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

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Abstract. The carbohydrate-protein linkage of the allergen Ag-54 in the mould *Cladosporium herbarum* was studied after alkaline-borohydride treatment of the glycoprotein. By incorporating tritium into the peptide-linked monosaccharide through the alkaline scission of the linkage, it was demonstrated that the highly branched galactomannan moiety of Ag-54 was linked to the protein through mannose. Threonine was identified as the carbohydrate-linked amino acid. In addition, a few glucose units were peptide-linked, but the corresponding amino acids were not identified. When deglycosylated with trifluoromethanesulfonic acid, Ag-54 retained only 5% of its allergenic activity as compared to the native Ag-54 when tested by rocket immunoelectrophoresis and autoradiography. Immunological identity between native and deglycosylated Ag-54 was observed. Deglycosylation also resulted in reduced stability towards denaturation by heat or urea.

Introduction

Over the last few years, it has been recognized that the glycoproteins represent an important group of IgE-binding molecules. Glycoprotein allergens and carbohydrate-rich allergen fractions from various sources have been identified and partly characterized [1-4]. In some cases the monosaccharide composition of the allergen fraction has been established [3, 5, 6], but only a few studies have been performed in order to investigate the mode of linkage between the monosaccharide constituents of allergens [7].

Previously we have investigated the carbohydrate moiety of the glycoprotein allergen Ag-54 from the mould *Cladosporium herbarum* [6, 8] corresponding to Cla h II according to the IUIS allergen nomenclature [9]. This allergen contained 19.5% protein and 80.2% carbohydrate made up of mannose, galactose and glucose in the proportion 1:0.6:1.3. The protein core possessed one highly branched galactomannan made up of more than 100 monosaccharide units. In addition, a few small oligoglucoside units seemed to be attached to the protein. The galactomannan consisted of *D*-mannose and *D*-glucose units in pyranose form having short side chains of one or two *D*-galacto-

furanose units attached to approximately every second unit in the main core. Mannose was both 1,2- and 1,6-linked while glucose was 1,4- and 1,6-linked. Some of the 1,6-linked galactofuranose branches were 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. The type of linkage between carbohydrate and protein was not identified.

The aim of the present work has been to investigate the carbohydrate-protein linkages of the glycoprotein and to study some biochemical properties of deglycosylated material.

Materials and Methods

Material

The allergen denoted Ag-54 obtained from *C. herbarum*. Link ex Fr. was isolated and purified as previously reported [6].

Rabbit Antiserum and Human Serum

A polyvalent rabbit antiserum (anti-ch-77) which contained high level of precipitating antibodies to Ag-54 and a human serum (TN) which contained high amounts of specific IgE to Ag-54 were employed as described earlier [6, 10].

*Determination of the Sugar-Linked Amino Acid***Alkaline-Borohydride Treatment in the Presence of PdCl₂ Catalyst**

Alkaline-borohydride treatment for splitting the protein-carbohydrate linkage was performed using PdCl₂ as catalyst basically as described by Downs et al. [11]. A sample of Ag-54 (2 mg) dissolved in 1 ml aqueous 0.1 mol/l sodium hydroxide containing 0.9 mol/l sodium borohydride was incubated at 50°C for 6 h. After cooling to room temperature 1 drop of N-octanol was added under magnetic stirring. 1 ml of 0.4 mol/l HCl was added under vigorous stirring, followed immediately by 100 µl of an aqueous solution of PdCl₂ 2H₂O (0.08 mol/l) prepared as described by Tanaka et al. [12]. 2 ml sodium borohydride (0.66 mol/l in 0.1 mol/l sodium hydroxide) and 2 ml PdCl₂ 2H₂O (0.016 mol/l in 0.8 mol/l HCl) were added dropwise and simultaneously under vigorous stirring. The mixture was evaporated under reduced pressure and hydrolyzed in 6 mol/l HCl (10 ml, Suprapure grade, Merck) at 110°C for 20 h without removal of the palladium black. After evaporation to dryness, amino acid analysis was performed as described below.

