

Studies on the carbohydrate moiety of Pla 1 1 allergen. Identification of a major N-glycan and significance for the immunoglobulin E-binding activity

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Summary

Background Pla 1 1, the major allergen of *Plantago lanceolata* pollen, is a glycoprotein that contains an N-glycosylation site. Carbohydrate moieties of many allergenic glycoproteins have been reported to be IgE-binding determinants responsible for cross-reactivity among different species.

Objective To identify the kind of linkages and the type of glycans present in Pla 1 1 and to investigate their contribution to the allergic response to this allergen.

Methods Pla 1 1 was deglycosylated by N-glycosidase A and the IgE-binding ability of the unglycosylated protein was evaluated by dot-blot. Identification of $\beta 1 \rightarrow 2$ xylose and/or $\alpha 1 \rightarrow 3$ fucose residues in Pla 1 1 N-glycan was carried out by incubation with specific antibodies from rabbit antiserum against HRP (anti-HRP). The contribution of this N-glycan to total IgE reactivity was analysed quantitatively by pre-incubation of Pla 1 1 with anti-HRP prior to incubation with sera. The role of the carbohydrate moiety of Pla 1 1 in cross-reactivity was studied by RAST using unrelated glycoproteins with known sugar composition and structure.

Results The effectiveness of N-glycosidase A to deglycosylate Pla 1 1 and the ineffectiveness of the treatment with PNGase F indicate that Pla 1 1 carries a complex N-glycan with an $\alpha 1 \rightarrow 3$ fucose residue in its structure. Furthermore, the presence of $\beta 1 \rightarrow 2$ xylose and/or $\alpha 1 \rightarrow 3$ fucose residues was identified in this N-glycan by means of an ELISA. Pre-incubation of Pla 1 1 with an anti-HRP antibody caused a weak but significant reduction in IgE reactivity. Some sera from *P. lanceolata*-allergic patients reacted positively with four glycoproteins that bear N-glycans of complex type but not with fetuine.

Conclusions Pla 1 1 is a glycoprotein that carries at least a complex, major N-linked glycan, with an $\alpha 1 \rightarrow 3$ fucose residue in its structure and probably also a $\beta 1 \rightarrow 2$ xylose. This glycan moiety does not seem to constitute a relevant allergenic epitope of Pla 1 1.

Keywords allergen, carbohydrate epitope, cross-reactivity, glycan, IgE, Pla 1 1, *Plantago lanceolata*

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Introduction

Cross-reactivity among pollen allergens is very frequent and may be caused by both peptide and carbohydrate epitopes [1, 2]. In the recent literature, the oligosaccharide moiety of several glycoproteins has been reported to be involved in the allergic response [3, 4]. In particular, the epitopes responsible for cross-reactions seem to belong to the complex N-linked glycans, which are present in plants and invertebrates (insects, molluscs, etc.) but not in mammals [5, 6]. This fact has led to the suggestion that structural parts of these N-glycans are immunogenic to the human immune system, constituting carbohydrate determinants capable of inducing an IgE response. Structurally, complex N-glycans are characterized by carrying a fucose residue $\alpha 1 \rightarrow 3$ linked to Asn-GlcNAc and/or xylose $\beta 1 \rightarrow 2$ linked

to mannose, and both residues are pivotal for IgE-binding epitopes [7, 8].

The contribution of carbohydrate epitopes to the total IgE-binding capacity of allergens has been found to be very relevant in some cases [3, 9]. Recently, the structure of the N-glycans of some allergens has been elucidated [10], although few N-glycans have been studied in terms of their clinical relevance. *In vivo* studies with glycan moieties are very scarce and the real significance of cross-reacting carbohydrate determinants (CCD) in inducing allergic symptoms is not clear [11].

