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Studies on the carbohydrate moieties of the timothy grass pollen allergen *Phl p I*

Timothy grass pollen was investigated in order to determine the carbohydrate moieties of its major grass group I (*Phl p I*) and to study its impact on allergenicity. Based on computer calculations one *N*-glycosylation site was deduced from the cDNA data of *Phl p I*. After two-dimensional polyacrylamide gel electrophoresis, followed by blotting of pollen extract and by use of the monoclonal antibody IG 12 we identified at least six isoallergens of *Phl p I* with the main spots at a molecular mass of 35–37 kDa and a *pI* range of 6.5–7.3. Deglycosylation by trifluoromethanesulfonic acid resulted in a decrease of about 2 kDa. Treatment with *N*-glycosidase A resulted in a partial deglycosylation, while *N*-glycosidase F and *O*-glycosidase had no effect. Ten lectins were investigated for their binding to *Phl p I* components: *Aleuria aurantia* agglutinin showed strong reactivity (indicating fucose residues), while *Galanthus nivalis* agglutinin (indicating mannose residues) and concanavalin A (indicating mannose, glucose or *N*-acetylglucosamine residues) showed weak binding. By neutral sugar analysis we determined similar contents of the monosaccharides in the isoallergens. In order to study the influence of the carbohydrate structures of *Phl p I* on IgE reactivity we tested some patient sera for their reactivity with intact and deglycosylated *Phl p I*. Even though most of the IgE antibodies bind at the protein core, we detected one serum that recognized carbohydrate moieties on the *Phl p I*.

1 Introduction

Carbohydrate structures of glycoproteins often play important physiological roles, e.g. in resistance to proteolytic degradation, transduction of information between cells and intercellular adhesion through ligand-receptor interactions [1–3]. They can represent strong antigens as in the case of the A, B, and H blood group substances. Some monosaccharides, e.g. xylose and arabinose, occur almost only in plants and are therefore potent antigens for animals and humans. Weber *et al.* [4] and Tretter *et al.* [5] demonstrated that phospholipase A₂ in bee venom possesses an important epitope for B-cell recognition and IgE reactivity formed by α 1–3 fucosylation of an innermost *N*-acetylglucosamine residue. Cross-reactivity among allergens of vegetable foods, pollen and bee venom was shown to be caused by similar carbohydrate structures [6]. Such reactions disappeared after the allergens had been oxidized by sodium periodate treatment [7]. In temperate grasses, e.g. timothy grass (*Phleum pratense*) and ryegrass (*Lolium perenne*), two groups of allergens, classified as grass group I and IV, comprise glycoproteins. More than 95% of grass pollen allergic patients reveal IgE antibodies against the group I allergens of ryegrass (major allergen) [8]. The carbohydrate content was determined to be 5% [9]. Johnson and Marsh [10] treated the allergens with different glycosi-

dases and performed skin prick testing. Since no difference in reactivity compared to the untreated extracts was observed, they excluded an essential influence of the carbohydrate structures on allergenicity. However, it is known that some allergic patients reveal IgE reactivities against carbohydrate structures.

We therefore investigated the importance of glycosylation in the group I allergens of timothy grass pollen (*Phl p I*). We identified the major allergenic components of *Phl p I* by 2-D immunoblotting techniques. The reactivity to the protein core was tested after pollen extract had been treated by several deglycosylation procedures. To study the binding to the carbohydrate structures, we performed hydrazinolysis and dotblotted the glycans. Besides investigating the carbohydrate moieties, we wanted to clarify whether glycosylation has an influence on allergenicity.

2 Materials and methods

2.1 Pollen extract and *Phl p I* fusion protein

One g of timothy grass pollen (ARTU Biologicals, Lelystad, The Netherlands) was incubated in 50 mL of 0.1 M NH₄HCO₃ buffer (pH 8.0) for 30 min. Afterwards the soluble fraction was isolated by centrifugation at 17 000 $\times$ g for 30 min and dialyzed against double distilled water overnight. The extract was lyophilized and stored at 4°C until use. (Typical protein concentrations: 10 μ g extract isolated from 1 g of pollen.) The unglycosylated *Phl p I* fusion protein [11] was obtained after induction of pPhlpl.p1 transfected *E. coli* K 12 SOLR cells (Stratagene, La Jolla, USA) with 2 mM of IPTG (isopropyl β -D-thiogalactopyranoside). After 2 h the cells were lysed with SDS-containing sample buffer.

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Abbreviations: AAA, *Aleuria aurantia* agglutinin; AP, alkaline phosphatase; DIG, digoxigenin; IgE, immunoglobulin E; mAb, monoclonal antibody; TBS, Tris buffered saline; TFMS, trifluoromethanesulfonic acid

Keywords: Allergen / Deglycosylation / Glycoprotein

2.2 Patient sera and monoclonal antibodies

For the identification of IgE reactive proteins we used sera taken from several grass-pollen-sensitive patients (Pharmacia CAP System, CAP score > IV; radio allergosorbent test for the quantitation of specific IgE, scores according to manufacturer's manual; Pharmacia Diagnostics AB, Uppsala, Sweden). The monoclonal antibodies Bo 1 and IG 12 were raised against crude timothy pollen extract. They are directed against grass group V and I, respectively [12, 11].

2.3 Western blot

Proteins (6 µg per cm gel) were separated by SDS-PAGE (12.5% T, 4% C; size 120 × 70 × 0.5 mm³) using the Laemmli buffer system [13]. The proteins were subsequently transferred onto nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany) by capillary force with a pressure of 0.2 Pa on a heating plate set to 50°C (thermoblotting) [14].

