

Carbohydrate moieties can induce mediator release: a detailed characterization of two major timothy grass pollen allergens

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Abstract

Specific IgE binding to carbohydrate moieties of glycosylated allergens has been known for years, but the importance of these structures for the elicitation of allergic reactions is still a matter of debate. Because of their conserved carbohydrate structures, especially N-glycans have always been prime candidates for IgE cross-reactivity between allergens from unrelated species. The aim of our study was to determine whether carbohydrate structures on glycoproteins can by themselves elucidate allergic reactions. We characterized in detail the carbohydrate moieties of the major allergens Phl p 1 and Phl p 13 of timothy grass pollen (*Phleum pratense* L.) by performing tryptic digests followed by HPLC, N-terminal sequencing, sugar analysis, MALDI-TOF- and ESI-ICR-FT-MS. Phl p 1 contains one N-glycan with one of the two glycoforms MMXF³ and MOXF³ and a single furanosidic arabinose, which is bound to a hydroxyproline residue in direct vicinity to the N-glycan. This O-glycosylation is probably due to an arabinosylation consensus sequence found in the N-terminal part of Phl p 1 and other group 1 allergens, but displayed no IgE-reactivity. Thus, Phl p 1 is monovalent with respect to its IgE-binding carbohydrate epitopes and showed no mediator release. In contrast, the carbohydrate moiety of Phl p 13, which carries four of the same N-glycans (like Phl p 1), can cross-link IgE-receptors via carbohydrate chains and elicits IL-4 release from basophils.

Keywords: allergy; arabinosylation; CCD; mass spectrometry; mediator release; N-glycosylation.

Introduction

The prevalence of allergic diseases has increased dramatically over the last decades. Today almost 25% of all western Europeans suffer from allergic symptoms, the severity ranging from mild hay fever to life threatening anaphylactic shock (Ring et al., 2001; Ring and Darsow, 2002). The most common type of hypersensitivity is type I allergy, which is mediated by IgE antibodies. In most

cases IgE recognizes epitopes on proteins and much work has been devoted to the mapping of peptide epitopes and recombinant expression of allergens, with the final goal of large scale production to improve specific immunotherapy (Ball et al., 1999; Schramm et al., 2001; Chapman et al., 2000).

In most of these studies the post-translational modifications of the allergenic proteins were not taken into account. A common modification is glycosylation and a number of allergens were shown to be glycoproteins. Carbohydrate structures of glycoproteins often have physiological functions, e.g. slowing the clearance or the enzymatic degradation of the protein (Lis and Sharon, 1993), and have been described as epitopes for B cells and antibodies (Batanero et al., 1999). IgE has been shown to bind to carbohydrates containing $\beta(1,2)$ -xylose and especially core- $\alpha(1,3)$ fucose (Tretter et al., 1993; Wilson and Altmann, 1998; van Ree et al., 2000; Ben-curova et al., 2004).

As these carbohydrate structures – especially plant N-glycans – are highly conserved, they represent possible cross-reactive epitopes of allergens from unrelated species (Kalkhofen et al., 1987; Koshte et al., 1989; Faye and Christpeels, 1998) and are therefore often referred to as ‘cross-reactive carbohydrate determinants’ (CCD; Wilson and Altmann, 1998). N-glycosylations can be predicted rather easily because of the known consensus sequence at the amino acid level (...Asn-X-Thr/Ser/Cys..., with X being any amino acid except proline). As many allergens have only one N-glycosylation site (e.g. they show IgE binding, but they are not able to crosslink IgE on mast cells and basophils (a prerequisite for histamine and IL-4 release from mast cells or release from basophils and the subsequent clinical symptoms), it has been suggested by some authors that the glycans are irrelevant for the elucidation of allergic symptoms (Aalberse et al., 1997; van der Veen et al., 1997). Only a few allergens were subjected to a detailed investigation of all their carbohydrate moieties (Swaerd-Normo et al., 1988). This is due to the lack of a consensus sequence for O-glycosylations. Especially in invertebrates and plants O-glycosylation is poorly characterized and most of the known allergenic glycoproteins are of invertebrate or plant origin. Interestingly, the recently published structure of the O-glycosylated mugwort allergen Art v 1 clearly indicates that the carbohydrate moiety considerably contributes to the IgE-reactivity (Himly et al., 2003).

Allergy to grass pollen is one of the most common forms of hypersensitivity and grass pollen allergens have been described to elicit cross-reactive IgE against carbohydrate structures (Petersen et al., 1996). For this study we have chosen the two glycoproteins Phl p 1 and Phl p 13, the group 1 and group 13 allergens of timothy

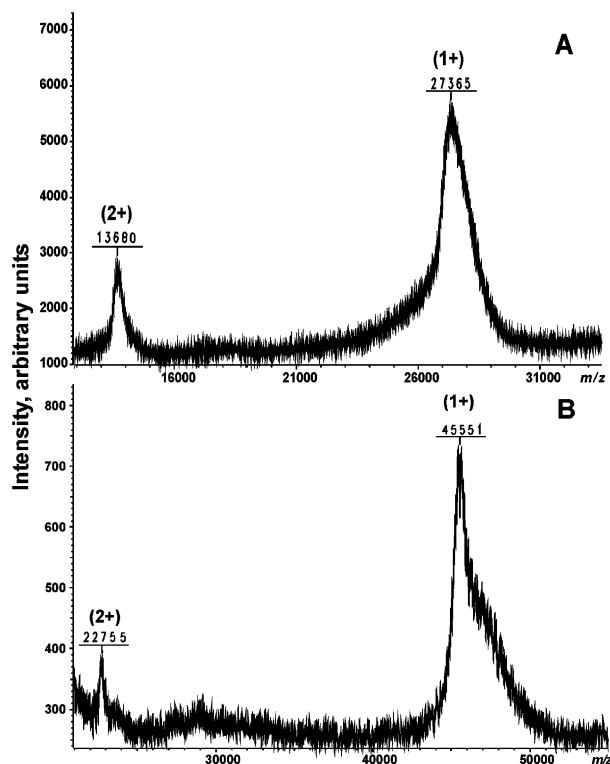


Figure 1 Positive ion MALDI-TOF mass spectra of natural Phl p 1 (A) and Phl p 13 (B).

grass pollen (*Phleum pratense* L.), since they are major allergens and their primary structures are known from N-terminal protein sequencing and from the cDNA sequence (Petersen et al., 1993, 1995b; Suck et al., 2000a/b). The 35 kDa (SDS-PAGE) glycoprotein Phl p 1 has one N-glycosylation site at position 9 of the mature protein and its carbohydrate moieties and their influence on allergenicity have already been studied to some extent (Petersen et al., 1995a): 20% of patients with allergy against grass pollen have IgE antibodies recognizing carbohydrate-containing epitopes. The other major grass pollen allergen we studied regarding its carbohydrate moieties was Phl p 13, a 55 kDa (SDS-PAGE) protein with six N-glycosylation consensus sites, thus being possibly polyvalent with respect to carbohydrate epitopes (Suck et al., 2000a/b).