The aim of this work was to obtain more in-depth knowledge of the structure of the carbohydrate moiety of Pla 1 1, the major allergen of *Plantago lanceolata* pollen, and its contribution to IgE response. This allergen was previously isolated from natural extracts and characterized, and monoclonal antibodies against it were produced in our laboratory [12, 13]. Pla 1 1 is a polymorphic protein that yields two bands with apparent molecular weight of 17 and 20 kDa when analysed on SDS-PAGE that, according to the results presented in this article,

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correspond to the unglycosylated and glycosylated forms of the allergen, respectively. In order to obtain valuable information about the oligosaccharide moiety of Pla 1 1, we carried out the study here described. Thus, the results reported in this article could be useful for two different applications: on one hand, to identify whether some structural elements of the Pla 1 1 glycan are allergenic determinants, and disclose possible sources of cross-reactivity; and, on the other hand, to enable the selection of a suitable system to produce the recombinant allergen. As mentioned above, N-glycans are important IgE epitopes of some allergens. Consequently, it is not possible to replace the natural allergen by an unglycosylated or differently glycosylated recombinant allergen for diagnosis [14].

Materials and methods

Purified proteins

The allergen Pla 1 1 was purified from *Plantago lanceolata* pollen extract by means of affinity chromatography using the monoclonal antibody 2A10, as previously described [13].

Horseradish peroxidase, ascorbate oxidase from *Cucurbita* spp., pineapple bromelain, honeybee venom phospholipase A₂, and fetuin from calf sera were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Human sera

Sera from 15 individuals allergic to plantain pollen, as demonstrated by clinical history, positive skin reaction and presence of serum specific IgE (RAST class 2–4), were acquired from Plasmalab International (Everett, WA, USA). For dot-blot, a serum pool made by mixing equal volume aliquots of the individual sera was used. All the serum samples were stored at -80°C .

Carbohydrate detection

Carbohydrate detection was carried out with the ECL Glycoprotein Detection System (Amersham Life Science, Little Chalfont, UK) using 10 μg of Pla 1 1, and following the instructions for the commercial procedure 'Total carbohydrate labelling in solution'. Briefly, glycoproteins were oxidized with metaperiodate, labelled with biotin and detected with streptavidin-horseradish peroxidase and ECL Western Blotting (Amersham Life Science) in dot-blot. A sample of transferrin was used as a positive control, and a sample of Pla 1 1, where the oxidation step was replaced by incubation with 100 mM acetate buffer, was used as the negative control.

Enzymatic deglycosylation

Pla 1 1 was subjected to treatment with different enzymes (NANase II, O-glycosidase DS, PNGase F) included in a commercial enzymatic deglycosylation kit (Bio-Rad, Hercules, CA, USA). The procedure was performed under reducing conditions, following the manufacturer's instructions. Fetuin was used as a positive control.

Pla 1 1 was also treated with N-glycosidase A. Two samples of 4 μg of glycoprotein were incubated in 10 mM sodium acetate buffer, 0.1 M 2-mercaptoethanol, 10 mM SDS, pH 5.1, for 15 min at 80°C . Afterwards, 1 mU of N-glycosidase A was added to one of the samples and both were incubated for 24 h at 37°C . The effectiveness of the deglycosylation procedures was

checked by dot-blot using the ECL glycoprotein detection kit as described above.

Polyacrylamide gel electrophoresis

Efficiency of deglycosylation reactions of Pla 1 1 was checked by SDS-PAGE using commercial Tricine gels elaborated in a 10 to 20% acrylamide gradient (Novex, Frankfurt, Germany). Electrophoresis was run for 90 min at 125 V using 0.1 M Tris-base, 0.1 M tricine, 0.1% SDS, pH 8.3, as the electrode buffer. Low-range SDS-PAGE standards (Bio-Rad) were used as molecular weight markers. Protein bands were silver stained with Gel code Silver Snap Stain Kit (Pierce, Rockford, IL, USA).

MALDI-TOF mass spectrometry

The matrix-assisted laser desorption/ionization experiments were performed on a BIFLEX III time-of-flight instrument (Bruker-Franzen Analytik, Bremen, Germany) operated in the positive mode. Samples were analysed in the linear mode, and typically 100 laser shots were accumulated into a single mass spectrum. External calibration was performed using myoglobin as the standard. A saturated solution of sinapinic acid in acetonitrile:water (1:2) with 0.1% trifluoroacetic acid was used as the matrix. Equal volumes of the matrix and the sample solution were combined and vortexed, and 1 μL of the mixture was spotted on the target and air-dried.