2.4 2-D PAGE blotting

2-D PAGE was performed according to Görg *et al.* [15]. In the first dimension IEF was performed in polyacrylamide strips (4% T, 4% C; size 240 × 80 × 0.5 mm³) with an immobilized pH gradient of 3.6–9.3, containing 8 M urea and 10 mM DTT. Two hundred µg of timothy grass pollen extract was applied per lane and separated at 3000 V and 2 mA for 7 h. After incubation for 30 min in an equilibration solution, containing 6 M urea, 65 mM DTT, and 260 mM iodacetamide, SDS-PAGE was carried out in the second dimension in discontinuous gels (8–13% T, 4% C; size 240 × 80 × 0.5 mm³) at 200 V and 30 mA. The pI values and molecular mass were determined by use of marker protein test mix 9 (pH range 3.5–10.5; Serva, Heidelberg, Germany) and low range SDS-PAGE standards (14.4–97.4 kDa; Bio-Rad, Richmond, USA), respectively. For immunological studies we performed thermoblotting. For structural determinations (amino acid analysis, carbohydrate determination) electroblotting was performed onto Immobilon P membrane (Millipore, Eschborn, Germany) for 30 min at 200 mA, using 10 mM CAPS (3-cyclohexylamino-1-propane-sulfonic acid), pH 11.0, as blotting buffer [16].

2.5 Immunological detections

For the identification of antigens and allergens the blotting membrane was incubated with supernatants of hybridomas and patient sera, respectively. As secondary antibodies we used alkaline phosphatase conjugated goat anti mouse IgG/IgM (Dianova, Hamburg, Germany) or goat anti human IgE (Tago, Hamburg, Germany), respectively. The binding patterns were visualized by a substrate solution consisting of 0.033% w/v nitro blue tetrazolium chloride (NBT) and 0.017% w/v 5-bromo-4-chloro-3-indolyl phosphate potassium salt (BCIP) (Serva) in 0.1 M Tris-buffered saline (pH 9.5) [17]. To study the carbohydrate moieties, blotted samples were incubated with several digoxigenin-labeled lectins (Boehringer Mannheim, Germany) [18]. Lectin binding was

visualized by alkaline phosphatase-labeled digoxigenin and an NBT/BCIP substrate. Specific binding to the lectins was shown when the immunoreactivity was inhibited by addition of the corresponding monosaccharides.

2.6 Carbohydrate detection and determination

Carbohydrate detection was performed by the glycan detection kit (Boehringer) according to the manufacturer's description [19]. Vicinal hydroxyl groups of carbohydrates were oxidized to aldehydes by 10 mM NaIO₄, diluted in 100 mM sodium acetate buffer (pH 5.5) for 20 min. Then the steroid hapten digoxigenin was covalently bound to the aldehyde groups *via* a hydrazide group. Glycoproteins were visualized by alkaline phosphatase-labeled anti-digoxigenin followed by incubation in NBT/BCIP substrate.

2.7 Deglycosylation procedures

Crude timothy grass pollen extract was treated with different endoglycosidases (Boehringer) and trifluoromethanesulfonic acid (TFMS; Deglycosylation Kit, Oxford Glycosystems, Oxford, UK) to destroy their carbohydrate structures. a) Recombinant N-glycosidase F from *Flavobacterium meningosepticum* (peptide-N⁴-(acetyl-β-glucosaminyl) asparagine amidase): 15 µg extract was diluted in 25 µL of 10 mM sodium phosphate buffer (pH 7.5), containing 1% v/v 2-mercaptoethanol, and was incubated for 3 min at 95°C. Afterwards 10 µL of N-glycosidase F (2 U) were added and digestion was performed at 37°C for 18 h. b) N-glycosidase A from sweet almonds (peptide-N⁴-(N-acetyl-β-glucosaminyl) asparagine amidase): 15 µg extract diluted in 25 µL of 10 mM sodium acetate buffer (pH 5.1), containing 0.5 M sodium thiocyanate and 0.1 M 2-mercapthoethanol. After 3 min incubation at 95°C, 10 µL of N-glycosidase A (0.5 mU) were added, followed by digestion at 37°C for 18 h. c) O-glycosidase from *Diplococcus pneumoniae* (O-glycopeptide endo-D-galactosyl-N-acetyl-α-galactosamino hydrolase): 15 µg extract diluted in 34.5 µL of 20 mM sodium phosphate buffer (pH 7.5), containing 1% v/v 2-mercaptoethanol. After 3 min incubation at 95°C, 0.5 µL of O-glycosidase (0.25 mU) was added. Digestion was performed at 37°C for 18 h. d) Neuraminidase from *Clostridium perfringens* (Acylneuraminyl hydrolase; Boehringer): 200 µg extract were diluted in 20 µL of 10 mM sodium acetic buffer (pH 5.0); 2 µL of neuraminidase (1 U) were added and the incubation was performed at 37°C for 15 h. e) TFMS treatment [20]: 100 µg of lyophilized extract were dissolved in 45 µL of TFMS under anhydrous conditions. After incubation at –20°C for 4 h the solution was neutralized by addition of 150 µL 60% pyridine-water solution and again stored for 15 min at –20°C. Finally, 400 µL of 0.5% w/v NH₄HCO₃ were added to precipitate the deglycosylated proteins. The suspension was dialyzed against double-distilled water. f) KOH treatment [21]: 200 µg of crude pollen extract were diluted in 15 µL of 50 mM KOH. After incubation for 12 h at room temperature the sample was directly applied for Western blotting. g) Periodate oxidation: After SDS-PAGE blotting of grass pollen extract, unblocked nitrocellulose strips were incubated in 10 mM

