

Post-translational modifications influence IgE reactivity to the major allergen Phl p 1 of timothy grass pollen

A. PETERSEN, G. SCHRAMM, M. SCHLAAK and W.-M. BECKER

Forschungszentrum Borstel, Biochemical and Molecular Allergology, Borstel, Germany

Summary

Background Grass group I consists of very potent allergenic components which are found in the pollen of all temperate grasses. Several post-translational modifications are predicted from the cDNA data.

Objective The aim of this study was to identify sequential IgE-binding sites on the allergen Phl p 1 and to determine their influence on IgE reactivity.

Methods Based on cDNA data and microsequencing results we synthesized overlapping decapeptides covering the complete Phl p 1 molecule and tested them for immunological reactivity by means of the PEPSCAN technique. In a dot test we determined the frequency of IgE reactivities to post-translationally modified structures (hydroxylated proline residues, carbohydrate structure, and disulphide formations).

Results Screening by overlapping peptides demonstrated an IgE binding site on the 10 N-terminal amino acids. Comprehensive studies showed that the two hydroxyproline residues of the native Phl p 1 allergen (at positions 5 and 8) and the N-glycan (at position 9) can result in an increased IgE reactivity; 3.3% of the sera exclusively bound to the hydroxyproline bearing peptide, while only 0.4% bound to the proline containing peptide. With regard to glycosylation, we estimated that 20% of sera recognized protein and carbohydrate epitopes, while one serum exclusively bound to the glycan. The formation of disulphide bonds has no detectable effect on the IgE reactivity to Phl p 1.

Conclusion Our results indicate that the post-translational modifications, the carbohydrate structure and the hydroxylation of proline residues, can enhance the IgE reactivity of Phl p 1.

Keywords: IgE reactivity, grass pollen allergen, PEPSCAN, post-translational modifications

Clinical and Experimental Allergy, Vol. 28, pp. 315–321. Submitted 21 September 1996; revised 24 June 1997; accepted 6 September 1997

Introduction

All the temperate grasses possess the important group I allergens and the majority (95%) of grass pollen allergic patients will demonstrate an IgE response to these [1]. For a better understanding of the specific interaction between an allergen and the immune response, the precise characterization of the IgE-binding epitopes is necessary. For diagnostics and immunotherapy, the use of recombinant allergens seems to be a promising concept, since they are well-defined components and can be easily quantified [2]. In initial

studies performed by Esch and Klapper [3,4], Lol p 1, the major allergen of ryegrass (*Lolium perenne*) from natural pollen extract, was isolated and cleaved by use of cyanogen bromide and trypsin. They identified IgE-reactive fragments which covered regions of the N- and C-terminus. Unfortunately, the length and the yield of fragments is poorly controlled by this technique. Since the introduction of cDNA cloning techniques to the field of allergy research, a number of allergen sequences has been deduced. We have used these techniques to elucidate the sequence of Phl p 1 (EMBL/Genbank, accession number Z27090 (1993) [5]), a major allergen of timothy grass pollen (*Phleum pratense*). Until now, grass group I sequence data have been available for ryegrass (Lol p 1 [6,7]), timothy grass (Phl p 1 [8]),

Correspondence: Dr A. Petersen, Forschungszentrum Borstel, Biochemical and Molecular Allergology, Parkallee 22, D-23845 Borstel, Germany.

velvet grass (Hol l 1 [9]), and canary grass (Pha a 1 [10]). Their sequences are similar and demonstrate an identity of >85% and homology >90%. Using recombinant Hol l 1, Schramm *et al.* [9] constructed recombinant fragments and deduced IgE-reactive epitopes from their immunoreactivity. This method is especially useful for roughly defining continuous (sequential) and discontinuous IgE-binding regions on the allergenic molecule. Ninety-two per cent of grass group I reactive patients demonstrated IgE binding to the 80 amino acids spanning the C-terminus, while 30–50% bound to N-terminal fragments of a similar size. Ball *et al.* [11] isolated an IgE-reactive hapten consisting of 15 amino acids by use of an epitope expression cDNA library from randomly fragmented cDNA coding for Phl p 1.

