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The Glycoprotein Allergen Ag-54 (Cla h II) from *Cladosporium herbarum*

Structural Studies of the Carbohydrate Moiety

Marit Swärd-Nordmo, Berit Smestad Paulsen, Jens Kristian Wold

Institute of Pharmacy, University of Oslo, Norway

Abstract. The carbohydrate moiety of an important allergen, Ag-54 in *Cladosporium herbarum* was studied by alkaline-borohydride treatment, gel filtration, high-performance liquid chromatography, methylation analysis, gas liquid chromatography and mass spectrometry. The Ag-54 protein core possessed a very limited number of sugar chains. The carbohydrate moiety consisted mainly of one large highly branched polysaccharide chain which accounted for nearly 75% of the total molecular weight of the glycoprotein. The carbohydrate moiety is made up of *D*-mannose and *D*-glucose units in pyranose form having *D*-galactofuranose side chains attached. Mannose is both 1,2- and 1,6-linked, while glucose is 1,4- and 1,6-linked. Some of the 1,6-linked galactofuranose side chains are bound through C-2 of the 1,6-linked mannose units, and the rest to C-3 of 1,6-linked mannose and 1,2-linked mannose units. A few oligoglucosidic chains of approximately 4 glucose units are also attached to the protein.

Introduction

Allergen extracts from fungal sources contain in general a high proportion of carbohydrate material [1, 2]. *Cladosporium herbarum* extracts from 10 different strains had a carbohydrate content ranging from 45 to 73% and differed extensively in immunological composition [3]. IgG-binding polysaccharides have been detected in several microfungi [4-6]. A galactomannan isolated from *Aspergillus fumigatus* was reported to possess antigenic activity [6]. Suzuki and Takeda [7] have studied antigenic galactomannans isolated from *Hormodendrum* strains [7], which are closely related to the genus *Cladosporium* [8].

So far, the IgE-binding activity has been associated almost exclusively with the protein components of allergen extracts. However, an increasing number of allergens are characterized as glycoproteins [9, 10]. Relatively few glycoprotein allergens have been subjected to structural studies [11-13] and only little attention has been paid to the carbohydrate moiety of IgE-binding compounds [12, 13].

One of the important allergens denoted Ag-54 in *C. herbarum* was isolated and purified [14]. This al-

lergen corresponds to Cla h II according to the IUIS allergen nomenclature [15]. The allergen was of glycoprotein nature and contained 19.5% protein and 80.2% carbohydrate material consisting of mannose, galactose and glucose in the proportion 1.0:0.6:1.3. Extensive information concerning the allergen structure is relevant for the knowledge of the molecular mechanisms involved in IgE-mediated allergy. The aim of the present study has been to characterize the carbohydrate moiety of Ag-54 in more detail by studying the mode of linkage between the monosaccharide constituents.

Materials and Methods

Material

The allergen denoted Ag-54 from *C. herbarum* Link ex. Fr was isolated and purified as described previously [14].

Release of Carbohydrate Chains: Alkaline-Borohydride Treatment

Alkaline-borohydride treatment for splitting the protein and carbohydrate moieties of the glycoprotein was performed basically as described previously [16]. To Ag-54 (0.7 mg) were added 0.5 ml

sodium borohydride (2 mol/l) and 0.5 ml sodium hydroxide (0.25 mol/l), and the mixture was incubated at 50°C for 6 h followed by decomposition of excess sodium borohydride by the addition of 3 mol/l HCl. The boric acid was removed as methyl borate by rotary evaporation with methanol. The protein and carbohydrate moieties were separated by ion exchange chromatography using DEAE-Sephadex A-25 (Pharmacia, Sweden). The sample was applied on the equilibrated column (1.5 × 10 cm) and the carbohydrate was eluted at 20°C with 0.025 mol/l ammonium bicarbonate buffer (pH 7.9) followed by elution of the protein moiety with 1 mol/l ammonium bicarbonate buffer (pH 7.9). The protein and carbohydrate fractions, respectively, were freeze-dried, dissolved in purified water and desalted or fractionated by gel filtration as described below. The desalted, freeze-dried protein moiety was analyzed for carbohydrate material after methanolysis, trimethylsilylation and gas liquid chromatography (GLC) as described previously [14].