The aim of this work was to characterize the carbohydrate structures of Phl p 1 and Phl p 13 and study their IgE-reactivity and potential for mediator release.

Results

General characterization of Phl p1 and Phl p 13

From SDS-PAGE the molecular masses of 35 kDa for Phl p 1 and 55 kDa for Phl p 13 were estimated. For more exact mass determination both proteins were subjected to mass spectrometry.

The mass spectra of nPhl p 1 and nPhl p 13 (Figure 1) comprise broad molecular ion regions centred around m/z 27365 and 45551, respectively, indicating that both natural isolates consist of different isoforms. Furthermore, the determined averaged molecular masses are

well above those calculated from the published amino acid sequences (25899 and 41693 Da; Petersen et al., 1993; Suck et al., 2000b), proving that both allergens carry considerable amounts of posttranslational modifications.

Structure of the carbohydrate moieties of Phl p 1 and Phl p 13

To determine the structure and binding sites of the glycans we decided to cleave the Phl p 1 molecule into tryptic peptides and then screen these fragments for glycopeptides by sugar analysis. All peptide-containing fractions were tested for carbohydrates.

0.5 mg of trypsin-digested nPhl p 1 was separated on a reversed-phase HPLC column (Figure 2). Only one peak contained sugars: N-acetylglucosamine, mannose, xylose, fucose and arabinose were found, roughly in a molar mass ratio of 2:2.5:1:1:1 (Figure 3). The glycopeptide was subjected to a second reversed-phase HPLC column and again only one peak was found (data not shown).

To check whether this glycopeptide contained the N-glycosylation site, the sample was investigated by N-terminal sequencing. The amino acid sequence of Phl p 1 (without post-translational modifications) was deduced from the cDNA sequence. Figure 4 shows the determined sequence of the glycopeptide in comparison with the N-terminal sequence of nPhl p 1 and rPhl p 1. The sequence corresponds to the expected peptide 4–17 with two hydroxylated proline residues. The glycopeptide was thus named gPep4–17. The amino acid at position 9 of the mature Phl p 1 could not be determined, indicating the presence of a posttranslational modification.

Mass spectrometric analysis of gPep4–17 by MALDI-TOF MS revealed four molecular ion peaks at m/z 2469.8, 2601.9, 2631.8 and 2764.0 (Figure 5A), indicating that the glycopeptide is heterogeneous with respect to the number of hexose ($\Delta m/z$ 162) and pentose ($\Delta m/z$ 132) residues. Considering the calculated mass of the peptide without any glycosylation (m/z 1462), the glycan of the glycopeptide with the lowest molecular mass (m/z 2469.9) should have a molecular mass of m/z 1026 ($=2470-1462+18$). In agreement with the GC/MS sugar analysis the glycan is composed of two N-acetylglucosamines, two mannoses, one fucose, and one xylose. The higher molecular components carry one additional pentose (probably the arabinose found) and/or mannose residue. To verify these findings, gPep4–17 was treated with N-glycosidases. Whereas the N-glycan could not be cleaved by N-glycosidase F (data not shown), the peptide was modified by N-glycosidase A as detected by reversed-phase HPLC and mass spectrometry. The mass spectrum (Figure 5B) comprises the expected peak of the pure peptide without any glycan at m/z 1462.5 and, surprisingly, a second peak at m/z 1594.5 indicating that one pentose is still linked to the peptide. To determine to which amino acid residue the pentose is linked, daughter ion scans of both molecular species were performed applying ESI-FT-ICR MS/MS with sustained off-resonance irradiation (SORI) collision-induced dissociation (CID). For easier interpretation the spectra were charge-deconvoluted (Figure 6A and B). From a comparison of

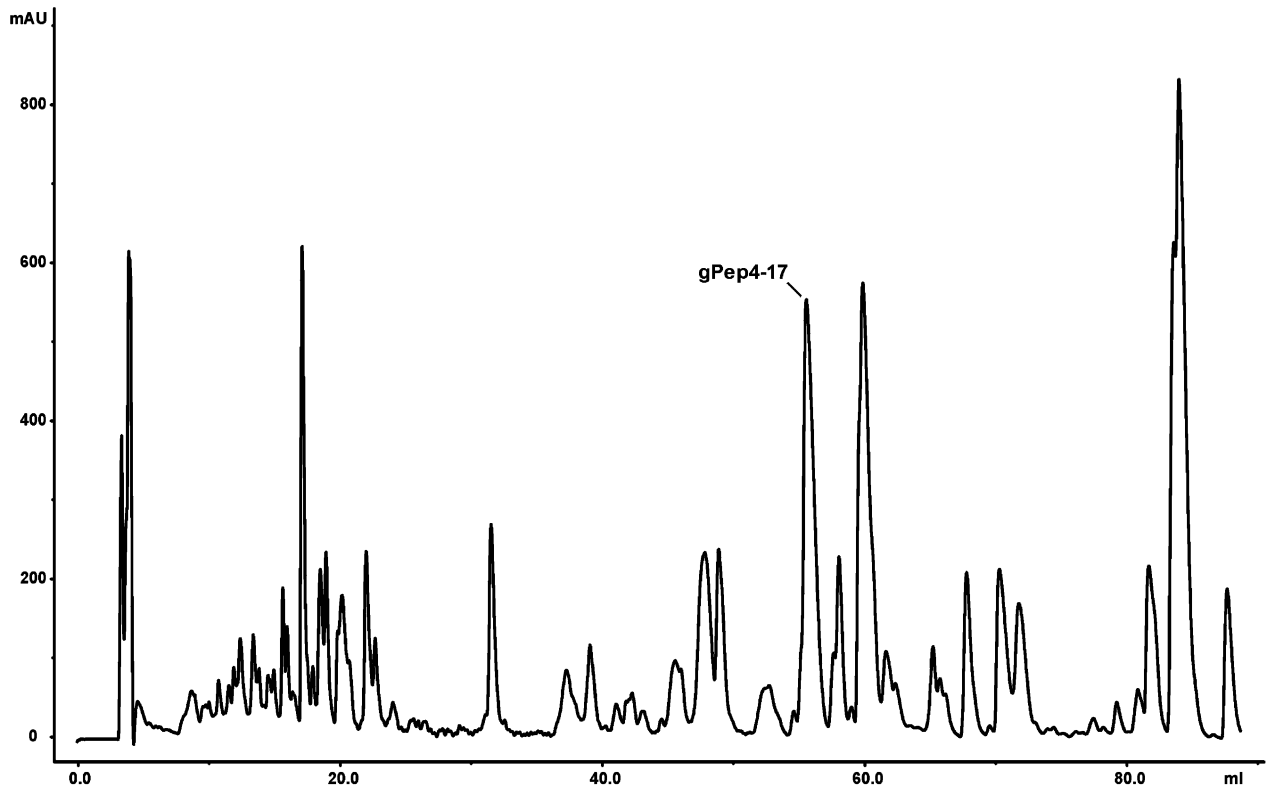


Figure 2 Reversed-phase HPLC separation of tryptically digested Phl p 1. 0.5 mg of tryptically digested natural Phl p 1 were separated to screen for glycopeptides.