A disadvantage of the recombinant allergens expressed in *E. coli* is their lack in post-translational modifications, e.g. glycosylations and the correct formation of disulphide bonds. However, grass group I allergens possess such structures. The carbohydrate moiety contributes 5% of the molecular weight and is *N*-glycosidically bound to amino acid residue 9 [12]. The *N*-linked oligosaccharides are of the complex-type with a conserved tri-mannosyl core, substituted with fucose α 1,3 bound at the innermost *N*-acetylglucosamine [13]. This structure is a part of an IgE-binding epitope [12,13]. The group I allergens contain seven cysteine residues which partly form intra and intermolecular disulphide bonds [unpublished data]. Finally, we identified two hydroxyproline residues in positions 5 and 8 by N-terminal microsequencing [14]. These findings suggest that the protein structure of Phl p 1 might be influenced by post-translational modifications.

Defining epitopes on allergens is difficult because they often depend on tertiary structure (discontinuous epitopes). These discontinuous epitopes can only be determined by highly sophisticated methods such as X-ray structure analysis and cocrystallization with antibodies. For defining continuous epitopes the PEPSCAN technique [15] is suitable.

In several studies continuous IgE-reactive epitopes were determined successfully by use of overlapping synthetic peptides. For example, in ovalbumin and hazelnut pollen allergen Cor a 1 IgE-binding epitopes were harboured, respectively, in the protein regions aa 1–10 and 29–34 [16,17]. We synthesized overlapping decapeptides of the Phl p 1 derived from the cDNA [5] and protein sequencing data [14] then screened for epitopes of human IgE. One IgE-reactive epitope was detected at the N-terminus consisting of 10 amino acids. Interestingly, the existence of two hydroxyproline residues within this region of the natural Phl p 1 allergen can evoke or enhance the IgE reactivity. Additionally the detection of post-translationally modified structures by patients' IgE was determined, to assess the importance of such modifications.

Materials and methods

Patient sera

For the identification of IgE-reactive proteins we used sera provided by the Medical Clinic in Borstel (Dr U. Lepp and Dr A. Bufo) and the Department of Allergology of the J.W. Goethe Medical University in Frankfurt (Dr R. Wiewrodt). The sera had to fulfil certain criteria: They had to have a specific IgE CAP score ≥ 4 (Pharmacia CAP System; Pharmacia Diagnostics AB, Uppsala, Sweden) and the Western blotting result had to be positive against Phl p 1.

To determine the frequency of IgE reactivity to post-translationally modified structures, we screened sera drawn from volunteer blood donor with undefined allergic history. These sera were kindly provided by the Institute for Immunology and Transfusion Medicine (Medical University of Lübeck).

Preparation of pollen extract

One gram of timothy pollen (ARTU Biologicals, Lelystad, The Netherlands) was suspended in 55 mL of 0.1 M NH_4HCO_3 buffer (pH 8.0) and incubated at room temperature for 30 min with agitation. After centrifugation for 30 min at 17 000 *g* (4 °C) the supernatant was dialysed against double-distilled water. After lyophilization the extract was stored at 4 °C until use.

Nucleotide sequence accession number

For the epitope mapping synthetic peptides were constructed according to the nucleotide sequence data of pPHLPI.p4, which had been submitted to Genbank/EMBL Data Library, accession number Z27090 (1993).

Computer analyses

Sequence data were analysed on GeneWorks and PCGENE software (Intelligenetics, Geel, Belgium) and on DNASIS and PROSIS-Software (Pharmacia LKB Biotechnology, Uppsala, Sweden). The antigenic index was calculated according to the Jameson and Wolf algorithm [18].

Pepscanning

To determine B-cell epitopes on the Phl p 1 we used the Pin technology [15] (Multipin non-cleavable peptide synthesis kit for B-cell epitope scanning, Chiron Mimotopes Peptide Systems, Clayton Victoria, Australia).

Synthesis of peptides to pins

Decapeptides with an increment of three amino acids were

synthesized to activated polypropylene pins with a spacer consisting of 1,6-diaminohexane and β -alanine utilizing 9-fluorenylmethoxycarbonyl amino acids (Fmoc).