Dot-blot

Samples (1 μg) were adsorbed to nitrocellulose membrane (Amersham Life Science) in dot-blot equipment (Bio-Rad) and incubated overnight at room temperature with a pool of sera from plantain-allergic patients diluted 1:5. IgE-binding proteins were detected by incubation with ^{125}I -labelled anti-human IgE mAb [15], followed by autoradiography exposure of the dried membrane for 3 days at -70°C .

ELISA

Different glycoproteins were adsorbed to polystyrene microtiter wells (EIA/RIA plates, Costar, Cambridge, MA, USA) at 1 $\mu\text{g}/\text{mL}$. After blocking with 1% BSA in 0.05% PBS-Tween 20, wells were sequentially incubated with an anti-horseradish peroxidase antibody developed in rabbit (anti-HRPO; Sigma-Aldrich) at 0.7 $\mu\text{g}/\text{mL}$ for 1 h and goat anti-rabbit IgG antibodies conjugated with peroxidase (Dako, Glostrup, Denmark). After 1 h at room temperature, a solution of *ortho*-phenyldiamine (OPD, Dako) was added to each well and left to react for 30 min at room temperature in the dark. The reaction was stopped with 2 N HCl and the absorbance measured at 490 nm with a reference filter at 650 nm in an automatic microplate reader.

RAST

Two different experiments of direct RAST were performed essentially according to the method described by Yman [16]. Samples were adsorbed to Maxisorp plates (Nalge Nunc, Roskilde, Denmark) with high protein binding capacity.

In one of the experiments, Pla 1 1 was adsorbed to wells at 1 $\mu\text{g}/\text{mL}$ and incubated, in duplicate, with individual sera from plantain-allergic patients (diluted 1:3), either with or without a previous incubation step with anti-HRPO antibody at 50 $\mu\text{g}/\text{mL}$ for 1 h. As a control, pre-incubation of human sera

with anti-*L. destructor* rabbit serum was carried out under the same conditions. IgE-binding to Pla 1 1 was detected with ^{125} I-labelled anti-human IgE mAb by adding approximately 100,000 cpm per well. After incubation for 2 h, bound radioactivity was counted in a gamma Walard Wizard 1470 counter.

IgE-binding capacity of complex N-glycans from different glycoproteins was evaluated by RAST as well. In this experiment, four glycoproteins (peroxidase, ascorbate oxidase, bromelain and phospholipase A₂) at 5 µg/mL and Pla 1 1 at 1 µg/mL were adsorbed to wells and incubated with the sera (diluted 1 : 3). The rest of the procedure was carried out as above.

Results

Structural characterization of Pla 1 1 carbohydrate moiety

Carbohydrate detection based on metaperiodate oxidation demonstrated that Pla 1 1 is a glycosylated protein (results not shown). In order to identify the types of glycans and links present, Pla 1 1 was treated with O-glycosidase and N-glycosidases enzymes. First, Pla 1 1 was treated with O-glycosidase DS and PNGase F. The efficiency of the deglycosylation reaction was analysed by SDS-PAGE (results not shown), and it could be observed that only fetuin, used as a positive control, showed a mobility shift. In contrast, Pla 1 1 did not suffer any alteration in its mobility. It has been described that PNGase F is unable to remove complex N-glycans with a fucose residue attached $\alpha 1 \rightarrow 3$ to the asparagine-linked N-acetylglucosamine [17]. Therefore, from its ineffectiveness to deglycosylate Pla 1 1, it could be suspected that this allergen carries a complex type N-linked glycan. To confirm this hypothesis, we treated Pla 1 1 with N-glycosidase A, which can cleave all types of asparagine bound N-glycans, including high mannose and complex types [18, 19]. This enzymatic treatment was very effective, as shown by SDS-PAGE (Fig. 1) and analysis in dot-blot with a commercial glycan detection kit as described in 'Materials and methods' (not shown). The two bands observed

Table 1. Results of an ELISA experiment in which Pla 1 1 and different glycoproteins were adsorbed to 96-well microtiter plates and incubated with an anti-horseradish peroxidase antibody developed in rabbit. As second antibody, an anti-rabbit IgG goat serum was used. Detection was accomplished with OPD. Values represent optical density at 490 nm

Glycoprotein	OD ₄₉₀
Pla 1 1	3.737
Bromelain	3.349
Ascorbate oxidase	3.666
Phospholipase A ₂	3.564
Fetuin	0.013

in the SDS-PAGE analysis of untreated Pla 1 1 control sample (Fig. 1, lane a) collapsed into a single band with the same mobility as the faster-moving band after the enzymatic treatment (Fig. 1, lane b), demonstrating that the upper band corresponds to the N-glycosylated form of the allergen that, consequently, bears a complex type glycan.