Alkaline-Borohydride Treatment without Catalyst

The amino acid composition of the protein moiety was investigated by performing alkaline-borohydride treatment in the absence of PdCl₂ as described previously [8]. The protein fraction obtained by anion exchange chromatography was submitted to amino acid analysis as described below.

*Determination of the Peptide-Linked Carbohydrate***Alkaline Degradation in the Presence of Tritiated Sodium Borohydride**

Tritium was incorporated into the peptide linked carbohydrate by dissolving the Ag-54 sample (12 mg) in a mixture (2 ml) of 0.125 mol/l sodium hydroxide and 1 mol/l sodium borohydride containing NaBT₄ (50 mCi). The mixture was heated to 50°C for 6 h, cooled to room temperature, and the reaction was stopped by addition of HCl to pH 4. After neutralization, the boric acid was removed as methyl borate by rotary evaporation with methanol, the residue was dissolved in 0.025 mol/l sodium bicarbonate buffer and applied on a preequilibrated column (1.5 × 10 cm) of DEAE-Sephadex A-25 (Pharmacia, Sweden). The neutral carbohydrate moiety was eluted with 0.025 mol/l sodium bicarbonate buffer pH 7.9 and the collected fraction was freeze-dried.

Detection of Radioactive Alditols

For detection of the labelled alditols formed during degradation with alkaline-tritiated sodium borohydride, the released carbohydrate moiety was hydrolyzed in 90% formic acid (1 ml) at 100°C for 1 h. Excess formic acid was removed by repeated evaporation with methanol under reduced pressure. The residue was dissolved in distilled water and the derived monosaccharide/radioactive alditol mixture was separated by thin layer chromatography (TLC) [13] using silica gel 60 TLC plastic sheet (Merck Art No. 5748) as stationary phase and a mobile phase containing isopropanol: 0.1 mol/l boric acid (85:15). Mannitol, galactitol and glucitol were used as reference substances and detected on the chromatogram by periodate-benzidine reagent [14]. The silica gel of the chromatographed sample corresponding to the position of the references were scraped off the TLC plate and transferred to scintillation vials. Alditols with incorporated tritium were detected by scintilla-

tion spectrometry in a Mark II scintillation counter after addition of 5 ml scintillation liquid (PPO 6 g/l, POPOP 0.2 g/l in toluene) to each sample vial.

Acetylation of the Alditols and Gas Liquid Chromatography

A part of the alditol sample obtained after the formic acid hydrolysis as described above was dissolved in anhydrous pyridine (0.5 ml) and acetic anhydride (0.5 ml) before heating at 100°C for 30 min. Excess reagents were removed by rotary evaporation with toluene. The alditol acetates dissolved in methanol were separated by gas liquid chromatography (GLC) using CP Sil 43 C B fused silica capillary column (0.32 mm × 26 m) H₂ as carrier gas (3.6 ml/min) and flame ionization detector. The alditol acetates were identified by their retention times and by coinjection with authentic references.

Amino Acid Analysis

The sample was hydrolyzed under vacuum in 6 mol/l HCl (Suprapure grade, Merck) at 110°C for 20 h. After removal of the HCl by rotary evaporation, the amino acid composition was determined using a Biotronic LC 5000 automatic amino acid analyzer.

Performic Acid Oxidation

Total half cystine was analysed as cysteic acid after performic acid oxidation of Ag-54 [15]. 150 µl of a mixture containing 90% formic acid: 35% hydrogen peroxide (9:1) was added to the sample (1 mg). The reaction mixture was kept at 0°C for 4 h, evaporated in a vacuum dessicator, dissolved in water and freeze-dried twice to remove excess reagent before hydrolysis and amino acid analysis, as described above.