Immunoscreening

The immunoscreening was performed by a Pin ELISA according to the manufacturer's instruction with some modifications. After a blocking step the pins were washed and then incubated with individual patient sera (1:20) overnight. The binding of the monoclonal antibodies and patients' IgE was demonstrated by alkaline phosphatase labelled mouse antihuman IgE (1:2000) (Allergopharma, Reinbek, Germany). p-Nitrophenyl phosphate (1 mg/mL in 0.1 M TBS; pH 9.5) was used as the substrate and absorbance was measured at 405 nm after a 1 h incubation period.

Automated peptide synthesis

In order to obtain higher yields, the peptides of interest (IPKVPPGPNTA and IPKVpPGpNTA) were synthesized on a 9050 Peptide Synthesizer (Millipore Corporation, Bedford, USA). Subsequently, peptides were purified by reverse phase HPLC and the sequences confirmed.

ELISA testing

For a detailed investigation of patients' IgE binding, we performed ELISA tests with the synthesized peptides coupled to the microtitreplate.

Microtitreplates (MaxiSorp, Nunc, Roskilde, Denmark) were pretreated by soaking in a solution of 0.05% (v/v) glutaraldehyde in 0.1 M carbonate buffer, pH 9, following which, they were thoroughly washed and dried. After incubating the plates overnight with 100 μ L of antigen dilution (20 μ g/mL in 0.1 M carbonate buffer, pH 9.6) per well, they were washed and blocked with TBS buffer containing 1% (v/v) goat serum. Patient sera (diluted 1:5 in TBS-buffer; pH 7.4) were incubated for 2 h at room temperature. Further steps were performed as for immunoscreening.

ELISA inhibition assays were performed under the same conditions, except the patient sera were preincubated with serial dilutions of inhibitors (to final concentrations of 0.1–500 μ g/mL) for 2 h, before the samples were transferred to a peptide-coated microtitreplate.

Dot blotting

Screening for IgE reactivity to post-translationally modified structures was performed by dot blotting onto CNBr-activated nitrocellulose strips [19]. To study the influence of disulphide formations affinity purified Phl p 1 was pretreated with reducing buffer (containing 60 mM

dithiothreitol and 280 mM iodacetamide) or non-reducing sample buffer (without dithiothreitol) at a concentration of 1 μ g/ μ L. To investigate carbohydrate recognition we used affinity purified Phl p 1 [14], chemically deglycosylated pollen extract (by trifluoromethane sulphonic acid) [12], recombinant Phl p 1 from expression in *E. coli* [5], phospholipase A₂ (PLA₂) from bee venom, horseradish peroxidase (HRP), and ovalbumin (Sigma-Aldrich Chemie, Deisenhofen, Germany) at concentrations of 1 μ g/ μ L. The two proteins PLA₂ and HRP were used, because they have a similar carbohydrate structure to Phl p 1 [12,13]: these possess N-linked oligosaccharides of the complex-type with a conserved tri-mannosyl core, substituted with fucose in the case of PLA₂ and fucose and xylose in HRP and Phl p 1. The glycoprotein ovalbumin lacks substitutions and was used as a control. The influence of hydroxylated proline residues was investigated using the peptides P and P' (refer to ELISA testing) at concentrations of 4 μ g/ μ L and comparing their IgE reactivities.

For immunological detection we used primary and secondary antibodies as described under immunoscreening. The binding patterns were visualized by a substrate solution consisting of 0.033% (w/v) nitro blue tetrazolium chloride and 0.017% (w/v) 5-bromo-4-chloro-3-indolyl phosphate potassium salt (Serva, Heidelberg, Germany) in 0.1 M Tris buffered saline (pH 9.5) [20].

Results

Determination of IgE-reactive epitopes by PEPSCAN

To identify IgE-reactive epitopes, we used individual patient sera that had shown positive results to Phl p 1 by Western blotting. Prior to use patient sera were depleted of IgG antibodies by adsorption to protein A sepharose.

A clear signal was determined at the N-terminal decapeptide, with a threefold absorbance over the background (Fig. 1). Since the proline residues at amino acid position 5 and 8 are post-translationally hydroxylated in natural Phl p 1, we synthesized the corresponding decapeptide with hydroxyproline residues and tested them for IgE reactivity.

Figure 1 shows the reactivity of two individual patient sera (G 53 and G 22) and one healthy control (NS) for the modified (hydroxyproline bearing peptides, 1'–3') and the unmodified N-terminal decapeptides (1–17). G 53 reacts strongly to the first peptide (1') giving a 30% greater signal than the unhydroxylated peptide (1).