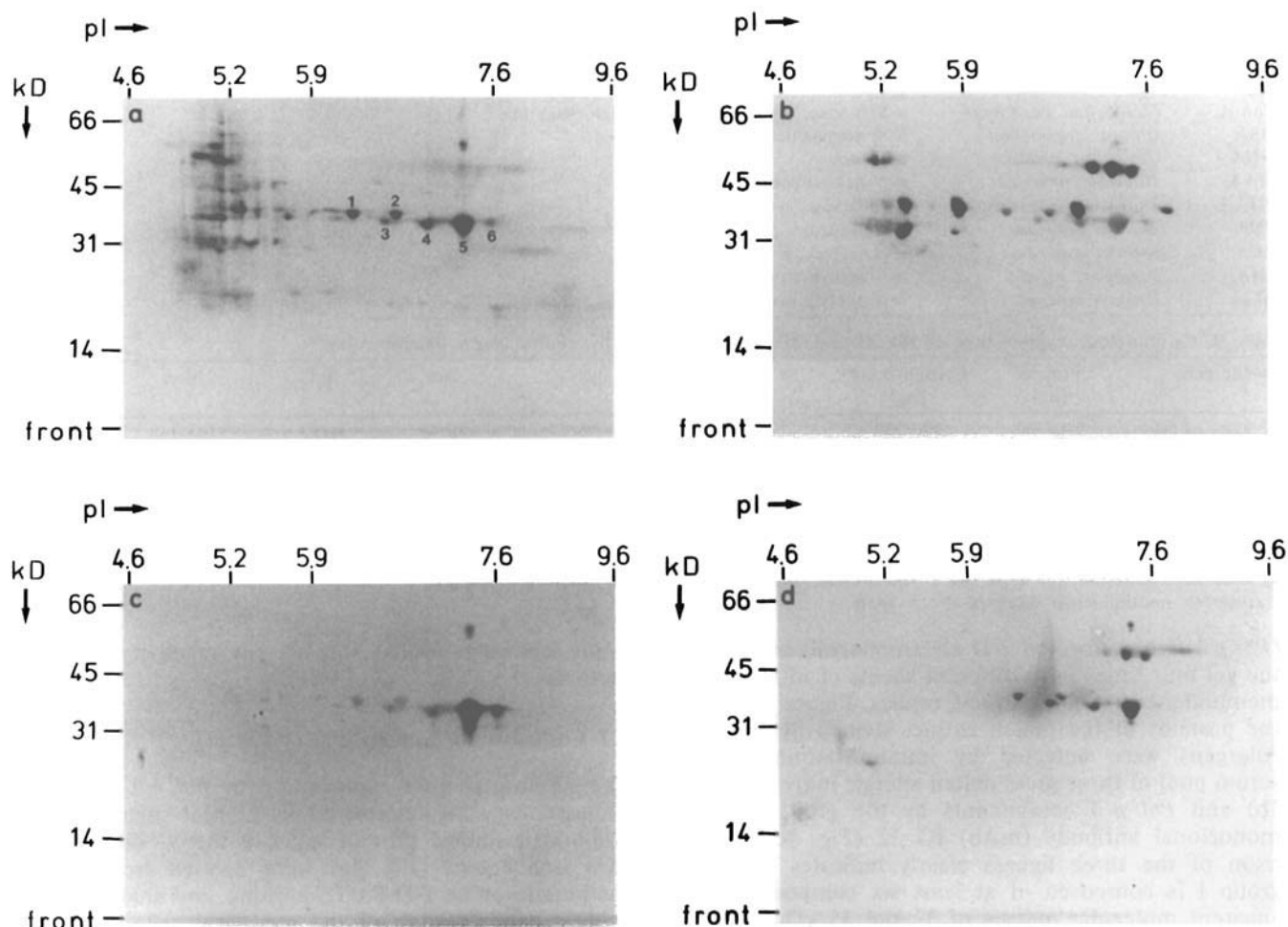


Figure 1. 2-D PAGE blotting of timothy grass pollen demonstrating the reactivity of allergen grass group I. (a) Protein staining with India ink, (b) binding pattern of a patients' serum pool (IgE), (c) binding pattern of mAb IG 12, (d) detection of lectin AAA-reactive structures. The six *Phl p* I isoallergens are marked.

sodium acetate buffer (pH 5.0), containing 50 mM NaIO₄. The incubation was performed in the dark for 2 h at room temperature. The reaction was stopped by addition of sorbitol to a final concentration of 0.015 M.

2.8 Analysis of *O*- and *N*-glycans

For the investigation of the carbohydrate structures two sets of 2.5 mg of pollen extract were dried exhaustively by lyophilization. Then the samples were diluted to the minimum amount of anhydrous hydrazine (50 µL) and incubated for 5 h at 60°C and 95°C for recovery of *O*- and *N*-glycans, respectively (*O*- and *N*-glycan Recovery Kit, Oxford Glycosystems) [22]. For removal of the released proteins the samples were passed over a glycan binding column according to the manufacturer's instructions. For immunological studies of the *N*- and *O*-glycans the eluted samples were dotted onto a CNBr-activated nitrocellulose membrane [23].