the respective C- and N-terminal fragment ions obtained from the precursor ion missing (A) and carrying the pentose (arabinose) (B) it follows unequivocally that the pentose is linked exclusively to the hydroxyproline at position 5 of the mature protein (see mass shifts of fragment b2 and y13). The mass spectrum of the liberated N-glycan (not shown) comprises two ion peaks at m/z 1026 and 1188, proving that in accordance to the above results the

N-glycan linked to Phl p 1 may carry two or three mannose residues.

The mass spectrum of the N-glycans liberated from the tryptic fragments of Phl p 13 (not shown) was identical to the spectrum for the N-glycan of Phl p 1 showing two ion peaks at m/z 1026 and 1188. In the mass spectra of the tryptic peptides of Phl p 13 peaks corresponding to masses of peptides containing an N-glycosylation site

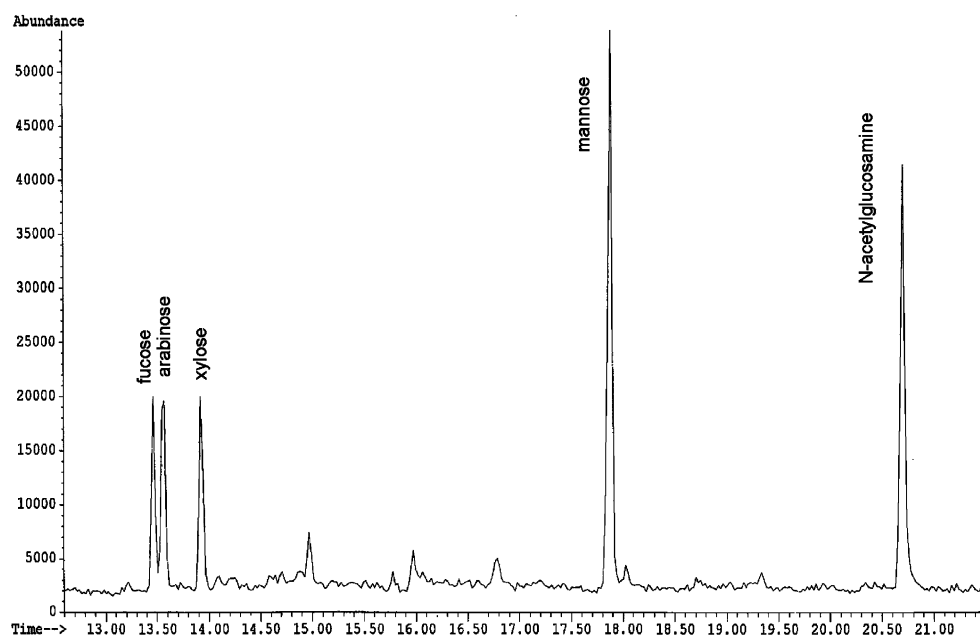


Figure 3 Carbohydrate analysis by GC-MS of the carbohydrate-containing tryptic fragment of Phl p 1 to determine the carbohydrate composition.

1 10 20
 VPPGP?ITATYGD **gPep 4-17**
 IPKVP~~PPGPN~~ITATYGDKWLD... **nPhl p 1**
 IPKVP~~PPGPN~~ITATYGDKWLD... **rPhl p 1**

Figure 4 Sequence of the glycopeptide 4-17 as determined by N-terminal sequencing compared with the N-terminal sequence of natural and recombinant Phl p 1.

gPep4-17: glycopeptide 4-17; nPhl p 1: natural Phl p 1 (Petersen et al., 1993); rPhl p 1: recombinant Phl p 1 (Petersen et al., 1995b); K: trypsin cleavage site; N-glycosylation motif underlined; P: hydroxyproline.

plus N-glycan (peptide mass+1026 or 1188 Da-18 Da) were found for the consensus sites at amino acid positions 72, 131, 370 and 390, whereas no such peaks were detected for the N-glycosylation sites at amino acid positions 228 and 332 (data not shown). No shifts of $\Delta m/z$ 132 (as in the spectrum of the gPep4-17) were found in the Phl p 13 spectra.

The glycopeptide gPep4-17 contains the monosaccharides xylose, fucose, arabinose, mannose and N-acetylglucosamine, an N-glycosylation motif and two hydroxyproline residues. A pentose (arabinose), bound to

the hydroxyproline at position 5, is present in some of the Phl p 1 molecules. The N-glycan can be cleaved from the peptide by N-glycosidase A and there are two forms of the N-glycan which (together with the absence or presence of bound pentose, e.g. arabinose) makes a total of four glycoforms of natural Phl p 1. Natural Phl p 13 carries four of the same N-glycans as Phl p 1 and no additional pentoses (arabinoses) were identified in Phl p 13.

IgE-reactivity toward fucose-containing glycans of timothy grass pollen extract

After determination of the carbohydrate structures of Phl p 1 and Phl p 13 their IgE binding potential was characterized. In pre-screening experiments IgE-antibodies against carbohydrate structures were found in the serum of patient Bo220, whereas antibodies of serum W recognized only protein epitopes. Both patients suffer from grass pollen allergy. The Bo220 serum showed the same binding pattern (regarding IgE) toward timothy grass pollen extract as the fucose-binding lectin AAA. Prominent bands can be seen at 55 kDa and 35 kDa, where Phl p