Influence of hydroxyproline residues on IgE reactivity

Patient sera were screened in an ELISA to estimate the importance of the N-terminal epitope and to study the modulating effect of hydroxyproline residues.

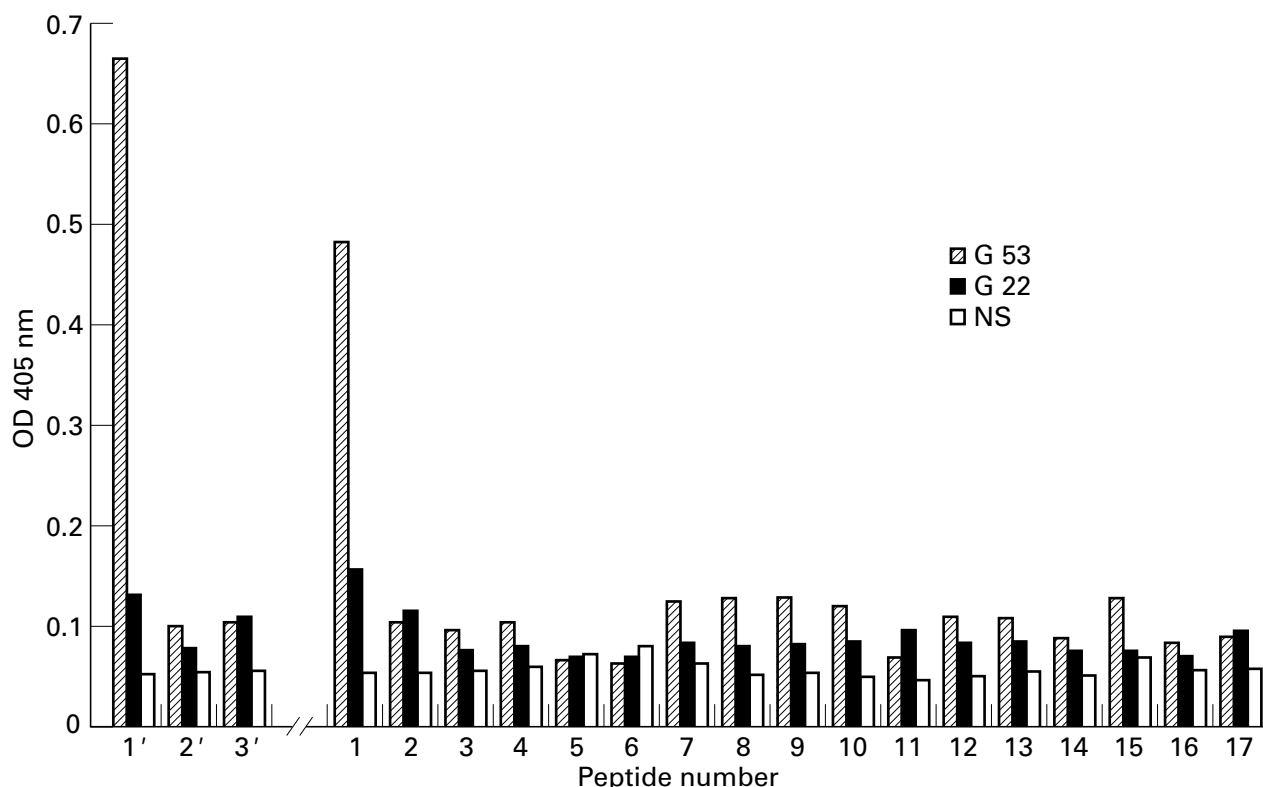


Fig 1. Epitope mapping of patients' IgE using overlapping decapeptides of the Phl p 1 N-terminal sequence (increments of 3 amino acid residues). 1'–3', decapeptides synthesized according to protein sequencing data (containing hydroxyproline residues in the corresponding positions); 1–17, decapeptides synthesized according to cDNA sequence data (without post-translational modifications). G 53 and G 22, sera of grass pollen allergic individuals; NS, healthy control.

Dodecapeptides of the Phl p 1 N-terminus were synthesized that differed only at positions 5 and 8, possessing either proline (P) or hydroxyproline residues (P').