2.9 Determination of the monosaccharides by neutral sugar analysis

Qualitative and quantitative determination of carbohydrate composition was performed according to Mankarios *et al.* [24]. The protein spots of fifteen 2-D separated

samples (each containing 400 µg of timothy pollen extract) were excised from PVDF membranes and treated for 48 h by 0.1 M HCl. Five hundred ng of allose were added as internal standard. The samples were evaporized and afterwards diluted in 0.3 M NaBH₄ solution, containing 0.25% w/v NaOH to reduce the monosaccharides to the corresponding alditol derivatives. Peracetylation was performed overnight with a mixture of pyridine:acetic anhydride (3:1). For identification and determination of the monosaccharides, the samples were separated by gas-phase chromatography (HP-5 cross-linked) on-line to mass spectrometry (MS Engine HP 5989A Mass Spectrometer, Hewlett-Packard, Avondale, PA, USA). To determine the protein concentration single spots were taken from PVDF blotting membrane and investigated by amino acid analysis (Dr. H. G. Meyer, Institut für Physiologische Chemie, Ruhr-Universität Bochum, Germany).

3 Results

3.1 Identification of *Phl p* I components by 2-D immunoblotting

To identify and characterize the components of crude timothy pollen extract referring to allergen grass group I

Table 1. Reactivity of lectin binding to group I allergens of timothy grass pollen

Lectin	Organism	Binding specificity for terminal sugar	Reactivity
AAA	<i>Aleuria aurantia</i>	α -Fucose	++
Con A	<i>Canavalia ensiformis</i>	α -Mannose, α -glucose, α -N-acetylglucosamine	(+)
DSA	<i>Datura stramonium</i>	β -N-Acetylglucosamine, β -galactose	—
GNA	<i>Galanthus nivalis</i>	α -Mannose	(+)
MAA	<i>Maackia amurensis</i>	α -N-Acetylneuraminic acid	—
PHA-L	<i>Phaseolus vulgaris</i>	α -Galactose, β -galactose	—
PNA	<i>Arachis hypogaea</i>	β -Galactose	—
RCA	<i>Ricinus communis</i>	α -Galactose, β -galactose	—
SNA	<i>Sambucus nigra</i>	α -N-Acetylneuraminic acid	—
WGA	<i>Triticum vulgaris</i>	β -N-Acetylglucosamine	—

Table 2. Carbohydrate composition of the 35 and 37 kDa isoallergens by neutral sugar determination^{a)}

Isoallergens	Protein [ng]	Carbohydrate [ng]	% Carbohydrates	Fuc	Ara	Xyl	Man	GlcNAc
37 kDa, pI 6.4	710/790	290/330	2.5–3.1	45/67	39/43	44/22	104/140	58/58
37 kDa, pI 6.6	390/740	184/232	1.7–3.2	30/36	6/22	21/0	98/105	29/69
35 kDa, pI 6.5	990/1480	768/551	2.5–5.2	147/66	104/72	169/55	258/217	90/141
35 kDa, pI 6.8	950/1140	835/971	4.9–6.8	208/117	174/127	164/108	300/315	125/168
35 kDa, pI 7.1	1890/2400	1772/1822	4.9–6.4	330/472	242/278	216/236	712/490	322/294
35 kDa, pI 7.3	530	390/411	5.0–5.2	126/50	55/33	40/62	109/170	60/96

a) Each determination was done twice. The protein concentrations refer to amino acid analysis of one spot, the carbohydrate concentrations refer to neutral sugar analysis of 15 spots.

(*Phl p I*) we performed 2-D electrophoresis and blotted the gel four times onto different sheets of nitrocellulose membrane to obtain identical replica. Figure 1a depicts the proteins of the pollen extract stained by India ink. Allergens were detected by immunostaining with a serum pool of three grass pollen allergic individuals (Fig. 1b) and *Phl p I* components by the group I-specific monoclonal antibody (mAb) IG 12 (Fig. 1c). Comparison of the three figures clearly indicates that grass group I is composed of at least six components with apparent molecular masses of 37 and 35 kDa and a *pI* ranging from 6.4–7.3. Additionally, a weak protein spot of about 60 kDa and *pI* 7.1 is detectable, which is considered to be a *Phl p I* dimer. Patients' IgE binds to all of these components (Fig. 1b). The group I allergens are glycoproteins as identified by glycan detection (data not shown) and with *Aleuria aurantia* agglutinin (AAA, Fig. 1d). In contrast to other allergens, e.g. grass group V (*Phl p V* consists of 38 and 32 kDa isoallergens), the group I allergens are intensely stained by India ink (Fig. 1a). To differentiate the glycan structure, nitrocellulose membranes were incubated with ten lectins after 2-D PAGE blotting. Only AAA (Fig. 1d) intensely binds to the 35 and 37 kDa components. However, stronger binding to glycoproteins of 50 kDa (*pI* 6.5–7.4) was observed. The AAA reactivity was inhibited by addition of fucose to the lectin solution (data not shown). Table 1 demonstrates the binding of the group I allergens to the lectins tested. Weak reactivities of concanavalin A (Con A) and *Galanthus nivalis* agglutinin (GNA), to each of the six *Phl p I* isoallergens, were also detected. In order to exclude that the microheterogeneity of the *Phl p I* allergens was caused by diversity of sialic acid residues in the carbohydrate moieties, we performed 2-D PAGE immunoblotting of neuraminidase-treated pollen extract. The binding pattern of mAb IG 12 was not altered compared to the pattern obtained without enzyme treatment, indicating that no sialic acid residues are present in the *Phl p I* allergens. This result is confirmed by the observation that neither lectin *Maackia amurensis* agglutinin (MAA) nor *Sambucus*

nigra agglutinin (SNA) showed any reactivity with the isoforms.