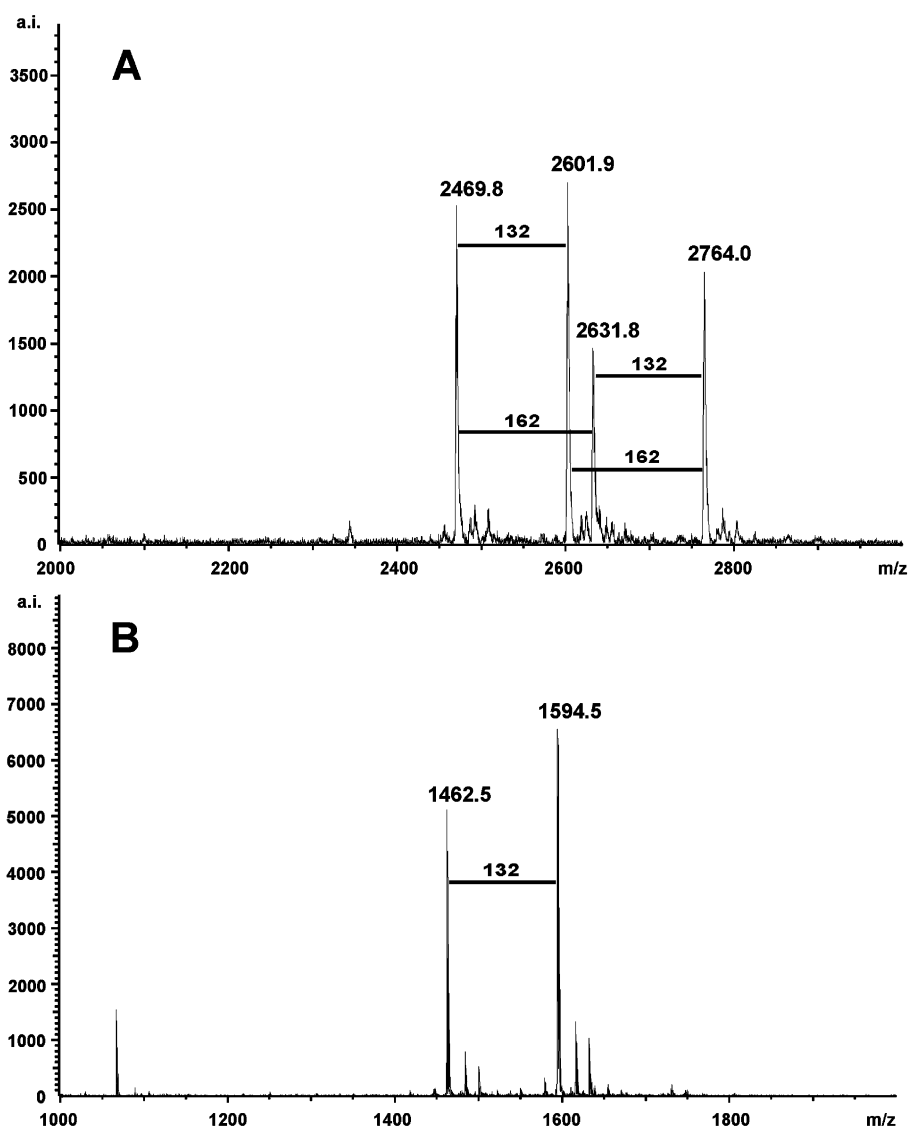


Figure 5 Positive ion MALDI-TOF mass spectrum of glycopeptide gPep4-17 (A) and N-glycosidase A-digested gPep4-17 (B).

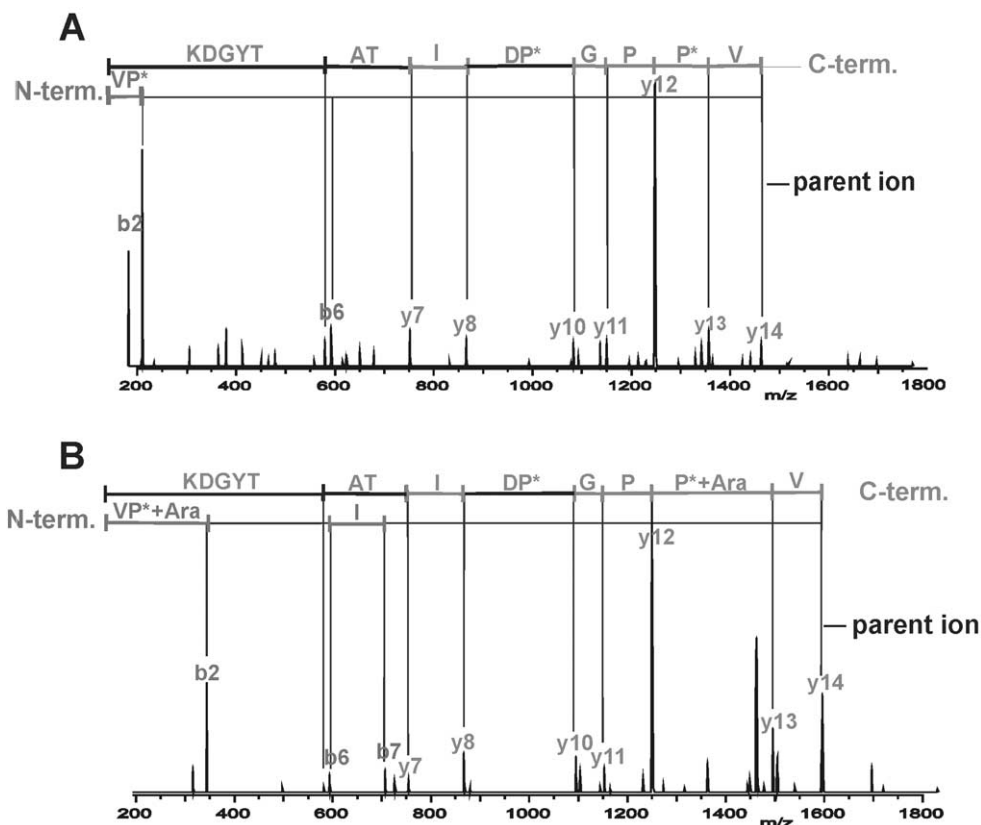


Figure 6 Charge deconvoluted ESI FT-ICR MS/MS spectra performed on the doubly protonated precursor ion of N-glycosidase A-digested gPep4-17 without (A) and carrying Ara (B) (see Figure 5). SORI-CID with argon as collision gas was applied. C- and N-terminal fragments are annotated on top of each spectrum. P*: hydroxyproline; Ara: arabinose.

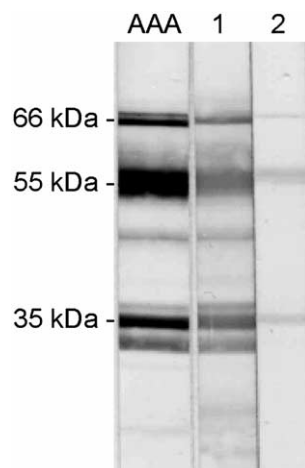


Figure 7 Binding of IgE to the carbohydrate structures of timothy grass pollen extract. Serum Bo220 was tested for IgE against the fucose-containing glycans. Lane AAA: lectin AAA; lane 1: serum Bo220; lane 2: Bo220 inhibited with bromelain N-glycan.