Moreover, to ascertain the presence of $\beta 1 \rightarrow 2$ xylose and/or $\alpha 1 \rightarrow 3$ fucose residues in the Pla 1 1 oligosaccharide, we tested this allergen in an ELISA with a specific anti-HRP IgG antibody developed in rabbit. This antibody reacts with $\beta 1 \rightarrow 2$ xylose and with $\alpha 1 \rightarrow 3$ fucose residues of different glycoproteins [10, 20, 21]. In this ELISA experiment (Table 1), all the control glycoproteins assayed with $\beta 1 \rightarrow 2$ xylose and/or $\alpha 1 \rightarrow 3$ fucose residues in their structural composition showed a positive reaction, and so did Pla 1 1. Fetuin, a glycoprotein with a high mannose type N-glycan, did not react with the antibody.

Mass spectrometry analysis of Pla 1 1

MALDI-TOF mass spectrum of purified Pla 1 1 (Fig. 2) showed two major overlapping peaks corresponding to the unglycosylated (14922 Da) and glycosylated forms (15891 Da). The relatively broad peaks observed suggest the existence of some degree of microheterogeneity in both forms. The difference between the molecular mass values determined at the peak apexes allows us to conclude that, on average, the modification of molecular mass due to glycosylation of Pla 1 1 is approximately 969 Da.

Contribution of the N-glycan of Pla 1 1 to the allergic response

To investigate whether the N-glycan of Pla 1 1 takes part in IgE-binding epitopes of Pla 1 1, we carried out different experiments. First, the capacity of Pla 1 1 to bind specific human IgE after the deglycosylation process was evaluated by dot-blot. Apparently, both Pla 1 1 and digested Pla 1 1 reacted in the same way with the pool of sera from patients sensitized to *Plantago lanceolata* (Fig. 3). Nevertheless, in a RAST experiment (Table 2), when Pla 1 1 was pre-incubated with the anti-HRPO antibody and later incubated with individual sera, a small reduction of IgE-reactivity was observed, in general, as compared with the allergen directly incubated with the sera. Statistical analysis by ANOVA with repeated measures demonstrated that the differences were significant, indicating that some minor allergenic determinants exist in the N-glycan of Pla 1 1. On the contrary, no significant differences were found with control samples pre-incubated with a rabbit serum against non-related antigens (anti-*L. destructor* rabbit serum; results not shown).

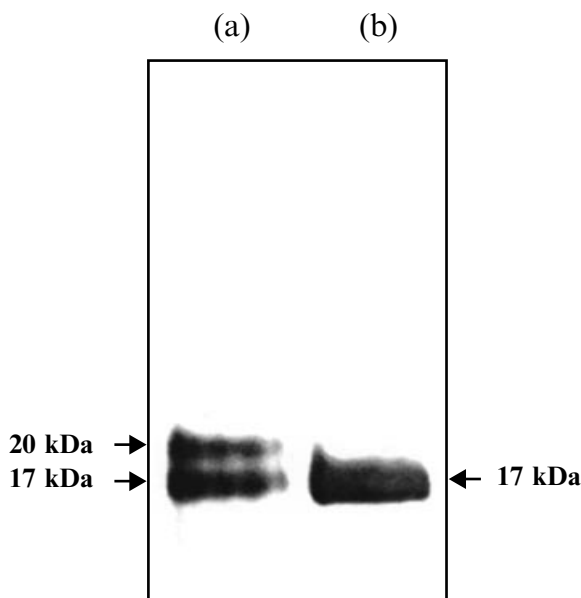


Fig. 1. SDS-PAGE analysis of (a) untreated Pla 1 1 and (b) Pla 1 1 treated with N-glycosidase A as described in 'Materials and methods'. Proteins were silver-stained.