3.2 Carbohydrate composition of *Phl p I*

The monosaccharide composition in *Phl p I* and their proportions were determined by neutral sugar analysis. Coomassie-stained protein spots of the 37 kDa and 35 kDa isoallergens (Fig. 1a) were excised from PVDF membranes, after 2-D PAGE blotting, and analyzed. The protein concentration of the spots was determined by amino acid analysis (Table 2). The degree of glycosylation varies between 1.7–6.8%. The ratios of the monosaccharides, comprising fucose, arabinose, xylose, mannose and N-acetylglucosamine, are similar among the isoallergens. In each of the isoallergens the concentration of mannose occurs at about twice the concentration of the other monosaccharides (fucose, arabinose, xylose, and N-acetylglucosamine). Within one isoallergen, arbitrary concentrations of fucose, arabinose, xylose, mannose and N-acetylglucosamine differ only slightly. Since arabinose and xylose form identical alditol derivatives upon reduction, we could not discriminate between these pentoses. Galactose was only detected in trace amounts. The determination of glucose was complicated because even the protein-free controls excised from the blotting membrane showed high concentrations of glucose. We determined 973, 565 and 1134 ng of glucose in negative controls (for each determination we used 15 membrane slices of comparable size to the largest protein spots). The contamination resulted from placing the PVDF blotting membrane onto a cellulose filter, soaked with buffer for semidry blotting. The use of a dialysis membrane between the filter and PVDF membrane, and thorough washing of the blotting membrane in double-distilled water did not diminish the glucose contamination and, therefore, high glucose concentrations were found in the *Phl p I* samples. The mean values of double determinations did not exceed the maximum value of the negative controls, indicating that there are no glucose residues in the carbohydrate structure of *Phl p I*.

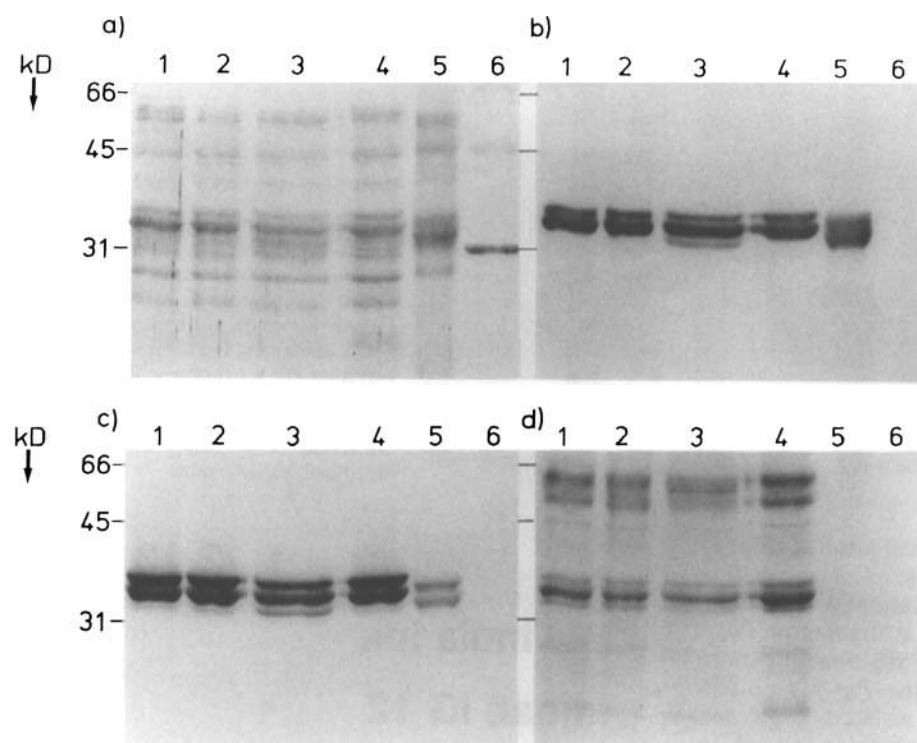


Figure 2. Effect of deglycosylation on the immunoreactivity of *Phl p I*. Western blots: (a) protein staining with India ink, and immunostaining: with (b) mAb IG 12, (c) patient's IgE, recognizing protein structures and (d) patient's IgE, recognizing carbohydrate structures. Timothy grass pollen extract: (1) untreated sample (control), (2) *N*-glycosidase F treatment, (3) *N*-glycosidase A treatment, (4) *O*-glycosidase digestion, (5) TFMS treatment. (6) Molecular mass marker.