Table 1 Inhibition ELISA with patients' sera against nPhl p 1.

Inhibitor	HC	Bo220	W
nPhl p 1	0.063	0.035	0.034
Bromelain N-glycan	0.066	0.037	0.254
No inhibitor	0.054	0.233	0.259

nPhl p 1, natural Phl p 1 (1 µg/ml); bromelain N-glycan, N-glycan of bromelain (1 µg/ml); HC, healthy control; Bo220 and W, patient sera. All values represent the OD at 450 nm.

13 and Phl p 1 are to be expected (Figure 7), and the corresponding bands react with monoclonal antibodies directed against Phl p 13 and Phl p 1, respectively (Petersen et al., 1995b, 2001). The IgE-reactivity of serum Bo220 could be completely inhibited by preincubation with Phl p 1 (positive control, minimum concentration for total inhibition was 1 µg/ml) and isolated bromelain N-glycan, which is structurally identical to the 1026 Da glycoform of the Phl p 1 and 13 (Table 1), whereas preincubation with the glycan had no effect on serum W (without reactivity toward carbohydrate structures). In all of six other carbohydrate-directed sera identified during the pre-screening, IgE-reactivity could also be completely blocked by bromelain N-glycan in the ELISA tests (data not shown).

Mediator release

To test whether IgE binding to the carbohydrate moieties of Phl p 1 and Phl p 13 can induce mediator release, IL-4 liberation from basophils was chosen as a test system. Since the O-glycosylation of Phl p 1 displayed no IgE-reactivity, only the ability of the N-glycan to cause mediator release was investigated by stimulating the cells with the following glycoproteins: an artificial glycoprotein (bromelain N-glycan-conjugated BSA with 5–6 glycan ligands per molecule), natural Phl p 1 containing one N-glycan, recombinant Phl p 1 (without carbohydrate moieties) or natural Phl p 13 with up to 4 N-glycans (Figure 8).

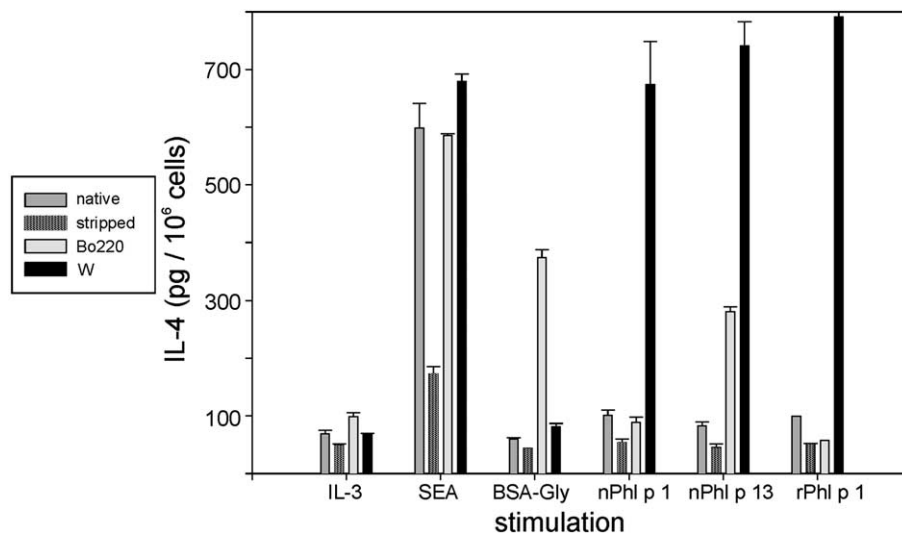


Figure 8 Mediator release from basophils induced by bromelain/Phl p 1 N-glycan.

Basophils were stimulated with: IL-3 (negative control); SEA (*Schistosoma* egg antigen, positive control); BSA-Gly (bromelain N-glycan conjugated BSA); nPhl p 1; nPhl p 13; rPhl p 1 (lacking the N-glycan).

The following basophil preparations were used: native: native cells as prepared from the donor; stripped: cells stripped off IgE; Bo220: cells resensitized with patient serum Bo220; W: resensitized with patient serum W.

All basophils except the stripped ones released IL-4 upon stimulation with the positive control SEA. When serum recognizing protein epitopes (serum W) was used for resensitization, IL-4 was released only when the basophils were stimulated with natural or recombinant Phl p 1 or natural Phl p 13 but not by the use of glycan-conjugated BSA. In contrast, after resensitization with Bo220 natural and recombinant Phl p 1 with one and no N-glycan, respectively, failed to cause significant IL-4 release. However, the IL-4 liberation was increased upon stimulation with bromelain N-glycan coupled BSA and nPhl p 13.

These results clearly indicate that carbohydrate-directed IgE can cause mediator release; some sera only contain carbohydrate-specific IgE directed against grass pollen extract – as far as detectable in our assays – and this IgE can be cross-linked to cause IL-4 release from basophils, provided there is more than one N-glycan on the cross-linking molecule.

Discussion

The glycoforms of Phl p 1

The allergen Phl p 1 is a glycoprotein containing the monosaccharides N-acetylglucosamine, mannose, xylose, fucose and arabinose. The expected asparagine residue at position 9 could not be determined by N-terminal sequencing, indicating a modification (e.g. an N-glycan), which is in accordance with the predicted N-glycosylation motif NIT.

Due to the highly conserved ways of biosynthesis N-glycans are not species-specific and have rather invariant structures, particularly plant N-glycans (Faye et al., 1989; Lis and Sharon, 1993). Therefore, the glycan structure of Phl p 1 can be concluded without doubt from the data presented here. N-glycosidase F does not cleave the N-glycan from the peptide backbone, but N-glycosidase A

does, which strongly indicates that fucose is α 1–3 bound to the core N-acetylglucosamine residue (Altmann et al., 1995; Figure 9). The composition of monosaccharides together with the masses of the N-glycans show that there are two variants (glycoforms): one is exactly the same as described for bromelain (Ishihara et al., 1979), which is present in about 65% of the Phl p 1 molecules. The other glycoform has one additional mannose (α 1–3) bound to the core mannose (Figure 9). Both of these complex-type N-glycans, termed M0XF³ and MMXF³, have been described to occur in pollen and non-pollen allergens (Priem et al., 1993; Wilson and Altmann, 1998; van Ree et al., 2000).