Reduction and Carboxymethylation

Carboxymethylation of Ag-54 was performed at pH 8.6 by dissolving the sample (1.3 mg) in a solution containing 6.7 mol/l guanidine chloride, 0.58 mol/l Tris-HCl and 5 mmol/l EDTA basically as described previously [16]. Dithiothreitol was added to a concentration of 2 mmol/l and the mixture was incubated under nitrogen at 37°C for 16 h. After addition of 10 µl of 50 mmol/l iodoacetic acid neutralized with sodium hydroxide the reaction was allowed to proceed in the dark for 40 min. The reaction product was dialysed against distilled water (membrane cut off 1,000 daltons) and freeze-dried before hydrolysis and amino acid analysis as described above.

Deglycosylation

The glycoprotein was freed from its carbohydrate moiety basically as described by Edge et al. [17]. A sample of Ag-54 (8 mg) was added to a mixture of anisole (1 ml) and trifluoromethanesulfonic acid (2 ml) precooled to 0°C, flushed with nitrogen and stirred for 3.5 h at 0°C. 50 ml diethylether containing 10% petrolether precooled to -40°C was added and the mixture was kept at -40°C for 1 h. After 20 min, 10 drops of pyridine were added to facilitate the precipitation of the deglycosylated protein. The precipitate was centrifuged and repeatedly washed with cold ether followed by dissolution in ethanol and rotary evaporation. The deglycosylated protein moiety was separated from the low molecular weight carbohydrate material by gel filtration on a Bio-gel P-4 (Bio-Rad Laboratories) column (1.5 × 12 cm) eluted with 0.125 mol/l ammonium bicarbonate buffer (pH 7.9). The protein material eluted in the void volume was freeze-dried. The protein content of the freeze-dried

material was determined by the method of Withaker et al. [18] and the carbohydrate content was analyzed by methanolysis as described below.

Quantitative Carbohydrate Analysis

The deglycosylated sample and the protein moiety obtained after alkaline-borohydride treatment were subjected to methanolysis (1 mol/l methanolic HCl, 24 h, 80°C) followed by GLC of the trimethylsilylated methylglycosides as described previously [19]. The Carlo Erba 4200 gas chromatograph was equipped with a flame ionization detector and a SE-30 glass capillary column (0.2 mm × 30 m) with H₂ used as carrier gas (1.2 ml/min).

Dansyl-Edman Degradation

A sample of 0.3 mg deglycosylated Ag-54 was submitted to N-terminal sequence analysis by the Edman [20] procedure as modified by Sletten et al. [21].

Thermal Denaturation

Samples of Ag-54 (100 ng/μl) and deglycosylated Ag-54 (90 ng/μl) respectively were heated to 60, 70 and 80°C for 5 min and 100°C for 5 min, 20 min and 1 h before immunoelectrophoretic experiments as described below.

Denaturation with Urea

Samples of Ag-54 (100 ng/μl) and deglycosylated Ag-54 (70 ng/μl) respectively were prepared in 8 mol/l urea containing 2-mercaptoethanol (0.6 mol/l). The mixture was incubated at 25°C for 1 h before immunoelectrophoretic experiments as described below.

Immunoelectrophoresis

Rocket radioimmunoelectrophoresis (RRIE) was performed as described elsewhere [19]. The antibody gel contained anti-ch-77 rabbit antiserum (7 μl/cm²) in 1% agarose (Bio Rad Laboratories) as described previously. The electrophoresis was carried out at 2 V/cm for 15 h with Tris-tricine buffer pH 8.6 (Bio-Rad Laboratories). The slides were incubated with TN patient serum before the autoradiography procedure as described earlier [7, 19].

Results

In order to establish whether the carbohydrate chains are bound to serine or threonine residues in the protein core through *O*-glycosidic linkages, an alkaline-borohydride treatment was performed both in the absence and in the presence of PdCl₂ as catalyst. In this reaction the α-amino-propenoic acid residues derived from serine will be hydrogenated quantitatively to give alanine both in the absence and presence of catalyst, whereas α-aminobutenoic acid residues from threonine will be converted into 2-amino-2-butanoic acid in the presence of catalyst [12]. Amino acid analysis of the protein core obtained after alkaline-borohydride treatment demonstrated a significant decrease in the threonine content as compared to the native glycoprotein. This was accompanied by a corre-