3.3 Deglycosylation experiments on *Phl p I*

The linkage of the carbohydrate chains to the protein backbone was determined in deglycosylation experiments. Figure 2b shows an SDS-PAGE blot, incubated with the *Phl p I* directed mAb IG 12. While untreated pollen extract (lane 1) reveals antigens of 37 and 35 kDa, the deglycosylated TFMS-treated sample (lane 5) shows protein bands of 35 and 33 kDa, the resultant shift of 2 kDa indicating a 5% carbohydrate content in the grass group I allergens. Deglycosylation by *N*-glycosidase F (lane 2) and *O*-glycosidase (lane 4) has no detectable effect compared to the untreated control. With *N*-glycosidase A treatment, an additional protein band of 33 kDa is visible, while the 37 and 35 kDa bands are slightly less intense. This band was not detected by lectin AAA, indicating that it lacks carbohydrates (data not shown). Thus *N*-glycosidase A causes an incomplete deglycosylation of *Phl p I* components. Figures 2c and d show the IgE binding of two sera from grass pollen allergic individuals. A similar binding pattern to that of mAb IG 12 was observed (Fig. 2c), identifying the 35 and 37 kDa bands as well as the deglycosylated 33 and 35 kDa bands after TFMS treatment. The IgE binding pattern, shown in Fig. 2d, is different in recognizing the 35 and 37 kDa carbohydrate-containing bands. No reactivity could be found in the TFMS-deglycosylated sample (lane 5) and in the 33 kDa protein band after *N*-glycosidase A treatment. Additionally, the serum recognizes allergens of 50 to 60 kDa which are known to be glycoproteins (*Phl p IV*). Deglycosylation of the group I allergens was also performed by alkaline treatment with resultant β -elimination. Timothy pollen extract was incubated with 50 mM potassium hydroxide prior to SDS-PAGE and blotting. The nitrocellulose blots were immunostained by mAb IG 12, lectins, and several patient sera.

Compared to the untreated controls no differences in the binding patterns were observed (data not shown).

In an attempt to detect patient sera recognizing epitopes on the carbohydrate structures, we treated nitrocellulose blots of timothy grass pollen extract with sodium periodate to destroy carbohydrate moieties. Figure 3 depicts the immunoreactivity, by Western blotting, of lectin AAA, mAb IG 12 and three patient sera. After sodium periodate-treatment no AAA reactivity could be observed. The binding reactivities of mAb IG 12 and two of the patient sera (lanes 6 and 7) showed no alteration while the patients' IgE tested in lane 8 lost its reactivity after periodate oxidation. Furthermore, we tested the samples for their binding to the recombinant *Phl p I* (Fig. 3c). The *Phl p I* fusion protein has a molecular mass of about 37 kDa, as identified by the mAb IG 12 (lane 2). (The smaller IG 12-reactive components, *Phl p I* fragments, result from enzymatic degradation during expression). Lectin AAA does not react with the recombinant *Phl p I*, indicating that it is unglycosylated. Two of the three patient sera (lanes 6 and 7) recognize the 37 kDa protein band, while no reactivity was found in the case of the serum shown in lane 8. Therefore, both periodate oxidation as well as use of the recombinant *Phl p I* fusion protein demonstrate that the IgE reactivities of that serum are directed against carbohydrate structures.

3.4 *N*- and *O*-glycans isolated from crude timothy pollen extract

In order to find out whether the carbohydrate chains could be probable binding sites for IgE antibodies, we separated the glycan structures from crude timothy pollen extract. The peptide/amino acid hydrazide mixture was dotted onto CNBr-activated nitrocellulose

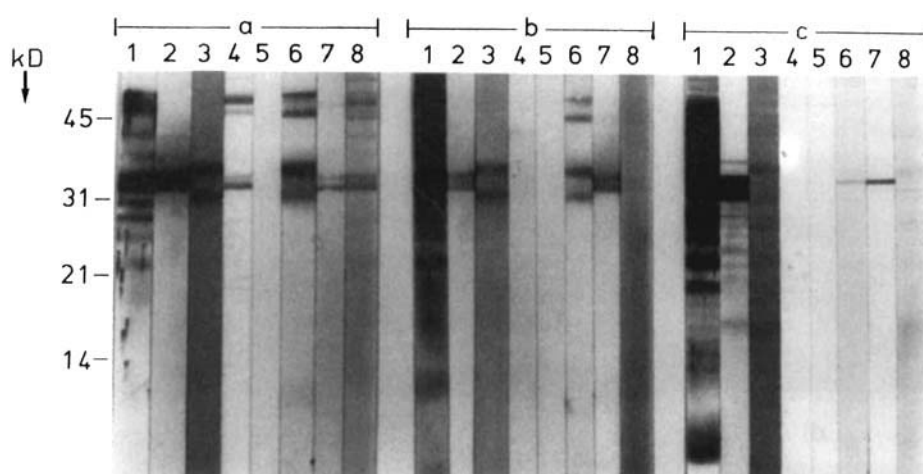


Figure 3. Identification of IgE-reactive structures on the *Phl p I* protein core. Western blots of timothy grass pollen extract: (a) untreated, (b) treated with sodium periodate, (c) blotting of *E. coli* lysate, containing the recombinant unglycosylated *Phl p I* fusion protein. (1) Protein staining with India ink, (2) mAb IG 12, (3) mAb Bo 1, (4) lectin AAA, (5) serum of a healthy donor, (6)–(8) individual patient sera.

membrane and used for immunological studies (Fig. 4). Reactivities to glycans are detectable after incubation with lectin AAA and with serum of patient 3. The lectin AAA (directed against carbohydrate structures of *Phl p I*) shows a faint reactivity with the TFMS-treated extract, while mAb IG 12 directed against the *Phl p I* protein moiety also binds weakly to the isolated *O*-glycans. These results indicate that residual peptides are left in the glycan fractions and that the TFMS fraction contains minimal amounts of carbohydrates. However, the sera of patients 1 and 2 only bind to crude and to TFMS-deglycosylated extract, whereas the serum of patient 3 reveals additional IgE reactivities against carbohydrate structures.