Furthermore, the data from mass spectrometry (especially the ESI-MS-MS analysis) demonstrated that the arabinose is not part of the N-glycan (in contrast to the glycan described for tomato (Priem et al., 1993), but is bound to the hydroxyproline residue at amino acid position 5 of the protein backbone (Figure 9). Generally, O-glycosylation at hydroxyproline residues such as in arabinogalactan proteins is common in the plant king

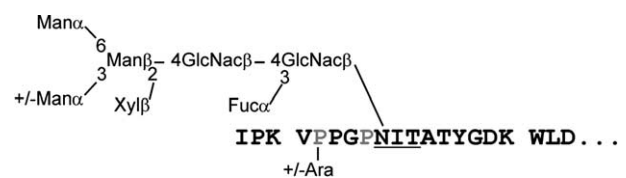


Figure 9 The glycoforms of natural Phl p 1 as deduced from our data and the literature.

Linkage positions of xylose and the branching mannose residues were deduced from published data; the tryptic glycopeptide 4–17 is indicated by gaps; Ara: arabinose; GlcNAc: N-acetylglucosamine; Fuc: fucose; Man: mannose; Xyl: xylose; P (in grey): hydroxyproline; NIT: N-glycosylation motif.

The 1026 Da glycoform (M0XF³, -Man α 1–3) is identical to the N-glycan of bromelain. The 1188 Da glycoform (MMXF³, +Man α 1–3) differs from the latter in only one additional mannose residue (162 Da).

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Phl p 1:IPKVPPGPNITATYGDKWLD...
Lol p 1:IAKVPPGPNITAEYGDKWLD...
Hol l 1:IAKVPPGPNITATYGDEWLD...
Pha a 1:IAKVPPGPNITAEYGDKWLD...
Ory s 1:HPKVPPGPNITTSYGDKWLE...
Zea m 1:PPKVPPGPNITTNYNGKWL...

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Figure 10 Comparison of the N-termini of several group 1 allergens.

Phl p 1: *Phleum pratense*; Lol p 1: *Lolium perenne*; Hol l 1: *Holcus lanatus*; Pha a 1: *Phalaris aquaticus*; Ory s 1: *Oryza sativa*; Zea m 1: *Zea mays*. Bold letters: conserved residues (in all shown group 1 allergens); KVPP: putative arabinosylation sequence; NIT: N-glycosylation sequence.

dom (Fötisch and Vieths, 2001). In analogy to the N-glycan, the structure of hydroxyproline-arabinose can be concluded from older data because it is a common structure in plants (Clarke et al., 1979; Shpak et al., 1999). However, only about 60% of the Phl p 1 molecules are O-glycosylated in this way, as far as can be estimated from the peak ratios. The arabinose residue does not interfere with N-terminal sequencing in contrast to the N-glycan, since the binding of furanosidic arabinose to hydroxyproline is labile under the fairly acidic conditions during the Edman degradation. An arabinosylation motif – variants of Pro-Hyp-Val-Tyr-Lys – has been described and tested with synthetic genes in tobacco (Shpak et al., 1999, 2001). Interestingly, the N-terminal sequence of Phl p 1 (...Lys-Val-Hyp-Pro...) has similarities to this arabinosylation consensus sequence, but it is inverted and there is no repetition of the motif, although this is considered necessary for arabinosylation. If the N-terminal sequence of Phl p 1 represents an arabinosylation motif for timothy grass, the lack of repetition might explain why only 60% of the molecules are O-glycosylated. There are no data, however, about the stability of hydroxyproline-arabinose under the conditions of MALDI-TOF experiments and the 40% of not arabinosylated Phl p 1 molecules might be artificial. The ratios of fucose and xylose to arabinose (1:1:1) found in the sugar analysis support the latter. The single arabinose residue attached to hydroxyproline is a new carbohydrate structure in an allergen, but has been described for proline-rich proteins (Clarke et al., 1979). The putative arabinosylation sequence is – along with other motifs – well conserved in the N-termini of group 1 allergens from several grass species (Figure 10), further indicating that KVPP(GP) might be a consensus sequence in grasses.

Thus there is a total of four glycoforms for the allergen Phl p 1: every molecule bears an N-glycan with (35%) or without an additional mannose residue (65%). 60% to 100% of the molecules are O-glycosylated. This hydroxyproline-arabinose region is a second possible epitope containing a carbohydrate structure in the Phl p 1 molecule, but no IgE-reactivity could be detected in our experiments (inhibition ELISAs).

The glycoforms of Phl p 13

Since the N-glycans of Phl p 13 have the same structure as the Phl p 1 N-glycan, the average (additional) mass of

one glycan would be roughly 1065 Da (65%+1008 Da; 35%+1170 Da). The average of the broad ion peak as determined by MALDI-TOF was 45551 Da and the calculated mass from the cDNA sequence of Phl p 13 is 41693.33 Da, which means that the molecule carries about 3858 Da of posttranslational modifications. Three N-glycans would have an average mass of 3195 Da, four glycans a mass of 4260. From these data it cannot be decided whether nPhl p 13 carries three or four N-glycans, taking into account that the mass of Phl p 13 deduced from the cDNA sequence comprises only one isoform, which might very well be not the most common isoform in the pollen. In the MALDI-TOF analysis of the tryptic peptides derived from Phl p 13, four of six potential N-glycosylation sites (at amino acid positions 72, 131, 370 and 390) were found to be glycosylated whereas no glycosylation could be detected for the consensus sites at amino acid positions 228 and 332. This is well in accordance with the mass of the complete molecule (45551 Da) which makes five or six N-glycans extremely unlikely. Other posttranslational modifications such as hydroxylation of proline residues or phosphorylation might cause additional minor changes in protein mass, but regarding our data it can be assumed that natural Phl p 13 is polyvalently N-glycosylated with four N-glycans of the MOXF³ or MMXF³ type.

132 Da (pentose, e.g. arabinose) shifts were not observed in the mass spectra of the Phl p 13 peptides. This, together with the fact that there is no (putative) arabinosylation consensus sequence in Phl p 13, makes the presence of hydroxyproline-arabinose in nPhl p 13 unlikely.

IgE-reactivity of the carbohydrate moieties

Both investigated glycoproteins were clearly detected as fucosylated by AAA in the immunoblots (55 kDa and 35 kDa). As has been demonstrated before (Tretter et al., 1993; van Ree et al., 2000), the N-glycans described above can be the cause for IgE cross-reactivity. Our experiments have not provided evidence that hydroxyproline-arabinose is a cross-reactive structure: the IgE-reactivity could be effectively blocked with the N-glycan alone in the inhibition ELISA experiments. Hydroxylation of proline residues, however, can be sufficient for an increased IgE-reactivity of Phl p 1 (Petersen et al., 1998) and arabinose might very well be part of IgE epitopes in some patients. It is rather unlikely that a putative arabinose-hydroxyproline epitope and the N-glycan of Phl p 1 can be bound by different IgE antibodies at the same time – and thus cause receptor cross-linking – due to the proximity of both structures. The additional glycosylation further increases the hydrophilic character of the molecule and, as water solubility is a crucial factor for the allergenic potential of a protein, the O-glycosylation might be a minor contribution to the allergenicity of nPhl p 1.

