acetolysed for 12 h at 40°, the profile ---○--- was obtained.

istics of the intact mannan. The presence of (1→2,3)-linked Man residues indicated either that other than (1→6) linkages were present in the main chain or that there was 2,3-branching in the side chains. The oligosaccharides in fraction II had no branch points but contained three types of linkage. This finding indicated that acetolysis had broken a (1→6) linkage next to the branch point and that the oligosaccharide consisted of both the side chain and the main chain, or that both (1→2) and (1→6) linkages were present in the main chain and the oligosaccharides contained only parts of the main chain. Fraction III contained branched and unbranched hexose residues, which supported the conclusions for fraction I.

Acetolysis of fractions I–IV for 12 h at 40° gave only oligosaccharide fractions similar to those obtained after prolonged acetolysis (Fig. 5). The molar ratios of the

TABLE V

Methylation analysis of fractions I–IV (Fig. 3)

Alditol acetates corresponding to	*Type of linkage*	T[a]	*Relative peak area of Fraction*			
			I	*II*	*III*	*IV*
3,4-Di-*O*-methylmannitol	(1→2,6)	2.07	1.1	0	1	0
4,6-Di-*O*-methylmannitol	(1→2,3)	1.89	1	0	0	0
2,3,4-Tri-*O*-methylmannitol	(1→6)	1.5	7.3	3	1.8	1
2,3,6-Tri-*O*-methylglucitol	(1→4)	1.35	3.9	1	1.3	0
3,4,6-Tri-*O*-methylmannitol	(1→2)	1.31	27.4	12.6	5.4	7.3
2,3,4,6-Tetra-*O*-methylmannitol/glucitol		1.0	6.2	3	1.8	1.6

[a] See Table II.

products of acetolysis are given in Table VI. The fractions were not purified by rechromatography and the molecular weights assigned are approximate. Acetolysis of fraction IV gave equimolar amounts of a pentasaccharide and a trisaccharide, and methylation analysis (Table V) revealed only (1→6) and (1→2) linkages which indicated the presence of [Man-(1→2)]$_2$-Man-(1→6)-[Man-(1→2)]$_4$-Man or [Man-(1→2)]$_4$-Man-(1→6)-[Man-(1→2)]$_2$-Man. Side chains of Gal*f* could be attached to this structure as proposed earlier[10].

N.m.r. analysis. — Both the native and partially hydrolysed polysaccharide were analysed variously by 1D and 2D ^{1}H-n.m.r. techniques (COSY, relayed, and double-relayed COSY) and 2D-n.O.e. spectroscopy was attempted. The signals for anomeric protons are designated A–P (Figs. 6 and 7) and the chemical shifts are given in Table VII. The spectrum (Fig. 6) of the partially hydrolysed material, which, according to methylation analysis[10], comprised (1→2)- and (1→6)-linked Man residues and some minor components, contained four major signals (E, I, K, P) together with numerous signals of lower intensity. ^{1}H-N.m.r. data on cell-wall mannans[11,12] and model com-