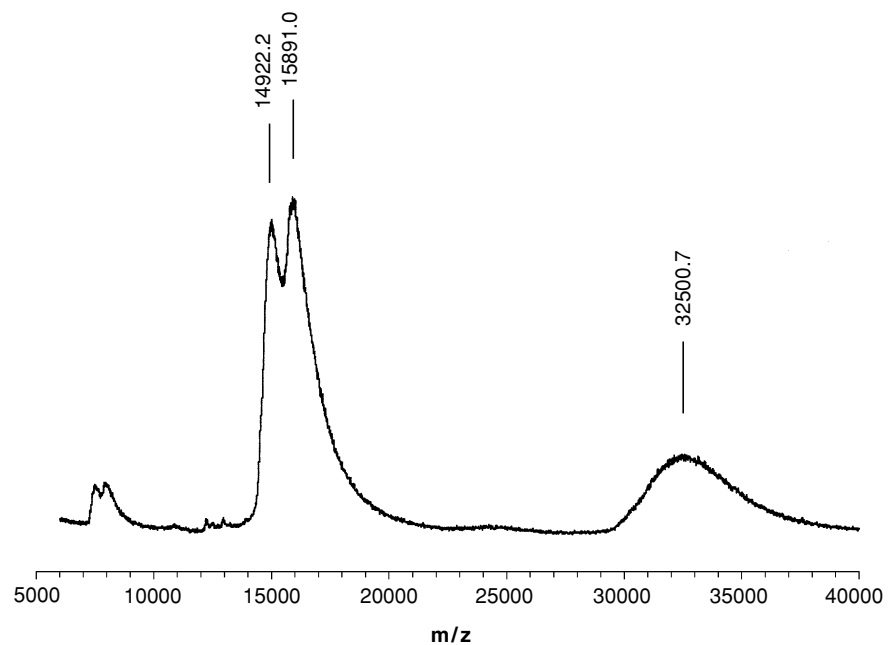


Fig. 2. MALDI-TOF mass spectrum of Pla I 1 allergen. A sample of mAb-affinity purified Pla I 1 was analysed as described in 'Materials and methods'.

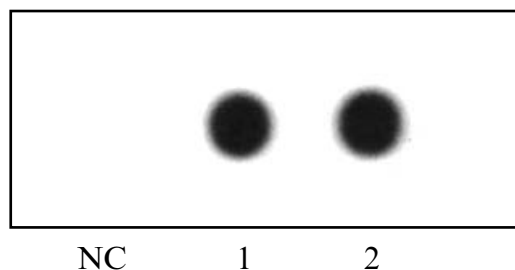


Fig. 3. Immunodetection with a pool of sera from patients allergic to *P. lanceolata*. IgE-binding to natural Pla I 1 (1) and deglycosylated Pla I 1 (2) was detected with ^{125}I -labelled anti-human IgE mAb. Bovine serum albumin was used as a negative control (NC).

Role of the carbohydrate moiety of Pla I 1 in cross-reactivity

In order to study the role of $\beta 1 \rightarrow 2$ xylose and $\alpha 1 \rightarrow 3$ fucose residues in potential allergenic cross-reactivities of Pla I 1, we evaluated the IgE-binding of four unrelated glycoproteins with known sugar composition and structure. The four glycoproteins chosen were horseradish peroxidase, ascorbate oxidase from *Cucurbita* spp., pineapple bromelain and honeybee venom phospholipase A₂, all of them with complex N-glycans. Fetuin was used as a negative control, as it is a glycoprotein with neither xylose nor fucose residues (data not shown). The assay was performed by RAST. Reactivity of the human IgE was evaluated against every glycoprotein and also against Pla I 1. Correlations between IgE responses to Pla I 1 and each glycoprotein were analysed statistically by Spearman test, and no significant correlation was found in any case. However, observing the graphic results in Fig. 4, it is possible to distinguish two groups of sera: one that yields points along the diagonal, and therefore with correlated IgE responses to Pla I 1 and other glycoproteins; and the other, the points of which are close to the Pla I 1 axis, for which there would not exist any correlation.