The immunoblot inhibition tests and ELISAs showed that patients' sera positive for timothy grass pollen in UniCap tests can have 'specific' IgEs directed exclusively against fucose-containing N-glycans. As such N-glycans are common in glycoproteins of plant or arthropod origin, this IgE is cross-reactive for a wide range of proteins.

Thus, a patient with high IgE titer against these carbohydrate structures will be tested positive in UniCap or similar tests for any extract containing such glycoproteins (van Ree, 2002).

The question whether this cross-reactivity causes the patients to suffer from allergic symptoms is of great relevance for clinical allergologists, since testing for IgE-reactivity is an important step in the process of detecting the allergen(s) causing symptoms. If, for example, the cross-reactivity has no clinical relevance, a patient with cross-reactive IgE would be tested falsely positive for a wide range of possible allergens. Regarding the biochemical structures involved, a difference in affinity between IgE directed against carbohydrate epitopes and protein specific IgE is not reasonable. In some cases the IgE-reactivity against carbohydrate structures obviously has clinical relevance: patient Bo220 is allergic to grass pollen (skin-prick-test and anamnesis) and the patient's IgE antibodies against timothy grass extract are directed against a carbohydrate structure. Natural Phl p 1 with its single N-glycan does not cause mediator release, but the polyvalent Phl p 13 and the N-glycan-BSA conjugate release IL-4 from basophils when used in our assays. Obviously, carbohydrate epitopes can cause FcεRI-cross-linking like any other epitope, as long as there are at least two of them present on the same molecule.

It is fair to say that it is still unclear why IgE-reactivity to carbohydrate epitopes has clinical relevance for some patients and no relevance for others. It is also fair to say that this is true for any other, non-carbohydrate epitope. In our experiments the carbohydrate-containing epitopes showed no difference to pure peptide epitopes: they show IgE binding and can cause mediator release if at least two of them are present on the same molecule. However, as the carbohydrate structures are rather invariant even between unrelated species, they are more likely to show cross-reactivity than the highly variable peptide epitopes.

Materials and methods

Allergen material and sera

Timothy grass pollen extract and the group 1 and group 13 allergens (Phl p 1 and Phl p 13) were prepared as previously described (Petersen et al., 1995b; Suck et al., 2000b). Pollen was obtained from Artu Biologicals (Lelystad, The Netherlands).

Bromelain N-glycan and bromelain N-glycan coupled to bovine serum albumine (BSA) were prepared as previously described (Wilson and Altmann, 1998).

Patients' sera were kindly provided by Dr. U. Lepp, Forschungszentrum Borstel, Clinical Ambulance, Borstel, Germany, Dr. J. Grabbe, Hautklinik, Medical University Lübeck, Lübeck, Germany and Dr. H. Aulepp, Hospital Borkum Riff, Bundesversicherungsanstalt, Borkum, Germany.

Enzymatic digestions

0.5 mg of natural Phl p 1 was digested with trypsin (sequencing grade; Roche Diagnostics, Mannheim, Germany) in 100 µl of 50 mM ammonium hydrogencarbonate at an enzyme:protein ratio of 1:20. Digestion was performed overnight at 37°C, in the absence of reducing agents. After incubation, samples were heated to 97°C for 5 min to inactivate remaining trypsin and

afterwards dried in a SpeedVac SPD12P (Thermo Savant, Holbrook, USA).

Natural Phl p 13 was digested as described above, but was reduced by adding 4 µl of 55 mM dithiothreitol (DTT) and incubation at 50°C for 1 h followed by addition of 4 µl 100 mM iodacetamide and incubation at room temperature in the dark for 1 h prior to drying of the sample.

Glycopeptide isolated from 0.5 mg of natural Phl p 1 was dissolved in 20 µl of 100 mM phosphate buffer, pH 5.6, digested with 0.1 mU of N-glycosidase A (Roche Diagnostics) at 37°C for 48 h and subsequently dried in a SpeedVac.

Additionally, Phl p 1 and Phl p 13 were subjected to in-gel digestion and their N-glycans were released (and purified) from the tryptic peptides as previously described (Kolarich and Altmann, 2000).

Isolation of glycopeptides

Chromatography was performed on an ÄktaPurifier10 with Unicorn software (Amersham Biosciences, Uppsala, Sweden). Tryptically digested Phl p 1 was separated on a HyPURITY Elite C18 5µ reversed-phase column (Thermo Hypersil-Keystone, Bellefonte, USA). Solvent A was Millipore water containing 0.1% (v/v) trifluoroacetic acid (TFA), solvent B 80% acetonitrile, isocratic grade containing 0.08% (v/v) TFA. A three step gradient was run: 0–8.5% B over 10 min, 8.5–42% B over 142 min and 42–60% B over 40 min with a flow-rate of 1 ml/min. UV absorption was detected at 214, 254 and 280 nm and fractions were collected in minute intervals. For the purpose of identifying glycopeptides, all peptide-containing fractions were dried in a SpeedVac. Subsequently, only neutral sugar containing fractions were pooled and dried.

Purity of the isolated glycopeptide was checked on a Source 5µ RPC reversed-phase column (Amersham Biosciences, Uppsala, Sweden), running a linear gradient from 0–100% B over 120 min, with solvent A (20 mM ammonium acetate, pH 7 containing 2% acetonitrile) and solvent B (20 mM ammonium acetate containing 70% acetonitrile).

After digestion of the glycopeptides by N-glycosidase A, samples were separated on the HyPURITY Elite C18 5 µ column, running a linear gradient from 0–100% B over 100 min, by the use of solvent A (water) and solvent B (80% acetonitrile). Peptide-containing fractions were pooled and dried.

Tryptically digested nPhl p 13 was subjected to a Superdex Peptide column (Amersham Biosciences) using 0.2 M ammonium hydrogencarbonate as solvent at a flow rate of 0.5 ml/min. Four pool fractions were collected from the numerous peptide-containing fractions and dried in a SpeedVac device.

Carbohydrate analysis

All peptides isolated from 0.5 mg tryptically digested Phl p 1 were incubated in 2 M TFA at 100°C for 4 h and dried in a SpeedVac. The samples were reduced overnight with 0.3 M NaBH₄, acidified with HCl, dried and washed three times with 5% acetic acid in methanol. Fifty µl pyridine and 50 µl acetic anhydride were added and the samples were incubated at 65°C for 2 h, dried in a nitrogen stream and diluted in chloroform.

GC-MS sugar analysis was carried out on a HP 5989 A mass spectrometer (Hewlett-Packard, Böblingen, Germany) as previously described (Kawahara et al., 2000).