Gel Filtration

The carbohydrate fraction from alkaline-borohydride treatment was applied on a pre-equilibrated Bio-Gel P-4 (Bio-Rad Laboratories) column (1.5 × 12 cm) and eluted at 20°C with 0.125 mol/l ammonium bicarbonate buffer (pH 7.9). Fractions of 0.4 ml were collected. The column was calibrated with dextrans (7,600 and 2,200 daltons) stachyose (667 daltons) and mannose (180 daltons) for estimation of molecular weight distributions. Fractions corresponding to the molecular weight ranges >4,000 daltons (void), 3,000–1,000 daltons and <667 daltons, respectively, were pooled, freeze-dried and further examined by methylation analysis as described below.

Mild Acid Hydrolysis

For removal of carbohydrate existing as furanose units, the high molecular weight fraction (void) derived after alkaline-borohydride treatment and gel filtration as described above was heated at 100°C for 2.5 h with 0.005 mol/l sulfuric acid (0.5 ml) basically as reported by Smestad Paulsen et al. [17]. The hydrolysate was neutralized with sodium bicarbonate (0.25 mol/l) and fractionated on a Bio-Gel P-4 column as described above. The low molecular weight fraction corresponding to the released furanose units, the intermediate molecular weight fraction and the high molecular weight fraction corresponding to the main core, were freeze-dried and analyzed for carbohydrate content after methanolysis, trimethylsilylation and GLC as described earlier [14]. The high molecular weight fraction was submitted to methylation analysis as described below.

Methylation Analysis for Determination of the Carbohydrate Structure

Native purified Ag-54 (0.2 mg), carbohydrate fractions derived from alkaline-borohydride treatment and the mild acid hydrolysis product of the high molecular weight fraction, respectively, were methylated according to the method of Hakomori [18] as described by Lindberg and Lönngren [19]. The samples, predried under vacuum, were added to 0.5 ml dimethylsulfoxide and flushed with nitrogen.

After 1 h with continuous magnetic stirring at room temperature, methylsulfinylcarbanion (0.5 ml), prepared as described by Conrad [20], was added and the mixtures were kept under nitrogen atmosphere and stirred over night at room temperature. The sam-

ples were cooled to 0°C and methyl iodide (0.5 ml) was added followed by nitrogen flushing and magnetic stirring for 2.5 h.

Excess reagents were removed by reverse-phase chromatography using SEP Pak C₁₈-cartridges (Waters Associates, Milford, Mass.) basically as described by Waeghe et al. [21]. After pretreatment of the cartridges with ethanol (40 ml), acetonitrile (2 ml) and distilled water (4 ml), respectively, the samples were diluted with 1.5 ml distilled water and loaded onto the column. The reagents were washed out of the column with distilled water (4 × 2 ml), 15% acetonitrile (2 × 2 ml) followed by 20% acetonitrile (2 × 2 ml) before the methylated carbohydrates were eluted from the column with 100% acetonitrile (2 ml) and subsequently with 100% ethanol (2 ml). The phenol-sulfuric acid method of Dubois et al. [22] was used for detection of the carbohydrate material. The methylated carbohydrates were evaporated to dryness under vacuum, and hydrolyzed with 90% formic acid (1 ml) at 100°C for 6 h. Any formyl esters were cleaved by addition of 1 ml of water and treatment at 100°C for 1 h.

Excess formic acid was removed as methyl formate by rotary evaporation with methanol. The derived partially methylated monosaccharides were reduced with sodium borodeuteride (1 mol/l, 1 ml) over night at room temperature. Excess borodeuteride was destroyed with acetic acid and the boric acid removed by rotary evaporation with methanol followed by toluene for drying the material. The partly methylated alditols were acetylated with acetic anhydride (1 ml) at 100°C for 1 h and the acetic anhydride was removed by evaporation with toluene. The residue was suspended in water and the mixture extracted with chloroform. The partially methylated alditol acetates were analyzed by gas-liquid chromatography/mass spectrometry (GLC/MS) using a Finnigan 9610 gas chromatograph equipped with a 0.2 × 30 m SE 54 glass capillary column coupled to a Finnigan Mat 4021 mass spectrometer employing an ion source temperature of 220°C and an ionization voltage of 70 eV. Retention times were calculated relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol (TMG) and compared to the corresponding values of reference mass spectra. The identity was confirmed by GLC on OV-1, SP-1000 and CP-Sil 88 glass capillary columns, respectively, as described by Lomax et al. [23].