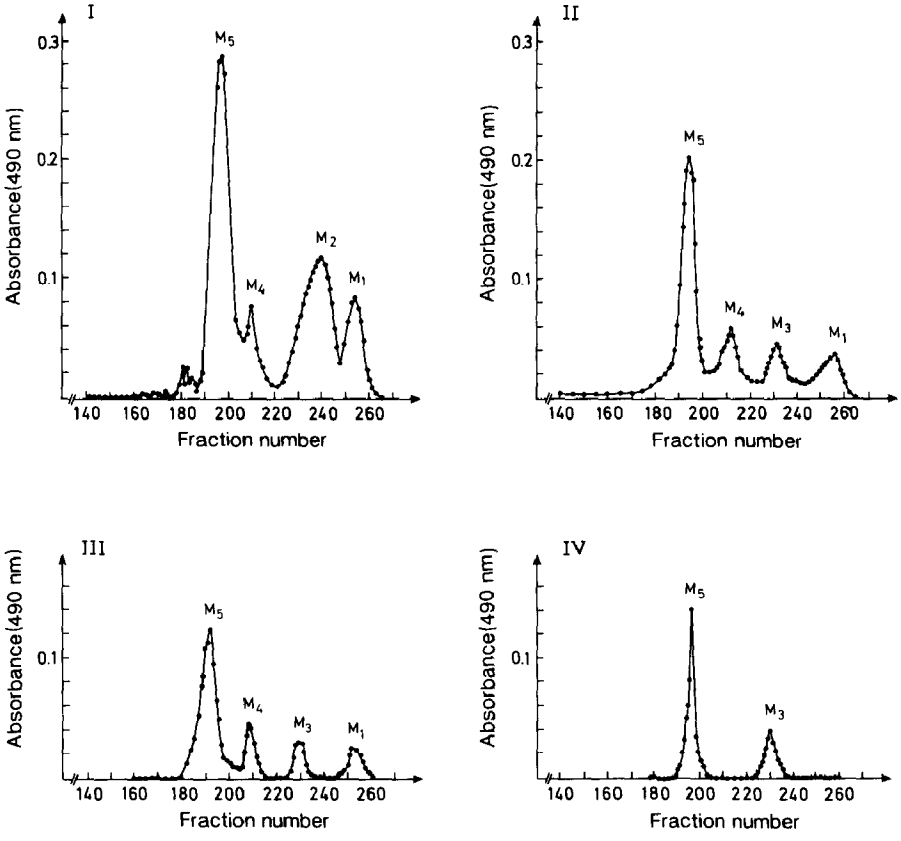

Fig. 5. Fractionation of the products obtained by acetolysis (12 h, 40°) of fractions I–IV (Fig. 3) on a column (2.5 × 95 cm) of Bio Gel P-2 (400 mesh).

TABLE VI

Products obtained by acetolysis (12 h, 40°) of fractions I–IV (Fig. 3)

Fraction	*D.p.*[a]	*Molar ratio*				
		M_1	M_2	M_3	M_4	M_5
I	12–13.5	1.7	2.4	0	0.2	1.2
II	9.5–10.5	2.2	0	0.9	0.6	1.2
III	8.5–9.5	1	0	0.3	0.3	0.9
IV	7.5–8.5	0	0	0.4	0	0.4

[a] Degree of polymerisation.

pounds are available to aid the assignment of signals in Fig. 6. The chemical shifts of the H-1 resonance in the α-**Man** residue in model compounds are as follows: δ 5.23 [Man-(1→2)-**Man**-(1→2)-Man], 5.13 [Man-(1→2)-**Man**-(1→6)-Man], 5.04 [**Man**-(1→2)-Man], and 4.91 [Man-(1→6)-**Man**-(1→6)-Man]. Branching at position 6 can be introduced most likely in the central Man residue in the second trisaccharide without markedly affecting the chemical shift of the H-1 resonance. The above chemical shifts correspond well to those of E, I, K, and P, and are assigned to residues in the side chain, branch points, terminals, and the main chain, respectively, of a structure with a (1→6)-linked main chain and side chains of (1→2)-linked α-Man residues; β-Man residues are not compatible with these chemical shifts.

TABLE VII

^{1}H-N.m.r. data for the carbohydrate moiety of Ag-54

Polysaccharide	*Atom*	*Chemical shift (p.p.m.)*							
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Native	H-1	5.28	5.25	5.23	5.21	5.20	5.19		5.11
	H-2	4.16		4.16		4.12	4.18		4.14
	H-3	4.08		4.08		4.10	4.09		4.10
	H-4								
Partially hydrolysed	H-1		5.25	5.23	5.21	5.20	5.18	5.13	
		I	*J*	*K*	*L*	*M*	*N*	*O*	*P*
Native	H-1	5.10	5.08	5.06	5.06	5.04	4.98	4.96	4.91
	H-2		4.14	4.12	4.10	4.12	3.58	4.20	4.01
	H-3		4.08	4.07		4.01	3.71		3.84
	H-4								
Partially hydrolysed	H-1	5.10	5.09	5.06			4.08	4.96	4.91