Table 2. IgE binding to Pla I 1 and Pla I 1 pre-incubated with anti-HRPO antibody. Results in cpm obtained in a RAST experiment. Pla I 1 was adsorbed to wells and incubated with sera from plantain-allergic patients. In the last column, the allergen Pla I 1 was pre-incubated with anti-HRPO prior to incubation with the sera. IgE binding was detected with ^{125}I -labelled anti-human IgE mAb. A negative serum (NC) was used to determine the non-specific binding. Statistical significance ($P = 0.0067$) was evaluated by ANOVA with repeated measures

Sample	Pla I 1	Pla I 1 + Anti-HRPO
1	4715	3734
2	15884	12382
3	12004	11288
4	1766	1368
5	10574	9515
7	4261	3478
8	2604	2010
9	8928	7610
10	4960	5042
11	8042	6462
12	9112	9858
13	8746	8944
14	1000	1032
20	2553	2598
21	1617	1673
NC	77	115

Discussion

In the last years, the role of glycans covalently linked to proteins in the allergic response has been clearly demonstrated [3, 4, 9, 10, 20]. Therefore, the study of glycans must be considered as an important part of the structural and immunochemical characterization of glycoprotein allergens. However, until now, the primary structure of N-glycans has only been

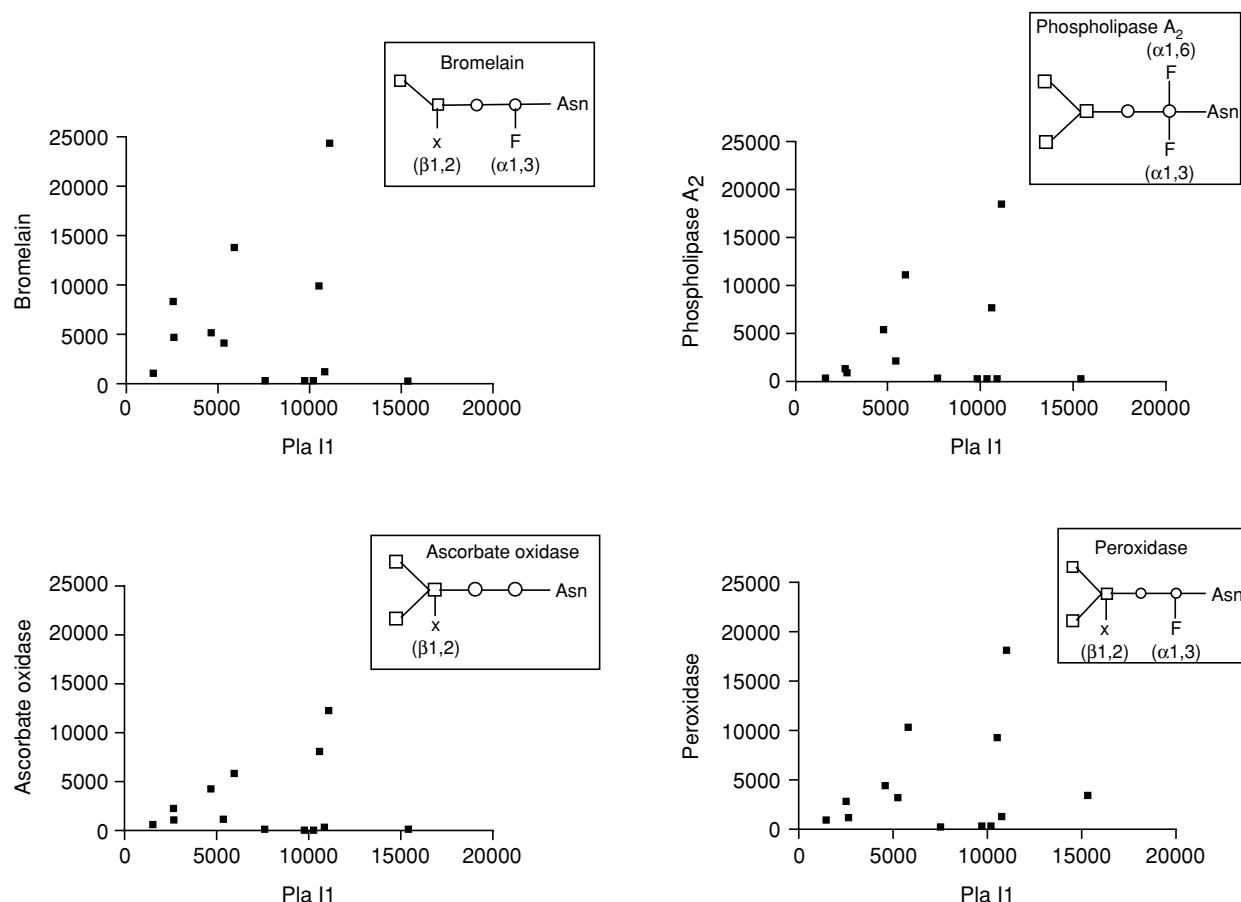


Fig. 4. Correlation of IgE-binding to different glycoproteins against IgE-binding to Pla I 1. Values represent cpm obtained in RAST analysis. Structure of N-glycans is represented as: $\square$ = mannose; $\circ$ = GlcNAc; $\times$ = xylose; F = fucose; Asn = asparagine.

established for some glycoallergens, such as Cry j 1 [3], Ole e 1 [10], Lol p 11 [10], Ara h 1 [10], Art v 2 [22], BG60 [23] and Api m 1 [24]. In this work, we intended to study the carbohydrate moiety of Pla I 1, the most relevant allergen of *Plantago lanceolata* from a clinical point of view. In the natural pollen extracts, this allergen exists in glycosylated and unglycosylated forms, in approximately the same proportion, as can be deduced from mass spectrometry or SDS-PAGE analyses [12, 13].

Although the results here described do not allow us yet to unambiguously assign a structure to Pla I 1 glycan, they provide relevant information on this subject. Thus, from enzymatic deglycosylation experiments, in which PNGase F and O-glycosidase did not cleave any oligosaccharide linked to the protein and only PNGase A could, it can be inferred that Pla I 1 bears a complex N-glycan with a fucose residue $\alpha 1 \rightarrow 3$ linked to GlcNAc. These results were corroborated by the positive reaction of the allergen Pla I 1 with an anti-HRP antibody that detects the presence of $\alpha 1 \rightarrow 3$ fucose and $\beta 1 \rightarrow 2$ xylose residues in glycoproteins. On the other hand, the N-glycan of Pla I 1 must be linked to the Asn residue at position 107 of the amino acid sequence, as it is the only potential N-glycosylation site (Calabozo et al., unpublished results).