Determination of Absolute Configuration

Dried Ag-54 and references, respectively, were heated at 80°C for 24 h with 1 mol/l HCl in anhydrous methanol (0.5 ml) using arabinitol as internal standard. After evaporation to dryness, the residual methylglycosides were converted to the corresponding (+)-2-butylglycosides by heating with 1 mol/l HCl in (+)-2-butanol (80°C, 8 h) basically as described by Gerwig et al. [24]. After solvent evaporation trimethylsilyl derivatives were prepared using pyridine:hexamethyldisilazane:trimethylchlorosilane (5:2:1) for 30 min at 25°C. The trimethylsilylated (+)-2-butylglycosides were chromatographed on a Carlo Erba 4200 gas chromatograph equipped with a flame ionization detector and a 15 m DB 5-15 W fused silica capillary column with H₂ as carrier gas (1.2 ml/min).

Molecular Weight Estimation: High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) was performed using a 300 × 7.5 mm TSK-G-2000 SW Blue Column (LKB, Sweden). Carbohydrate moieties (25 µg) released from the protein core by alkaline-borohydride treatment as described above and carbohydrate moieties submitted to mild acid hydrolysis (25 µg),

Table I. Methylation analysis of Ag-54 carbohydrate moiety giving the type of linkages of the constituent monosaccharides

Alditol acetate corresponding to	Type of linkage	R _t ¹	Primary fragments	Relative peak area				
				GLC/MS system ²			Lomax system [23]	
				MW < 667	MW > 4000 and native glyco- protein	mild acid hydro- lysis	MW > 4000 and native glyco- protein	mild acid hydro- lysis ³
Branch points								
2,4-di- <i>O</i> -methylmannitol/glucitol	1, 3, 6	1.66	118, 189		1	0.2	1/1 ⁴	0.2/0.2 ⁴
3,4-di- <i>O</i> -methylmannitol	1, 2, 6	1.62	189, 190		6.3	1	2.7	0.4
4,6-di- <i>O</i> -methylmannitol	1, 2, 3	1.49	45, 161, 262		2.6	0.8	2	0.6
Unbranched chain groups								
2,3,4,tri- <i>O</i> -methylmannitol	1, 6	1.35	118, 162, 189, 233		2.5	4.9	1.9	3.7
2,3,5-tri- <i>O</i> -methylgalactitol	1, 6	1.41	117, 118, 233		2.3	0.1	1.4	0.06
2,3,6-tri- <i>O</i> -methylglucitol	1, 4	1.29	45, 118, 162, 233	trace	1.4	1.4	1.2	1.2
3,4,6-tri- <i>O</i> -methylmannitol	1, 2	1.25	45, 161, 190		7.6	8.3	4.8	5.2
End groups								
2,3,4,6-tetra- <i>O</i> -methylmannitol/glucitol	1 _p	1.0	45, 118, 161, 162, 205	trace	1	1	0.5/0.5 ⁴	0.5/0.5 ⁴
2,3,5,6-tetra- <i>O</i> -methylgalactitol	1 _f	1.02	45, 89, 118, 162, 205, 278		2.3	0.8	4	1.4

¹ Retention value relative to 2,3,4,6-tetra-*O*-methyl-*D*-glucitol 1,5-diacetate in the GLC/MS system.² Area of the peak relative to 2,3,4,6-tetra-*O*-methyl-*D*-mannitol/glucitol 1,5-diacetate.³ Calculated values.⁴ The identity of both has not been confirmed by MS.

respectively, were injected in a 20- μ l vol and eluted with 50 mmol/l phosphate buffer (pH 6.8) containing 0.1 mol/l NaCl at a flow rate of 0.5 ml/min. The column was calibrated with dextrans (19,100, 16,400, 2,300 daltons), stachyose (667 daltons) and maltotriose (505 daltons). The elution of carbohydrates was monitored with an RI detector (5902 Interference Refractometer).