Table 1 depicts the IgE reactivity of 14 individual patient sera, which had been selected because of their positive reaction to Phl p 1 by Western blotting. Sera of 10 healthy donors were taken to determine the mean value and the standard deviation for background reactivity. (A positive reaction was defined as being threefold greater than that of the negative controls.) The IgE reactivity differs for both the P' and P peptides. One serum (F 11) bound to both peptides, while two other sera (G 626, G 34) showed a positive reaction only with peptide P'. Ovalbumin was used as the negative control.

In order to directly compare the IgE-binding activity of the peptides P and P' we performed an ELISA inhibition test.

Figure 2 shows the results of the test for patient serum F 11: Inhibition of the binding of patient's IgE was tested by increasing concentrations of the potential inhibitors, pollen extract (g6, positive control), ovalbumin (negative control), and the peptides P and P'. Inhibition by P' was observed at concentrations which were one hundredth that of P.

Screening for IgE binding to post-translationally modified regions of Phl p 1

A total of 274 grass group I reactive individuals was analysed for their recognition of post-translational modified structures. In order to identify epitopes generated by disulphide formations we used affinity purified Phl p 1 under nonreducing vs reducing conditions. Binding to the hydroxylated proline residues was investigated by comparing the reactivities between peptides P' and P. IgE binding to the carbohydrate moieties was determined by use of the following antigens: natural affinity purified Phl p 1 and the glycoproteins phospholipase A₂ from bee venom and horseradish peroxidase, which have similar carbohydrate structures, while recombinant Phl p 1 from *E. coli* and TFMS-deglycosylated Phl p 1 lack the carbohydrate residues.

Figure 3 shows a part of the screening results. From the 274 sera tested no differences in IgE reactivities were observed between affinity purified Phl p 1 treated under reduced and non-reduced conditions. Thus the disulphide bridges in Phl p 1 do not alter the IgE binding. Twelve (4.4%) recognized the N-terminal dodecapeptides P and/or

Table 1. Effect of hydroxylated proline residues on the IgE reactivity of individual patient sera determined by ELISA. The N-terminal proline containing peptide P (IPKVPPGPNITA) and the hydroxyproline containing peptide P' (IPKVpPGpNITA) were used as antigens. The results of ten healthy controls are given as mean values and standard deviation. Strong IgE reactivities (> threefold increase in absorbance) are indicated in bold.

Serum	Ovalbumin	P	P'
10 healthy controls	0.190 ± 0.095	0.141 ± 0.037	0.143 ± 0.032
G 626	0.275	0.160	0.696
F 38	0.248	0.214	0.209
W	0.227	0.109	0.386
G 34	0.257	0.139	0.464
F 14	0.219	0.223	0.413
G 56	0.208	0.184	0.210
F 11	0.193	0.433	1.824
G 961	0.204	0.147	0.139
G 540	0.197	0.180	0.161
G 53	0.250	0.258	0.332
F 9	0.230	0.343	0.358
G 638	0.202	0.200	0.199
G 625	0.215	0.168	0.157
F 3	0.217	0.245	0.227

P'. Nine sera exclusively bound to the hydroxyproline bearing peptide, while only one serum detected the unmodified peptide P. Two sera recognized both peptides. With regard to reactivity towards the carbohydrate moiety we determined one serum (0.4%) that exclusively bound to samples containing glycosylated Phl p 1 and also to PLA₂ and HRP which have similar carbohydrate chains (Fig. 3, PS 3). Fifty-seven sera (20.8%) recognized epitopes on the carbohydrate moiety and on the protein backbone. (216 sera (78.8%) detected epitopes only on the protein structure.)

In summary, the post-translational modifications at the N-terminus of Phl p 1, two hydroxylated proline residues and one N-glycan, clearly enhance the IgE reactivity.

Discussion

The aim of our study was to precisely define continuous epitopes on the major grass pollen allergen Phl p 1, which is a prerequisite for gaining a deeper insight into the antigen-antibody-interaction. According to van Regenmortel [21] at least 15 amino acid residues of the antigen are involved in antigen-antibody-binding as determined by X-ray studies. On the other hand ELISA shows that only 5–8 amino acids are functionally necessary to enable antibody binding. In preliminary tests we used two monoclonal antibodies directed against Phl p 1 and we were able to harbour their epitopes on a length six amino acids (data not shown).