The molecular mass of the oligosaccharide chain of Pla I 1 deduced from the difference in molecular mass of the glycosylated and non-glycosylated forms of the allergen, as determined by MALDI-TOF mass spectrometry, was 969 Da. It should be

noted that glycosylated Pla I 1 rendered a broad peak in mass spectrometry, so the estimated molecular mass could be affected by some inaccuracy. In addition, this fact suggests that a certain degree of microheterogeneity might exist in the carbohydrate moiety of Pla I 1, a feature that does not seem to be very uncommon among glycoallergens [3, 10, 22–24], and it could be that Pla I 1 contains several variants of N-glycans. Of all complex N-glycans of plants described so far, the most similar value is that of pineapple bromelain (1008.9 Da), which has a structure $\text{Man}_2\text{XylFucGlcNAc}_2$. Taking into consideration the observations mentioned above, this structure could be suggested as the most probable one for the major N-glycan of Pla I 1.

With regard to the role of this N-glycan in IgE reactivity, our results demonstrate that this moiety of Pla I 1 does not constitute an important allergenic epitope. Thus, application of qualitative techniques, such as dot-blot, did not enable us to detect any significant loss of IgE reactivity after the deglycosylation treatment. These results were in accordance with our previous findings by immunoblot after SDS-PAGE [13], showing that the protein band corresponding to the unglycosylated allergen (17 kDa) apparently had the same IgE-binding capacity as the protein band of the glycosylated protein (20 kDa). However, when more quantitative assays were used, we found that the carbohydrate moiety of Pla I 1 possessed at least one allergenic determinant. The experiment consisted of pre-incubating Pla I 1

with an anti-HRP antibody before incubation with sera. This antibody has been reported to react with $\beta 1 \rightarrow 2$ xylose and $\alpha 1 \rightarrow 3$ fucose residues [10, 20, 21], and this was confirmed by us in an ELISA in which the antibody reacted with all glycoproteins that contain fucose and xylose in their structure, but not with fetuin that lacks these residues. Therefore, using this antibody at a high concentration, the binding of other antibodies to these residues could be blocked and, if they formed part of allergenic epitopes, a reduction in the IgE-binding would be observed. For some sera, such as 1, 2, 4, 7 and 11 (Table 2), a noticeable decrease of about 20% was detected, but it was not so for the rest of the sera. Globally considering all the results, the statistical analysis demonstrated that the small differences in IgE reactivity detected after incubation with anti-HRP were significant.