Experimental and Results

The protein-carbohydrate bond was split by alkaline sodium borohydride treatment. No carbohydrate material could be detected in the released protein moiety. The carbohydrate moiety was fractionated by gel filtration to get an approximate estimate of the oligosaccharide size. A part of the high molecular weight fraction was hydrolyzed under mild conditions before the samples were submitted to methylation analysis to determine the position of the linkages between component monosaccharide residues in the carbohydrate moiety. The oligosaccharide fractions were transformed to polyalkoxide ions and methylated before

hydrolysis. The derived partly methylated monosaccharides were converted into the corresponding alditol acetates before GLC/MS analysis. The high molecular weight fraction (MW > 4,000 daltons) contained significant amounts of carbohydrates while nothing but trace amounts of glucose could be detected in the low molecular weight fraction (MW < 667 daltons). No carbohydrate material was found in the intermediate molecular weight fraction (MW 3,000–1,000 daltons). The components present and their mode of linkages in the different fractions are listed in table I. The chromatogram of the partly methylated alditol acetates before and after mild acid hydrolysis is shown in figure 1.

In order to estimate the molecular weight of the carbohydrate moiety released from the protein core by alkaline-borohydride treatment, the sample was analyzed by HPLC. The molecular weight of the carbohydrate was found to be 19,000 daltons based on dextran references as shown in table II. To estimate the molecular weight of the main chain as compared

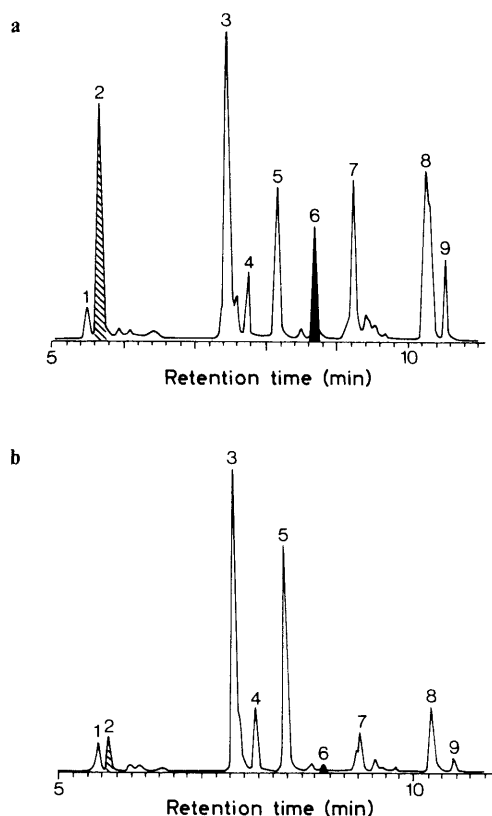


Fig. 1. GLC pattern obtained after methylation analysis of the Ag-54 carbohydrate moiety (SE 54 glass capillary column). The peaks represent the partially methylated alditol acetates corresponding to: 1 = 2,3,4,6-Tetra-*O*-methylmannitol/glucitol; 2 = 2,3,5,6-tetra-*O*-methylgalactitol; 3 = 3,4,6-tri-*O*-methylmannitol; 4 = 2,3,6-tri-*O*-methylglucitol; 5 = 2,3,4-tri-*O*-methylmannitol; 6 = 2,3,5-tri-*O*-methylgalactitol; 7 = 4,6-di-*O*-methylmannitol; 8 = 3,4-di-*O*-methylmannitol; 9 = 2,4-di-*O*-methylmannitol/glucitol. **a** Fraction >4,000 daltons before mild acid hydrolysis. **b** Fraction >4,000 daltons after mild acid hydrolysis.

to that of the branches, the galactofuranose side chains were removed by mild acid hydrolysis. The HPLC results gave an apparent molecular weight of 9,000 daltons, indicating that the main chain accounted for half of the molecular weight of the total carbohydrate moiety.