Table I. Destruction of threonine and formation of 2-amino-2-butanoic acid after alkaline borohydride treatment of Ag-54 with PdCl₂ as catalyst

	Res/100			
	native Ag-54	alkaline-boro-hydride treatment with PdCl ₂ catalyst	alkaline-boro-hydride treatment without catalyst	ΔRes/100
Serine	8.2	n.d.	8.3	+0.1
Threonine	8.1	6.8	6.7	-1.3
Alanine	10.6	n.d.	11.1	+0.5
2-Amino-2-butanoic acid	0	1.6	n.d.	+1.6

n.d. = Not determined.

Table II. Tritium activity in cpm detected by scintillation spectrometry of the alditols released after alkaline degradation of Ag-54 in the presence of excess borohydride containing tritium, hydrolysis and separation of the alditols by TLC

Sample corresponding to reference	cpm
Mannitol	1,050
Galactitol	200
Glucitol	2,150
Silica gel (blank)	170

sponding formation of 2-amino-2-butanoic acid as shown in table I, strongly indicating that threonine is involved in the attachment of the carbohydrate unit. Both the serine and the alanine content remained largely unaffected by the alkaline-borohydride treatment. No carbohydrate material could be detected when the protein core was analyzed by methanolysis, trimethylsilylation and GLC, indicating that the glycoprotein did not contain alkali-stable peptide-glycoside linkages.

By performing the alkaline degradation in the presence of excess borohydride containing tritium, the carbohydrate residues liberated would be expected to terminate in a sugar alcohol with tritium incorporated into the C-1 position. After hydrolysis of the released carbohydrate chain, the radioactive alditols separated by TLC were detected by scintillation spectrometry. The tritium activity shown in table II indicates that both mannose and glucose but not gal-

Table III. Carbohydrate and protein contents of native and deglycosylated Ag-54

	Native % of dry weight	Deglycosylated % of dry weight
Carbohydrate	80.2	5.9
Protein	19.5	94.1
Man:gal:glc	1:0.6:1.3	1:0:0.3

The carbohydrates were quantified by methanolysis and GLC of the trimethylsilylated methylglycosides.

Table IV. Amino acid composition of native and deglycosylated Ag-54

Amino acid	Res/100	
	native	deglycosylated
Aspartic acid/asparagine	13.1	13.4
Threonine	8.1	8.2
Serine	8.2	8.3
Glutamic acid/glutamine	10.0	9.8
Proline	4.1	3.9
Glycine	14.8	14.7
Alanine	10.6	10.9
½-Cysteine	1.3	1.3
Valine	7.5	7.3
Methionine	0.2	trace
Isoleucine	3.5	3.8
Leucine	2.9	2.8
Tyrosine	1.1	0.1
Phenylalanine	3.3	3.2
Histidine	4.5	4.3
Lysine	5.8	5.6
Arginine	3.2	3.7

Table V. Allergenic activity of deglycosylated Ag-54 expressed as percent of a Ag-54 reference determined by RRIE using selected patient serum containing high amounts of specific IgE to Ag-54 only

	Percent of Ag-54 reference	
	determined as dry weight material	corresponding to protein material
Native	100	100
Deglycosylated	5	1

1
Val-Lys-Ala-Val-Ala-Val-Ile-Arg-Gly-Asp-Ser-Asn-
15
Val-Lys-Gly-Thr-Val-Thr-Phe-Glu-
20

Fig. 1. N-terminal sequence analysis of deglycosylated Ag-54 by Dansyl-Edman degradation.

actose, participate in the glycopeptide linkages. The content of peptide-linked glucose appears to be twice the amount of peptide-linked mannose. GLC of the alditol acetates confirmed that mannitol was present in the sample, but neither galactitol nor glucitol could be detected.

Performic acid oxidation of the glycoprotein and subsequent hydrolysis indicated that the cysteine content of the glycoprotein is 1 per 100 residues. No modification of the cysteinyl residues was observed after reduction and carboxymethylation of the glycoprotein. Amino acid analysis of the sample did not reveal any occurrence of S-carboxymethyl-cysteine, suggesting that the sulfhydryl group of the cysteine residue must be blocked.