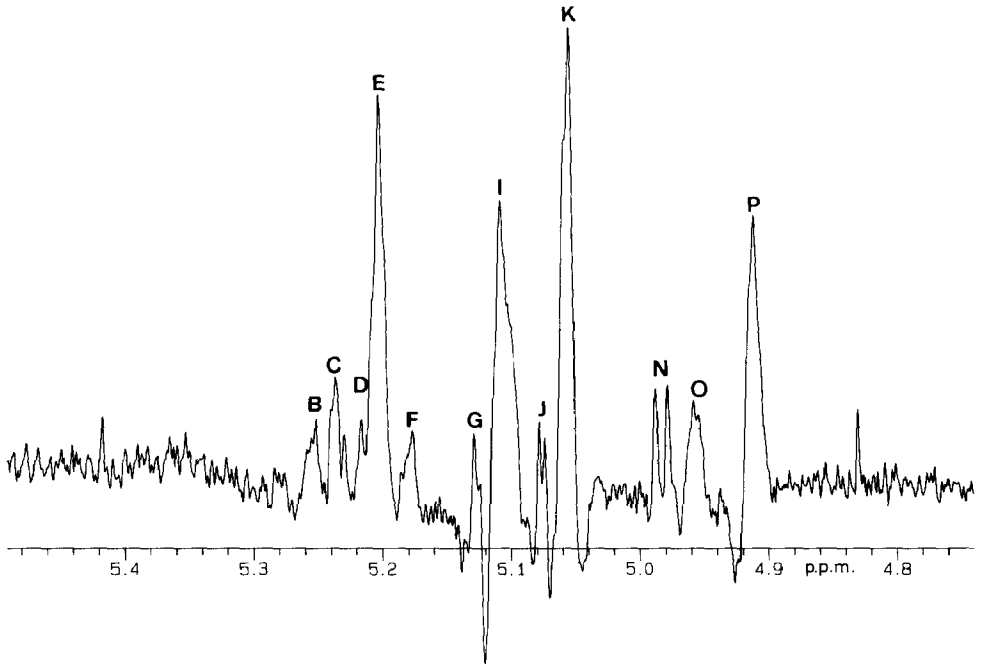

Fig. 6. Resolution-enhanced ^{1}H-n.m.r. spectrum (region for H-1 resonances) of the partially hydrolysed carbohydrate material derived from Ag-54.

In the native material, terminal and (1→6)-linked Gal*f* residues are present[10] and attached mainly to positions 2 and 3 of (1→6)-linked Man residues, position 3 of (1→2)-linked Man residues, and position 6 of other Gal*f* residues. Signals A, H, and J of the native material (Fig. 7) had $J_{1,2}$ values of ~2 Hz, appear at chemical shifts where none was present for the partially hydrolysed material, and are assigned to β-D-Gal*f*-residues (*cf.* $J_{1,2}$ 3.5 Hz, for α-D-Gal*f*[13]).

Molecular models show that H-1 in the β-D-Gal*f* residues are near to protons in neighbouring residues, which may induce an upfield shift[14,15] in the H-1 resonance. The signal for H-1 in β-D-Gal*f* is at δ 5.27, *i.e.*, to lower field of the signals H and J, but not of A. It is possible that when a β-D-Gal*f* residue is attached to position 3 of a (1→2)-linked Man residue, steric crowding occurs which changes the conformation and hence the chemical shift of the H-1 resonance.

Of the remaining H-1 signals in Fig. 7, some had chemical shifts that were the same as those for the partially hydrolysed material (Fig. 6). This finding implies partial substitution of the majority of the Man residues, since at least seven Man signals were identified (E, F, K, L, M, O, and P). 2D-N.m.r. experiments showed that the largest signal in Fig. 7 contained two components (K and L). Substitution of the (1→2)-linked Man residues should yield signals F and K (L) and, of the (1→6)-linked Man residues, signals M and O. The possibility that M was derived from (1→2)-linked and K from (1→6)-linked residues cannot be excluded. The $J_{1,2}$ value of 3.8 Hz for N, together with