4 Discussion

Our aim was to study the carbohydrate moieties of the grass group I allergens in timothy grass pollen and to clarify their allergenic role. The group I allergens occur as a number of isoallergens, differing slightly in molecular mass and *pI*. This microheterogeneity was first observed by Johnson and Marsh [25] for the corresponding allergen of ryegrass pollen (*Lol p I*). The *pI* variability might be caused by single amino acid exchanges or by post-translational modifications. In carbohydrate structures, sialic acid residues can cause *pI* differences. Since we did not identify sialic acid residues, neither by use of lectins MAA and SNA nor by neuraminidase treatment of the extract, we assume that the carbohydrate structures in *Phl p I* are not responsible for the microheterogeneity. On the other hand the carbohydrate compositions of the isoallergens are very similar and therefore will not cause such variations in *pI*. We found a carbohydrate content of about 5% by deglycosylation with TFMS and of 1.7–6.8% by neutral sugar analysis. Because of the low sample concentrations and the isochronous assembly and disassembly of the carbohydrate chains, a more precise value for the glycosylation could not be achieved. Our results correspond to the carbohydrate content of 5% determined for *Lol p I* [9]. The group I allergens of different grass species reveal similar *N*-terminal protein sequences [26]. Deducing the complete protein sequence from the cDNA data of *Lol p I*, *Hol I I* and *Phl p I*, we calculated > 90% sequence

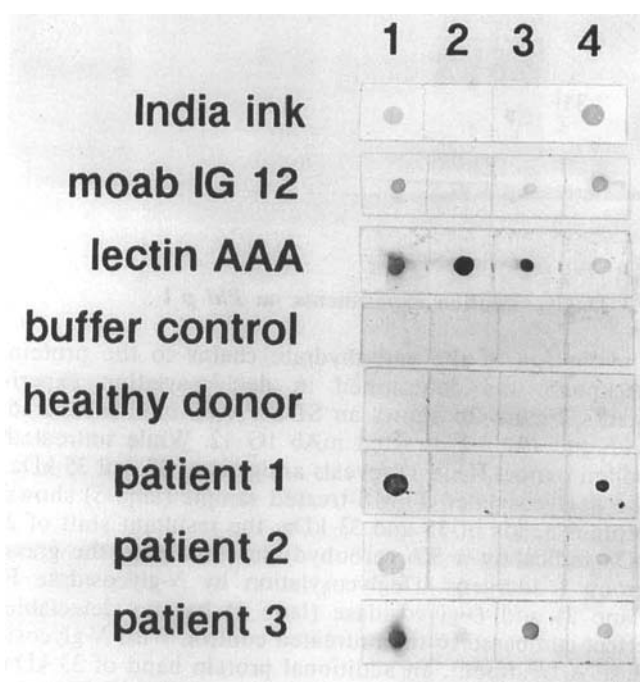


Figure 4. Isolated *N*- and *O*-glycans obtained after hydrazinolysis of timothy pollen extract. (1) Crude timothy pollen extract, (2) *N*- and (3) *O*-glycans isolated from pollen extract, (4) TFMS-deglycosylated pollen extract.

homology and > 85% sequence identity [11]. From computer calculations only one *N*-glycosylation site is predicted at position 9. This was confirmed by the observation that no amino acid residue could be determined at this position by protein sequencing due to the modification of the amino acid by the attached carbohydrate. The carbohydrate structure was investigated using several endoglycosidases and lectins. *N*-glycosidase A resulted in a partial degradation of *Phl p I* to a 33 kDa unglycosylated component corresponding to the TFMS-deglycosylated sample. By contrast, treatment with *N*-glycosidase F showed no effect. According to Tretter *et al.* [27] *N*-glycosidase A can degrade *N*-linked glycans with a fucose residue, linking α 1–3 to asparagine-*N*-acetylglucosamine, while *N*-glycosidase F fails to degrade this

linkage. Since we identified fucose residues as part of the group I molecules, such a carbohydrate structure seems to be present in *Phl p I*. Additionally, we identified accessible mannose residues by the lectins *Galanthus nivalis* (GNA) and Con A and by neutral sugar analysis. Neither digestion by *O*-glycosidase nor KOH treatment resulted in a detectable alteration of the *Phl p I* carbohydrate moieties, proving that *O*-glycans are not present. Haavik *et al.* [28] found a 20% carbohydrate content in timothy grass pollen extract, consisting of 7% arabinose and 13% galactose. They assumed that *O*-glycans might be covalently linked to the protein core via hydroxyproline residues. By neutral sugar analysis we also detected arabinose residues, but only traces of galactose. Previously, we had identified two hydroxyproline residues in the *Phl p I* allergens by microsequencing [29]. Since these hydroxyproline residues were clearly detectable, we assume that they are not glycosylated, or only to a low extent. The high arabinose and galactose content is due to oligosaccharides in the pollen extract (unpublished data).