Therefore, we concluded that a length of 10 amino acids seems suitable for epitope scanning. Since the peptides are coupled via a spacer of 1,6-diaminohexane and β -alanine to the polypropylene pins, no steric hinderance of the amino acids located at the carboxy end of the peptides should occur. Compared with the epitope mapping strategies of Esch and Klapper [3,4] and Schramm *et al.* [9], respectively, using natural or recombinant fragments of group I allergens, the advantages of the pepscanning technique are the precise identification of continuous epitopes, since peptides of each length and sequence can be synthesized.

For the determination of IgE-binding epitopes we used short peptides. We assume that it is not the complete, natural protein but its' immunoreactive fragments that play an important role in the allergic reaction. Under physiological conditions the pollen grains release their contents after contact with the nasal mucosa. The released components are then denaturated and cleaved into IgE-reactive peptides by various enzymes of the nasal secretion [22], before they reach the mast cells. Therefore, the structure of these peptides differs from that of the complete, natural protein in the pollen grains. Thus, the relevant B-cell epitopes are the antigenic structures on these fragments. Whether these epitopes already exist on the complete natural allergen molecule, and 'survive' denaturation and fragmentation and remains as a stable IgE-reactive epitope depends on the structure. Cryptic epitopes on the other hand are inaccessible in the natural molecule but become accessible for antibodies after denaturation and/or fragmentation. By SDS-PAGE the protein conformation is disrupted. Since nearly all of the grass pollen allergic patients can be detected by Western blotting, continuous IgE-reactive epitopes should be present on the Phl p 1 molecule. However, during blotting a partial renaturation of the proteins might occur [23].

We identified one epitope covering the N-terminus of Phl p 1 (aa 1–10: IPKVPPGPNI). The peculiarity of this structure is the 3 proline residues in a close proximity. Since each proline residue forms a kink in the protein conformation, this part of the Phl p 1 molecule seems to be very striking. Besides its unique structure, N-terminal sequences are often antigenic [24], presumably their accessibility enables antibody attachment. According to Schramm *et al.* [9] 26% of the group I reactive patients recognized a recombinant fusion protein of the 30 amino acid N-terminus. Interestingly, the Phl p 1 N-terminus bears some post-translational modifications as identified by protein sequencing [14]: two hydroxylated proline residues at positions 5 and 8 and one glycosylation site at position 9 to which a glycan of about 2 kDa is conjugated. These modifications change this part of the molecule to a more hydrophilic structure. As shown by the screening, the carbohydrate structure is recognized by 20%

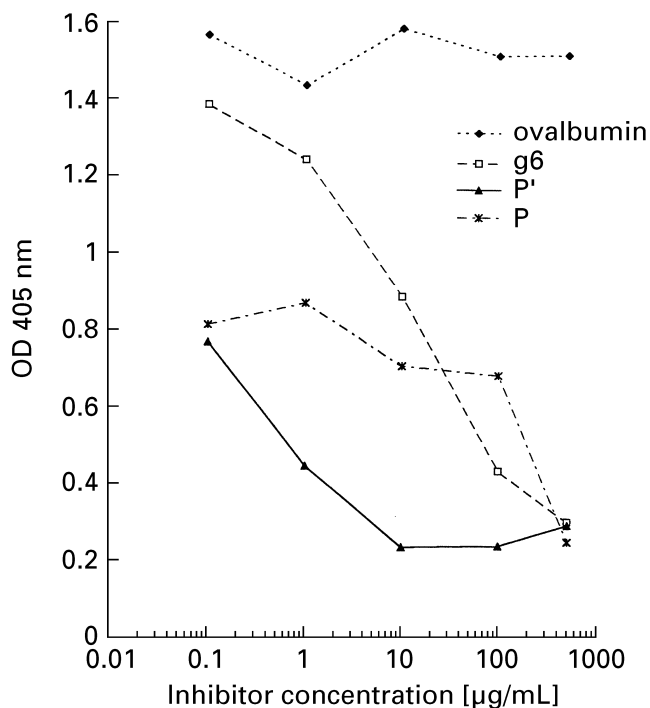


Fig. 2. ELISA inhibition assay demonstrating the enhanced IgE reactivity of patient serum F 11 due to hydroxylated proline residues. Serum was preincubated with serial dilutions of inhibitors (ovalbumin, g6 (crude timothy grass pollen extract), dodecapeptide P', and dodecapeptide P). Afterwards the samples were transferred to a microtitreplate which was coated by peptide P'.

of the Phl p 1 reactive individuals. Previously, we [13] were able to show that this carbohydrate moiety bears an α 1,3-fucosylation to the innermost N-acetylglucosamine, a carbohydrate configuration which is quite common and known for its IgE-binding reactivity.