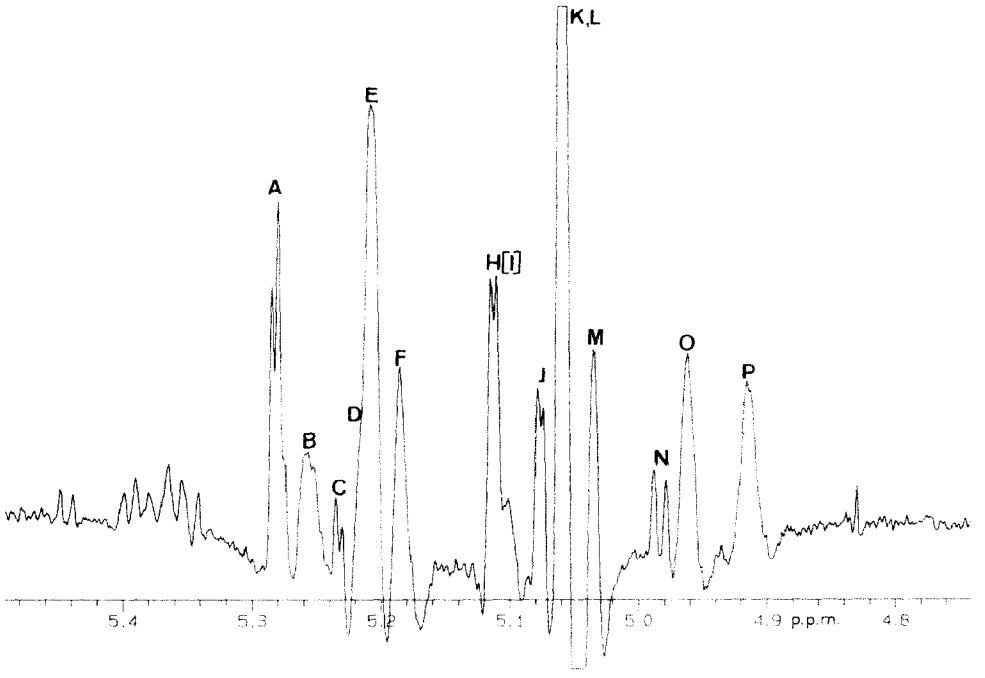

Fig. 7. Resolution-enhanced ^{1}H-n.m.r. spectrum (region for H-1 resonances) of the carbohydrate moiety of Ag-54.

the chemical shifts of signals for H-1,2,3, suggested that N be attributed to a terminal α-D-Glc*p* residue. Furthermore, from the chemical shift of the H-1 resonance, this residue should be attached to position 6 of the next sugar residue[14].

The n.O.e. spectrum of the native material was difficult to interpret because of the crowding of the signals. The Gal*f* residue that gave signal A had an n.O.e. contact at δ 4.08, which could be to H-3 in signal F or K. However, an internal n.O.e. to H-3 appeared at the same chemical shift and made such an assignment uncertain. Signals H and J displayed n.O.e. signals at δ 4.1 but, as this region contained overlapped signals, no conclusions could be drawn. Most Man signals also showed n.O.e.'s in crowded regions. The α-D-Glc*p* group had an n.O.e. contact with an unidentified signal at δ 3.62.

In conclusion, the n.m.r. data of the galactoglucomannan allowed for the possible existence of a structure built of a backbone of (1→6)-linked Man and some Glc, with oligosaccharides of (1→2)-linked Man residues attached in addition to the Gal*f* side chains established earlier[10]. Acetolysis of the native galactoglucomannan yielded both linear and branched oligosaccharides. The presence of (1→2)-, (1→6)-, and (1→2,3)-linked Man together with (1→4)-linked Glc indicated the possibility that acetolysis had taken place next to the branch-point residue or that the main chain might not be analogous to the uniform backbone of (1→6)-linked Man characteristic of many yeast mannans. The polysaccharide moiety of Ag-54 may bear some resemblance to the cell-wall mannan of *Hansenula wingei*[16] in having both (1→6)- and (1→2)- or (1→3)-

linked Man in the main chain. However, a repeating unit could not be suggested from the structural requirements outlined above, which indicated that the polysaccharide is more complex than had been proposed[10].

EXPERIMENTAL

The allergen denoted Ag-54 was isolated from the mycelium and conidia of *C. herbarum.* Link ex. Fr. as described[8].

Treatment of Ag-54 with alkaline borohydride. — Ag-54 (5–15 mg) was treated with M $NaBH_4$ in 0.125M NaOH (1 mL) at 50° for 6 h. The pH of the cooled solution was adjusted to 3 with HCl, the mixture was neutralised, and the boric acid was removed as methyl borate by evaporation with methanol. The carbohydrate components were isolated by ion-exchange chromatography on DEAE Sephadex A-25 [Pharmacia; elution with 0.025M ammonium hydrogencarbonate buffer (pH 7.9)] and freeze-dried.

Fractionation of the carbohydrate components. — The foregoing mixture of carbohydrates was eluted from a column (1.5 × 13 cm) of Bio Gel P-4 200–400 mesh (Bio Rad Laboratories) with 0.075M ammonium hydrogencarbonate buffer (pH 7.9) (0.4-mL fractions). The column was calibrated with dextrans (mol. wts. 16400 and 2360), maltoheptaose (mol. wt. 1153), maltopentaose (mol. wt. 829), and glucose (mol. wt. 180). The phenol–sulfuric acid method[17] was used to obtain the carbohydrate elution profile. The fractions corresponding to each peak were combined, freeze-dried, and subjected to methanolysis and methylation analysis.