Dansyl-Edman degradation of the deglycosylated material showed that valine is the N-terminal amino acid. The sequence of the initial 20 amino acids resulting from the analysis is indicated in figure 1. Deglycosylation of Ag-54 reduced the carbohydrate content from 80.2 to 5.9% of the dry weight material as presented in table III. The relative proportions of the monosaccharides were altered as galactose was totally removed. The amino acid composition of the deglycosylated material as compared to native glycoprotein is given in table IV. The tyrosine content was significantly lower in the deglycosylated sample.

A mixture of deglycosylated and native Ag-54 gave rise to increased precipitate height in rocket immunoelectrophoresis and subsequent autoradiography as shown in figure 2, indicating the existence of immunological identity between native and deglycosylated Ag-54. RRIE for estimation of the allergenic activity (table V) showed that the amount of deglycosylated material had to be increased 20 times compared to the native glycoprotein to obtain the same activity when expressed as dry weight material. This corresponded to a 100-fold increase of the protein content.

No allergenic activity could be detected either in the carbohydrate moiety or the protein core obtained after alkaline borohydride treatment of the glycoprotein when tested by RRIE.

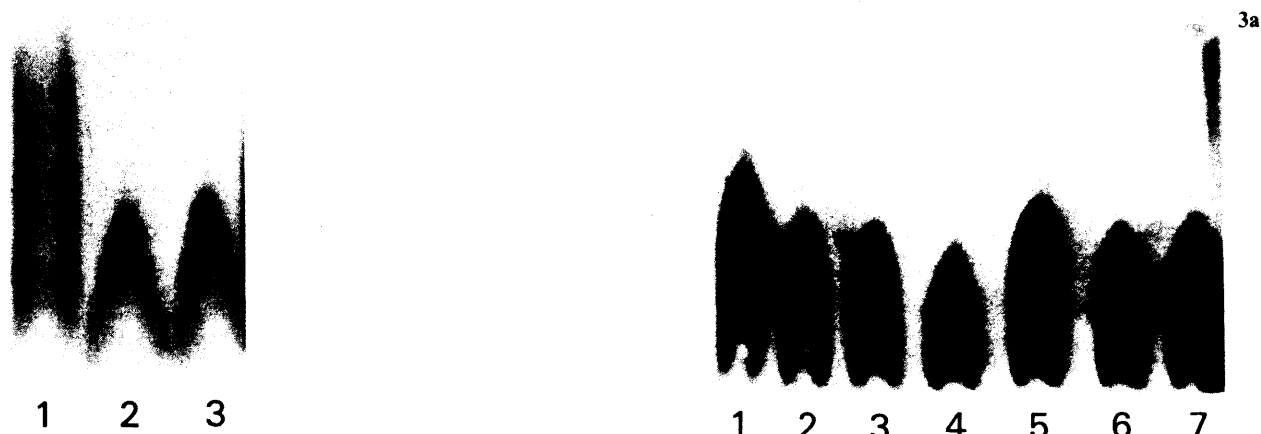


Fig. 2. Native Ag-54 and deglycosylated Ag-54 examined by autoradiography experiments after RIE in 1% agarose containing $7 \mu\text{l}/\text{cm}^2$ of anti-Ch-77 rabbit antiserum. The electrophoresis was carried out at 2 V/cm for 15 h with Tris-tricine buffer pH 8.6. Autoradiography was performed using selected patient serum (TN) with high amounts of IgE to Ag-54 followed by incubation with ^{125}I -anti-IgE. 1 = 0.1 μg native Ag-54 + 1.7 μg deglycosylated Ag-54; 2 = 1.7 μg deglycosylated Ag-54; 3 = 0.1 μg native Ag-54.