N-terminal sequencing

Glycopeptides purified by reversed-phase HPLC were sequenced by Edman degradation on a 476A protein sequencer with online PTH analyzer (Applied Biosystems, Weiterstadt, Germany) as previously described (Petersen et al., 1995b).

Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was used for molecular mass determination of nPhl p1 and nPhl p13 and of glycopeptides obtained from HPLC fractions after digestion with trypsin. The measurements were performed in the positive ion mode on a Reflex III (Bruker-Daltonik, Bremen, Germany) in the linear- and reflector-TOF configuration applying an acceleration voltage of 20 kV. Appropriate mixtures of proteins and peptides of known masses were used for external mass scale calibration. The spectra shown are the sum of at least 60 single laser shot experiments. Samples, dissolved in a 2:1 mixture of 0.1% trifluoroacetic acid and acetonitrile, were mixed with a saturated matrix solution (3,5-dimethoxy-4-hydroxy cinnamic acid, SA, in the same solvent). 0.5 µl aliquots of sample solution were deposited on the metallic sample holder dried in a stream of air and analyzed immediately after evaporation.

N-glycosidase A-digested glycopeptide gPep4-17 was mass analyzed using an Electro Spray Fourier Transform Ion Cyclotron Resonance ESI-FT-ICR mass spectrometer (APEX II, Bruker Daltonics, Billerica, USA) equipped with a 7 Tesla actively shielded magnet and an Apollo ion source. Samples were dissolved in a 50:50:0.001 (v/v) mixture of methanol, water, and acetic acid and sprayed with a flow rate of 2 µl/min. Doubly protonated precursor ions were selected for sustained off-resonance irradiation collision-induced dissociation (SORI-CID) MS/MS analysis with argon as collision gas.

The released and purified glycans of Phl p 1 and Phl p 13 were subjected to MALDI-TOF analysis as previously described (Kolarich and Altmann, 2000).

Inhibition ELISA

MaxiSorb ELISA plates (Nunc, Roskilde, Denmark) were coated with natural Phl p 1, each well loaded with 150 µl coating buffer (0.1 M sodium carbonate buffer, pH 9.6), containing 1 µg natural Phl p 1 and incubated for 4 h incubation at room temperature. Subsequently the coating buffer was removed and the remaining free binding sites blocked with 150 µl Tris-buffered saline, 3% (w/v) milk powder. After incubating the plates for 1 h, the blocking buffer was removed and the wells filled with 150 µl of patients' sera, diluted 1:20 in TTBS (0.1 M Tris-buffered saline with 0.05% Tween, pH 7.4). The diluted sera had been pre-incubated for 2 h at 37°C with 1 µg/ml of natural Phl p 1 or bromelain N-glycan, respectively. (The experiment was repeated several times using inhibitor concentrations ranging from 5 to 0.5 µg/ml.) The plates were then incubated overnight at 4°C. Subsequently the sera were removed, the wells washed four times and filled with 150 µl alkaline phosphatase-conjugated mouse anti-human IgE. The plates were incubated for 2 h at room temperature and after four washing steps (3 times pH 7.4, 1 time pH 9.5) bound IgE was visualized with 150 µl of 0.1 M Tris-buffered saline, pH 9.5, containing *p*-nitrophenylphosphate (1 mg/l); the plates were incubated for 2 h at 37°C. Optical densities were measured with a Dynatech MR-7000 ELISA reader (Acterna, Germantown, USA) at 405 nm.

Western blotting and immunological detection

Timothy grass pollen extract (6 µg protein per cm gel) was separated by SDS-PAGE (12.5% T, 4% C) using the Laemmli buffer system and subsequently transferred onto nitrocellulose membrane, 0.45 µm pore diameter (Schleicher and Schüll, Dassel, Germany) by semi-dry electroblotting for 30 min at 200 mA, using the Kyhse-Andersen buffer system (Kyhse-Andersen, 1984).

After blotting, the membranes were blocked for 30 min in TTBS, dried and cut into strips. The membrane strips were incubated overnight at room temperature in patients' sera, diluted 1:20 in TTBS. For immunoblot inhibition the diluted sera were preincubated with 1 µg of bromelain N-glycan for 1 h at 37°C. The strips were washed three times in TTBS and incubated for 2 h at room temperature with alkaline phosphatase-conjugated mouse anti-human IgE (Allergopharma, Reinfeld, Germany), diluted 1:2000 in TTBS. Subsequently the strips were washed again.

Fucosylation was detected with alkaline phosphatase conjugated AAA (*Aleuria aurantia* agglutinin; Vector, Burlingame, USA), a lectin specific for α1-6 and α1-3 bound fucose. AAA was used at a concentration of 0.5 µg/ml in TTBS. The strips were incubated in the lectin solution for 4 h at room temperature.

After washing, 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) in 0.1 M Tris-buffered saline, pH 9.5.

For inhibition assays the diluted sera were incubated with 1 µg/ml bromelain N-glycan for 2 h at 37°C.

Mediator release

Basophils from a non-atopic donor were isolated and bound IgE was stripped as previously described (Haisch et al., 1999). Briefly, EDTA-blood was subjected to Ficoll density centrifugation and subsequent counterflow elutriation, both performed at 4°C. Further purification was performed using the MACS basophil isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions, but without addition of EDTA to the PBS-BSA buffer system. Cell number and purity were checked by trypan blue staining and counting in a Neubauer chamber.

Stripping of the basophils was done following a modified protocol according to Pruzansky et al. (1983): the cells were resuspended in 200 µl of cold LS-buffer (10 mM lactic acid, 140 mM NaCl, 5 mM KCl) and kept on ice for 3 min (carefully vortexed every minute). Afterwards the cells were neutralized with 4 ml Hanks' Balanced Salt Solution (HBSS) containing 0.25% BSA. The basophils were resensitized with patients' sera (W and Bo220), diluted 1:10 in medium, for 1 h at 37°C, 6% CO₂.

Basophils were stimulated over night at 37°C, 6% CO₂ with natural or recombinant Phl p 1 (the latter lacking glycosylations), natural Phl p 13 (up to 4 N-glycans) or the bromelain N-glycan coupled to BSA with 5-6 ligands per molecule. Anti-human IgE and *Schistosoma mansoni* egg antigen (SEA), which always triggers IL-4 release from IgE carrying basophils, served as positive controls. 2500 resensitized cells per well were stimulated with 14 nmol of the particular protein. Untreated donor basophils and stripped (not resensitized) cells served as negative controls.

IL-4 release was measured using the IL-4 Eli-pair Kit (Diacclone, Besançon, France) and a Dynatech MR-7000 ELISA reader (Acterna).

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