Partial acid hydrolysis. — The protein-free carbohydrate fraction (5 mg) was heated at 100° for 2.5 h with 5mM sulfuric acid (1 mL) as reported[10]. The mixture was cooled, neutralised with sodium hydrogencarbonate, and desalted on a column of Bio-Gel P-4 as described above.

Methanolysis. — Quantitative monosaccharide composition was determined by methanolysis (M and 4 M HCl, in dry methanol, 80°, 24 h), followed by trimethylsilylation and g.l.c. as described[2]. The gas chromatograph was equipped with a flame-ionisation detector and a DB-5 fused-silica capillary column (0.2 mm × 15 m), and hydrogen was the carrier gas.

Methylation analysis. — Methylation was effected by the Hakomori method[18] as described by Lindberg and Lönngren[19]. Methyl sulfoxide (0.25 mL) was added to each dry carbohydrate fraction, and the solution was flushed with nitrogen and stirred at room temperature for 2 h. After addition of a solution (0.25 mL) of methylsulfinylmethanide prepared as described by Conrad[20], the mixture was stirred under nitrogen overnight at room temperature, then cooled (ice bath). Methyl iodide (0.25 mL) was added followed by nitrogen flushing and stirring for 2.5 h. Excesses of reagents were removed by reverse-phase chromatography using Sep Pak C_{18}-cartridges (Waters Associates), as described by Waeghe *et al.*[21], and the methylated carbohydrates were hydrolysed with aqueous 90% formic acid (0.5 mL) at 100° for 6 h. After addition of methanol and evaporation of excess of formic acid, the partially methylated monosaccharides were reduced with M sodium borodeuteride (1 mL) overnight at room temperature.

Excess of borodeuteride was destroyed by the addition of acetic acid, and the boric acid was removed by evaporation with methanol and then toluene. The partially methylated alditols were treated with acetic anhydride (0.3 mL) for 1 h at 100°, and the acetic anhydride was removed by co-evaporation with toluene. The residue was analysed by g.l.c.–m.s., using an ion-source temperature of 220° and an ionisation voltage of 70 eV. The chromatograph was equipped with a DB-5 fused-silica column (0.2 mm × 30 m), and retention values were calculated relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Identities were confirmed by g.l.c.–m.s. employing an SP 2340 capillary column (0.2 mm × 12 m). Erythritol tetra-acetate and *myo*-inositol hexa-acetate were used as internal references.

Acetolysis. — A solution of the protein-free carbohydrate (8 mg) in 10:10:1 acetic anhydride–acetic acid–sulfuric acid (1 mL) was kept at 40° for 12 h as described by Kobayashi *et al.*[22]. A 100:100:1 mixture did not effect acetolysis. The reaction was terminated by the addition of pyridine (0.25 mL) to the cooled solution, the mixture was concentrated, the residue was extracted with chloroform, and the extract was washed several times with water and then concentrated. To a solution of the residue in dry methanol (1 mL) was added methanolic M sodium methoxide slowly until precipitation of *O*-deacetylated acetolysis products occurred. The mixture was left at room temperature for 30 min, neutralised with aqueous 50% acetic acid, and concentrated.

The residue was loaded on to a column (2.5 × 95 cm) of Bio Gel P-2 (400 mesh), which had been calibrated with malto-oligosaccharides, and eluted with 0.075M ammonium hydrogencarbonate buffer (pH 7.9, 10 mL/h) that contained 2% of 1-butanol. Fractions of 1.5 mL were collected. Eluates corresponding to the various peaks were combined and concentrated, and the products were rechromatographed. The appropriate fractions were combined, freeze-dried, and analysed by t.l.c. on Silica Gel 60 (Merck, 5748), using 1-butanol–ethanol–water (5:3:2), with detection by the periodate–benzidine reagent[23].

N.m.r. spectroscopy. — The 400-MHz ^{1}H-n.m.r. spectra were recorded at 50° with a JEOL GX400 spectrometer on solutions in D_2O (internal sodium 3-trimethylsilylpropanoate-d_4). All 2D experiments were performed with JEOL standard pulse sequences and had a total of 128 spectra, each accumulated 32 times, except for the NOESY experiment, which was accumulated 64 times. Each spectrum consisted of 512 data points, and the 128 spectra were zero-filled into a 256 × 512 data matrix with a frequency range of 800 Hz in each dimension. For the H-relayed H,H-COSY experiment, a delay time of 70 ms was used; for the NOESY experiment, the mixing time was 200 ms. The data matrix was processed with a trapezoidal filter function, followed by calculation of a power spectrum and symmetrisation.

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