The absolute configuration of the constituent monosaccharides was elucidated after butanolysis with the chiral reagent (+)-2-butanol and GLC separation of the trimethylsilylated (+)-2-butylglycosides on a nonchiral stationary phase. The gas chromatogram (fig. 2) demonstrates the occurrence of *D*-mannose, *D*-galactose and *D*-glucose. No *L*-enantiomers could be detected.

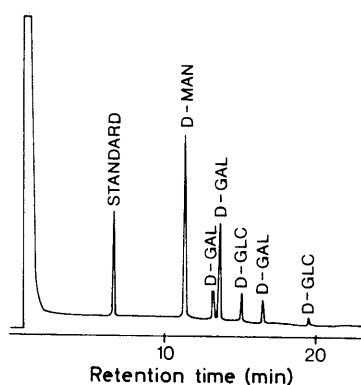


Fig. 2. GLC separation of the trimethylsilylated (+)-2-butyl glycosides of the monosaccharides occurring in the Ag-54 carbohydrate moiety, indicating the absolute configuration.

Table II. Molecular weight estimation by HPLC of the Ag-54 carbohydrate moiety released by alkaline-borohydride treatment and of the carbohydrate backbone remaining after removal of the furanose side chains

	Apparent molecular weight	Estimated number of monosaccharide units
Carbohydrate moiety, branched	19,000	117
Carbohydrate backbone	9,000	55

A LKB Ultrapack TSK-G 2000 SW Blue column precalibrated with dextrans was employed.

Discussion

The methylation analysis of the various fractions of carbohydrate present in Ag-54 from *C. herbarum* demonstrated that the carbohydrate moiety mainly consisted of a galactomannan containing a few glucose units. The partly methylated alditol acetates were identified both by their retention times and their mass spectra. As response factors for the various methylated alditol acetates were not available, the 2,3,4,6-tetra-*O*-methylmannitol/glucitol peak area was used as a unit to indicate their relative proportions. The quantity of this compound seemed to be unaltered after the acid treatment of the carbohydrate

moiety. The multiple-column approach of Lomax et al. [23] was used to confirm the identity and to quantify the partly methylated alditol acetates. This method of quantification probably represents a more reliable estimation of the relative proportions of the methylated alditol acetates than peak areas from GLC/MS analysis (table I). By the Lomax method it was demonstrated that 2,3,4,6-tetra-*O*-methylmannitol and 2,3,4,6-tetra-*O*-methylglucitol, corresponding to pyranosidic mannose and glucose end groups, respectively, were present in equal amounts. The presence of 2,3,5,6-tetra-*O*-methylgalactitol reveals terminal galactofuranose. The high proportion of this indicates that galactofuranosyl represents the main terminal sugar present in the polymer. Apart from being end groups, galactofuranose is also 1,6-linked in the chain, as demonstrated by the formation of 2,3,5-tri-*O*-methylgalactitol in the methylation analysis. Terminal galactofuranose and 1,6-linked galactofuranose were found in the proportion 2,8:1, indicating short branches. 1,2-linked mannose accounts for more than half of the pyranosidic sugar moieties in the unbranched chain, the rest being 1,6-linked mannose and 1,4-linked glucose.

The high proportion of di-*O*-methyl compounds formed in the methylation analysis indicates that the carbohydrate moiety must be highly branched. The di-*O*-methylalditolacetates corresponding to 1, 3, 6-linked mannose and 1,3,6-linked glucose could not be separated by GLC/MS but was found to be present in equal proportions by the Lomax method. The content of units representing branch points is fairly equal to the total amount of pyranosidic sugar moieties occurring in the unbranched chain, suggesting one branch for every second to third unit in the main core. One should, however, expect to find terminal groups and branch points in equal amounts. This lack of consistency may be due to the acid lability of furanosidic sugars, resulting in degradation to some extent of galactofuranose during the formic acid hydrolysis, and also to the fact that tetra-*O*-methyl derivatives are more volatile than di-*O*-methyl derivatives.