To screen patient sera for IgE reactivity to carbohydrate structures we investigated the immunoreactivity to the deglycosylated proteins (TFMS-treated sample) as well as to the glycans separated from the proteins after hydrazinolysis. One of about 30 patient sera tested so far revealed IgE reactivity to the glycans (Fig. 4, patient serum 3), while the other sera bound to the protein moieties (crude or deglycosylated extract). This individual suffers from many allergies, presumably caused by cross-reactivity to similar carbohydrate structures. Therefore, the glycans might play a role in grass pollen allergy and have to be considered when planning a hyposensitization therapy. The role of carbohydrate-directed IgE antibodies is being discussed controversially. While Aalberse *et al.* [7] assume that such IgE antibodies do not cause histamine release and clinical symptoms, many of these patients suffer from severe allergies. A histamine release test with basophiles of the patient in question might help to decide whether the carbohydrate-binding IgE antibodies have a functional effect. However, the investigations of Weber *et al.* [4] and Tretter *et al.* [5] clearly indicate that the carbohydrate epitopes in phospholipase A₂ play an important role in the immediate type hypersensitivity to bee venom. According to Chou and Fasman [30] antigenic binding sites correlate with hydrophilic protein structures. Since post-translational modifications, *e.g.* glycosylation and phosphorylation, enhance the hydrophilicity of proteins and are normally found on the surface, they might be targets for antibody binding and therefore should be further investigated. For an improved diagnostic and hyposensitization therapy it might be better to use recombinant proteins produced in eucaryotic cells, *e.g.* yeast, than to use unglycosylated recombinant proteins produced in bacterial systems.

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5 References

- [1] Gesundheit, N., Fink, D. L., Silverman, L. A., Weintraub, B. D., *J. Biol. Chem.* 1987, 262, 5197–5200.
- [2] Ashwell, G., Harford, J., *Annu. Rev. Biochem.* 1982, 51, 531–554.
- [3] Potskaldy, J. M., Rouiller, D. G., Grunberger, G., Baxter, R. C., McElduff, A., Gordon, P., *J. Biol. Chem.* 1986, 261, 14076–14081.
- [4] Weber, A., Schröder, H., Thalberg, K., März, L., *Allergy* 1987, 42, 464–470.
- [5] Tretter, V., Altmann, F., Kubelka, V., März, L., Becker, W.-M., *Int. Arch. Allergy Immunol.* 1993, 102, 259–266.
- [6] Aalberse, R. C., Koshte, V., Clemens, J. G. J., *Int. Arch. Allergy Appl. Immunol.* 1981, 66, 259–260.
- [7] Aalberse, R. C., Koshte, V., Clemens, J. G. J., *J. Allergy Clin. Immunol.* 1981, 68, 356–364.
- [8] Freidhoff, L. R., Ehrlich-Kautzky, E., John, M. S., *J. Allergy Clin. Immunol.* 1986, 78, 1190–1200.
- [9] Howlett, B. J., Clarke, A. E., *Biochem. J.* 1981, 197, 695–706.
- [10] Johnson, P., Marsh, D. G., *Immunochemistry* 1966, 3, 101–110.
- [11] Petersen, A., Schramm, G., Bufer, A., Schlaak, M., Becker, W.-M., *J. Allergy Clin. Immunol.* 1995, 95, in print.
- [12] Becker, W.-M., Schaubschläger, W., Westphal, W., Schlaak, M., in: Chmiel, H., Hammes, W. P., Baily, J. E. (Eds.), *Biochemical Engineering*, Gustav-Fischer Verlag, Stuttgart 1988, pp. 440–442.
- [13] Laemmli, U. K., *Nature* 1970, 227, 680–685.
- [14] Petersen, A., Becker, W.-M., Schlaak, M., *Int. Arch. Allergy Immunol.* 1992, 98, 105–109.
- [15] Görg, A., Postel, W., Günther, S., *Electrophoresis* 1988, 9, 531–546.
- [16] Matsudaira, P., *J. Biol. Chem.* 1987, 262, 10035–10038.
- [17] Leary, J. J., Brigati, D. J., Ward, D. C., *Proc. Natl. Acad. Sci. USA* 1983, 80, 4045–4049.
- [18] Haselbeck, A., Schickaneder, E., Von der Eltz, H., Hösel, W., *Anal. Biochem.* 1990, 191, 25–30.
- [19] O'Shannessy, D., Voorstad, P. J., Quarles, R. H., *Anal. Biochem.* 1987, 163, 204–209.
- [20] Sojar, H. T., Bahl, O. P., *Methods Enzymol.* 1987, 138, 341–350.
- [21] Vailes, L. D., Li, Y., Bao, Y., DeGroot, H., Aalberse, R. C., Chapman, M. D., *J. Allergy Clin. Immunol.* 1994, 93, 22–33.
- [22] Patel, T., Bruce, J., Merry, A., Bigge, C., Wormald, M., Jaques, A., Parekh, R., *Biochemistry* 1993, 32, 679–693.
- [23] Demeulemester, C., Peltre, G., Laurent, M., Pankelueux, D., David, B., *Electrophoresis* 1987, 8, 71–73.
- [24] Mankarios, A. T., Jones, C. F. G., Jarvis, M. C., Threlfall, D. R., Friend, J., *Phytochemistry* 1979, 18, 419–422.
- [25] Johnson, P., Marsh, D. G., *Nature* 1965, 206, 935–937.
- [26] Matthiesen, F., Nielsen, A. K., Søgaard, T. J., Klysner, S., Löwenstein, H., *Allergy* 1992, 47, 31.
- [27] Tretter, V., Altmann, F., März, L., *Eur. J. Biochem.* 1991, 199, 647–652.
- [28] Haavik, S., Smestad Paulsen, B., Wold, J. K., Grimmer, Ø., *Phytochemistry* 1982, 21, 1913–1919.
- [29] Petersen, A., Becker, W.-M., Schlaak, M., *J. Allergy Clin. Immunol.* 1993, 92, 789–796.
- [30] Chou, P. Y., Fasman, G. D., *Adv. Enzymol. Relat. Subj. Biochem.* 1978, 47, 45–148.