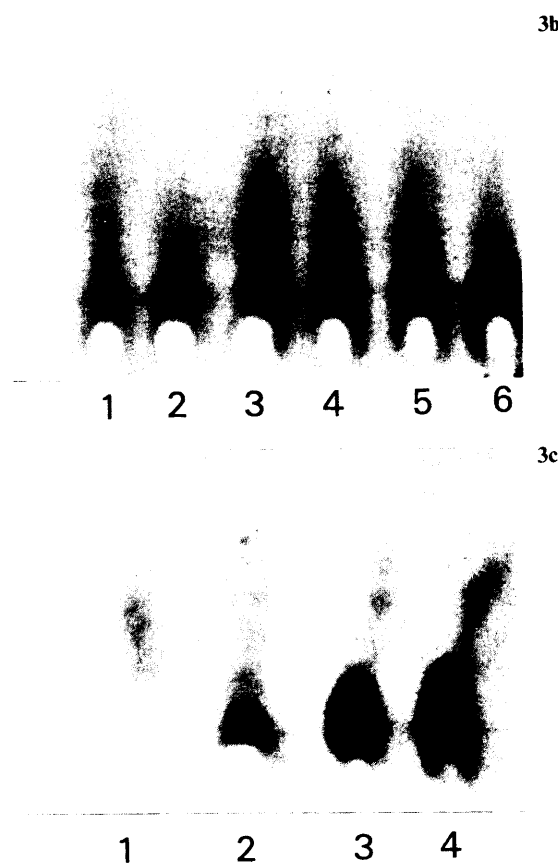


Fig. 3. Autoradiography experiments after RIE of native Ag-54 (0.1 μg) and deglycosylated Ag-54 (2.7 μg). The electrophoresis was performed at 2 V/cm for 15 h with Tris-tricine buffer pH 8.6 into 1% agarose containing $7 \mu\text{l}/\text{cm}^2$ of anti-Ch-77 rabbit antiserum. Autoradiography was carried out using selected patient serum (TN) with high amounts of IgE to Ag-54 followed by incubation with ^{125}I -anti-IgE. **a** Ag-54 heated for various periods of time 1 = 100°C, 20 min; 2 = 100°C, 5 min; 3 = 80°C, 5 min; 4 = 70°C, 5 min; 5 = 60°C, 5 min; 6 = untreated material; 7 = untreated material. **b** Deglycosylated Ag-54 heated for various periods of time. 1 = 100°C, 20 min; 2 = 100°C, 5 min; 3 = 80°C, 5 min; 4 = 70°C, 5 min; 5 = 60°C, 5 min; 6 = untreated material. **c** Urea denaturation. 1 = Deglycosylated Ag-54 incubated with 8 mol/l urea, 1 h, 25°C; 2 = deglycosylated Ag-54, untreated; 3 = Ag-54 incubated with 8 mol/l urea, 1 h, 25°C; 4 = Ag-54, untreated.

Both Ag-54 and deglycosylated Ag-54 retained its IgE binding capacity after heating up to 100°C for 20 min as shown in figure 3a, b. Exposure to 100°C for 1 h reduced the allergenic activity of both Ag-54 and of the deglycosylated material by approximately 30%. No decline in allergenic activity was observed after denaturation of Ag-54 with urea while urea treatment of deglycosylated Ag-54 resulted in complete loss of allergenic activity (fig. 3c).

Discussion

The carbohydrate moiety of Ag-54 consists of a highly branched galactomannan with a molecular weight of approximately 19,000 daltons and a few small oligoglucosidic units [8]. The establishment of threonine as a carbohydrate-linked amino acid and mannose as a peptide-linked monosaccharide suggests an *O*-glycosidic linkage between one residue of

threonine and a mannose unit of the galactomannan. Both *O*-mannosyl-serine and *O*-mannosyl-threonine glycopeptide bonds have been detected in fungal glycoproteins [22, 23]. In our experiments, however, there was no evidence of serine being linked to carbohydrate. Galactose was not found to participate in carbohydrate-peptide linkage. The identity of the amino acids carrying the small oligoglucosidic units was not determined. However, the lability of the carbohydrate-peptide bond towards alkali suggests that threonine or serine participates in the glucosyl bond, though glucose linked to these two amino acids has to our knowledge not yet been reported.