The lability of galactofuranose was utilized for selective removal of the galactofuranosyl units by hydrolyzing the carbohydrate material obtained by the alkaline-borohydride treatment under mild acidic conditions before fractionation and methylation analysis. By this method it was possible to demonstrate that both the 1,6-linked galactofuranose and terminal galactofuranose originated from the branches and not

from the main core, since the main core was still present in the high molecular weight fraction. No increase in 2,3,4,6-tetra-*O*-methylmannitol/glucitol could be detected, confirming that the main core had not been cleaved during mild acid hydrolysis. The unaltered content of terminal pyranosidic sugars also indicates that the side chains most probably are entirely furanosidic. Methanolysis of the low molecular weight fraction corresponding to released furanose units derived from mild acid hydrolysis showed that galactose was the only sugar present. No carbohydrates were detected in the intermediate molecular weight fraction (MW 3,000–1,000 daltons), while mannose and glucose were found in the same proportion in the high molecular weight fraction (MW > 4,000) as in the native Ag-54. Methylation analysis of the acid-treated material revealed some residual terminal galactofuranose most probably due to incomplete removal of the side chains. These could probably have been split off quantitatively by increasing the duration of the hydrolysis, but then the risk of cleaving the pyranosides is growing. The removal of the branches resulted in a reduction of the amount of units with branch points, most clearly reflected by the decrease of the 3,4-di-*O*-methylmannitol. This was accompanied by a simultaneous increase of the amount of tri-*O*-methyl sugar units. The 2,3,4-tri-*O*-methylmannitol increased most extensively in the methylated mild acid hydrolyzed material, while only a slight increase of the amount of 3,4,6-tri-*O*-methylmannitol was observed. Both 3,4-di-*O*-methylmannitol and 2,4-di-*O*-methylmannitol would give rise to 2,3,4-tri-*O*-methylmannitol if their corresponding sugar moieties in the native material had branch points linked at C-2 and C-3, respectively. 4,6-di-*O*-methylmannitol would give rise to 3,4,6-tri-*O*-methylmannitol if the corresponding sugar moiety had a branch point at the C-3 position. The results obtained make it possible to conclude that nearly half of the total amount of branches are linked to position C-2 of the 1,6-linked mannose, while the rest is linked to position C-3 of the 1,6-linked mannose and the 1,2-linked mannose and probably not to position C-3 of the 1,6-linked glucose, since no increase in tri-*O*-methylglucitols could be detected after mild acid hydrolysis.

The molecular weight of the carbohydrate moiety was estimated by HPLC of the sample after removal of the protein by alkaline-borohydride treatment. The results confirmed that the carbohydrate moiety is made up of more than 100 units. Previously the gly-

coprotein molecular weight was estimated by different methods suggesting a value of 19,000–25,000 daltons [14, 25]. HPLC using protein references gave an apparent glycoprotein molecular weight of 22,000 daltons [14]. The present HPLC results indicate that each glycoprotein has a long carbohydrate moiety attached. In addition, one or two small oligoglucosidic units consisting of up to four sugars may be linked to the protein as indicated by the methylation analysis of the low molecular weight fraction (table I). HPLC of the sample after mild acid hydrolysis demonstrated that the main core had a molecular weight of 9,000 daltons, corresponding to approximately 50 sugar units. According to the methylation analysis, there should be one branch for every second to third unit in the main core and a proportion of terminal galactofuranose:1,6-linked galactofuranose equal to 2,8:1. Based on this it can roughly be suggested that the branches consist of disaccharide and monosaccharide units in fairly equal proportions.

In conclusion, it has been demonstrated that the Ag-54 protein core possesses a very limited number of sugar chains. The carbohydrate moiety consists mainly of one large polysaccharide which is highly branched with short side chains. This moiety accounts for nearly 75% of the total molecular weight of the glycoprotein. In addition a few small oligoglucosidic units are possibly linked to the protein. The carbohydrate moiety of the Ag-54 allergen must be expected to have a profound effect upon the three-dimensional structure of the glycoprotein molecule and consequently influence its biological function.

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Correspondence to: Dr. Marit Swärd-Nordmo
Nycomed AS
Research and Development Section
PO box 4220, Torshov
N-0401 Oslo 4 (Norway)