Based on all the above findings, it is possible to conclude that sugar residues of Pla l 1 participate in epitopes that are minor allergenic determinants, contributing in a low proportion to the total IgE-binding capacity of the allergen.

On the other hand, the existence of reactive IgE against sugar epitopes was further demonstrated by the reaction of sera from *P. lanceolata*-allergic patients with unrelated glycoproteins. An approach to characterizing N-glycans of allergens is the use of commercial N-glycoproteins with known carbohydrate composition and structure [4, 9, 10, 25]. We tested four complex N-glycoproteins with xylose and/or fucose residues and one N-glycoprotein with only mannose on the core (fetuin). Several of the sera tested displayed reactivity with all the glycoproteins that bear complex N-glycans but not with fetuin. In addition, although a lack of correlation was found when the statistical analysis was applied to the whole group of sera, the IgE response to glycoproteins of positive sera seemed to correlate with the response to Pla l 1. Therefore, it could be that, among the overall population of patients sensitized to Pla l 1, a subgroup of patients has specific IgE directed to carbohydrate epitopes of Pla l 1 that would be responsible for the observed *in vitro* cross-reactivities with unrelated glycoproteins. From these results, nevertheless, it is not possible to establish any conclusion on the oligosaccharide structure of Pla l 1, as cross-reactivity was observed with the four complex N-glycoproteins tested.

Interestingly, based on the results presented here, the N-glycan of Pla l 1 does not seem to be an important allergenic epitope, yet IgE from some sera strongly recognized epitopes on other glycoproteins. One possible explanation could be that those patients had been sensitized to carbohydrates from other allergens. The presence of CCD shared by taxonomically unrelated allergens has been described and their involvement in cross-reactivity has been suggested [26, 27]. In addition, taking into account that monosensitization is very uncommon in patients allergic to *P. lanceolata* [28, 29], the positive reaction to N-glycans may be probably due to cross-reactions. So, although some of the sera recognize carbohydrate epitopes of Pla l 1, as we have been able to prove, it could be that the affinity of IgE for these epitopes is lower than the affinity for the rest of epitopes present in the Pla l 1 structure, or perhaps that the accessibility of carbohydrate epitopes in Pla l 1 is sterically hindered and, consequently, the contribution of Pla l 1 N-glycan in allergic reactions is not clinically relevant.

Even though an allergen displays *in vitro* high IgE-reactivity against N-glycans, it does not necessarily mean that those sugar epitopes are able to induce clinical symptoms [11]. For instance,

after extensive studies [17, 30] including *in vivo* assays, it has been concluded that the N-glycans of honeybee venom phospholipase A₂ are less important than was previously thought [31]. The capacity of CCD to induce clinical manifestations of allergy is practically not documented, although its capacity to release histamine *in vitro* has been elucidated in Ole e 1 [32] and Api g 1 [9]. Therefore, although the role of CCD in allergic reactions is not clear, the idea that carbohydrate epitopes could interfere in diagnosis and immunotherapy is progressively more accepted [33], and probably CCD together with the panallergens, such as profilin [34] and Bet v 1 [35], are responsible for the immense majority of cross-reactions between pollens from unrelated species.

Hence, studies on the contribution of the carbohydrates in IgE reactivity must be included in the characterization of an allergen, in particular in cases where N-glycan belongs to the complex type. Perhaps a good alternative to avoid false positive diagnoses in those allergens in which carbohydrate epitopes are not very relevant is to replace natural extracts by recombinant allergens that do not carry complex N-glycans [36, 37]. Thus, taking into account that Pla l 1 is clinically the most relevant allergen of *P. lanceolata* and that its N-glycan is not an important epitope for IgE, we suggest that the use in diagnostic tests of recombinant Pla l 1, without complex N-glycan as part of its structure, could help to identify allergic patients specifically sensitized to *P. lanceolata* pollen.

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