The observation that the sulfhydryl group of the cysteine residue of Ag-54 is blocked allows for the possible existence of a thioglycosidic linkage. Such a linkage would also be susceptible to the alkaline-borohydride treatment, resulting in the formation of 1-deoxy-1-thioglucitol and alanine [24]. Since the alkaline degradation procedure caused no loss of serine, the almost 0.5% increase in alanine may originate from cysteine residues carrying S-glycosyl groups. A glycopeptide comprising glucose linked to cysteine has been isolated from erythrocyte membranes [24] and the same thioglycosidic bond has been suggested to exist in allergen M from cod [25]. However, 1-deoxy-1-thioglucitol has an R_F value different from glucitol in our TLC system and this matter was not further investigated. Isolation and characterization of the linkage fragments containing the respective monosaccharide and amino acid are necessary to confirm the nature of the carbohydrate-peptide linkage in Ag-54. The existence of more than one type of carbohydrate-peptide linkage in a single glycoprotein has been established previously [26, 27].

Deglycosylation of the glycoprotein did not totally remove the carbohydrate moiety, though the major part of the sugar was split off. The reason for the incomplete deglycosylation is not clear. Previously, it has been demonstrated that the galactofuranose units of Ag-54 are labile to mild acid hydrolysis [8]. During deglycosylation, the galactofuranosidic side chains of the galactomannan polysaccharide moiety as well as a large part of the main core were split off. Based on this, the deglycosylated material can be expected to consist of one small unbranched mannan and one or a few oligoglucosidic units linked to the protein. The carbohydrate content could be reduced further by using longer reaction periods or higher temperatures

during deglycosylation, but this resulted in more extensive damage to the protein.

The decline of the IgE-binding capacity of the deglycosylated Ag-54 was evident. This may in part be due to damage to the protein moiety of the allergen. However, the amino acid composition except for the tyrosine content was fairly unaffected by the method used. Damage to tyrosine during deglycosylation has been reported by Edge et al. [17]. Removal of the major part of the carbohydrate moiety of Ag-54 will have an effect on the conformation of the glycoprotein. Unless the biological activity of the allergen is entirely dependent on the primary structure of the protein, one must expect that such a thorough encroachment in the glycoprotein nature will affect the ability to bind IgE and that the reduced allergenic activity at least in part results from the absence of the main part of the carbohydrate moiety.

The total loss of allergenic activity after alkaline-borohydride treatment indicated that the IgE binding ability of Ag-54 could not be ascribed to the carbohydrate moiety alone, though more sensitive test systems could have been used. Except for possible conformational changes, the carbohydrate moiety split off by alkaline-borohydride treatment is only slightly modified by the formation of an alditol at the reducing end of the carbohydrate. A previous investigation showed that the treatment did not significantly damage the carbohydrate moiety of Ag-54 [8]. On the other hand, the protein moiety was probably thoroughly modified during the alkaline-borohydride treatment, resulting in loss of allergenic activity.

Both native and deglycosylated Ag-54 were stable towards extensive heating, as no significant difference between the two samples could be detected with respect to IgE-binding capacity. When treated with urea as a denaturing agent, the native Ag-54 showed a greater stability than the deglycosylated material with respect to allergenic activity.

Separate experiments in our laboratory have shown that native Ag-54 has retained its allergenic activity unaltered for at least 5 years when stored at -20°C while the deglycosylated allergen lost most of its activity after 2 years of storage under the same conditions. This is in keeping with the findings of other authors who have reported on the protective and stabilizing effect exerted by the carbohydrate residues of glycosylated polypeptide chains [28–30].

In conclusion it has been demonstrated that the galactomannan moiety of Ag-54 is *O*-glycosidically

linked to threonine through mannose and that a few glucose units are peptide-linked. Removal of the main part of the carbohydrate moiety resulted in decline of allergenic activity and reduced stability of the glycoprotein.

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