Although we determined the partial formation of intra and intermolecular disulphide bonds in Phl p 1 (data not shown), these modifications showed no effect on the IgE reactivity. This is in contrast to allergens like Lol p 11 [25], where the disulphide bonds form important B-cell epitopes.

The carbohydrate structure and the hydroxylated proline residues contribute to the IgE reactivity of the Phl p 1 N-terminus. However, this region was not predicted as antigenic binding site, neither by the algorithm of Hopp and Woods [26] nor by the calculations according to Jameson and Wolf [18]. Since the reliability of such determinations is only 50–60% [27] and both calculations do not consider variations caused by post-translational modifications. Therefore, the influence of such structures on the allergenicity must be examined.

In conclusion, our studies indicate that single post-translational modifications can alter the IgE reactivity of allergens or just short peptides. This has to be taken into account,

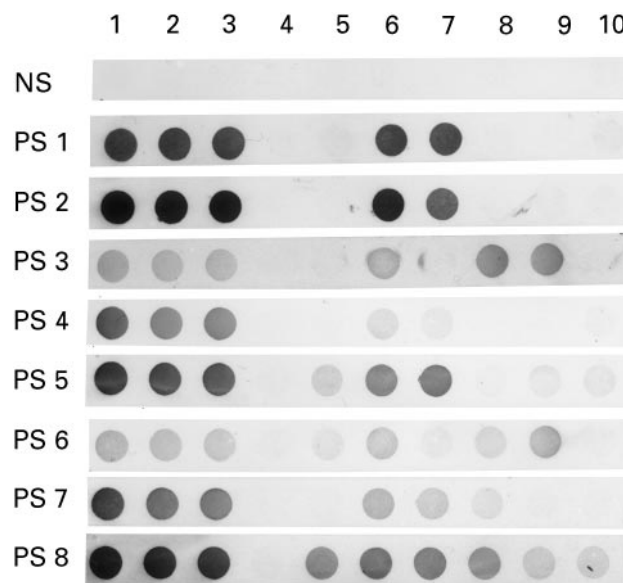


Fig. 3. Screening for patient sera (PS) that recognize post-translationally modified structures in a dot test. Untreated crude timothy pollen extract (1); affinity purified Phl p 1 treated with SDS sample buffer under reducing (2) and non-reducing conditions (3); dodecapeptides P (4) and P' (5); affinity purified nPhl p 1 (6); rPhl p 1 from *E. coli* (7); standard glycoproteins phospholipase A2 (8), horseradish peroxidase (9), and ovalbumin (10). (NS was analysed with a serum from a healthy control.)

when recombinant allergens are used for diagnostics and immunotherapy.

Acknowledgements

The authors would like to thank Dr L. Bock for the automated synthesis of the N-terminal peptides and Dr Brigitte K. Flesch for critical reading of the manuscript. We are also grateful to Mrs Maren Hohn and Mrs Kerstin Ponellis for their excellent technical assistance.

References

- Freidhoff LR, Ehrlich-Kautzky E, John MS. A study of the immune response to *Lolium perenne* (rye) pollen and its components, Lol p I Lol p II (Rye I Rye II) *J Allergy Clin Immunol* 1986; 78:1190–200.
- Scheiner O, Kraft D. Basic and practical aspects of recombinant allergens. *Allergy* 1995; 50:384–91.
- Esch RE, Klapper DG. Identification and localization of allergenic determinants on grass group I antigens using monoclonal antibodies. *J Immunol* 1989; 142:179–84.
- Esch RE, Klapper DG. Isolation and characterization of a major cross-reactive grass group I allergenic determinant. *Mol Immunol* 1989; 26:557–61.

- 5 Petersen A, Bufe A, Schramm G, Schlaak M, Becker WM. Structural investigations of the major allergen Phl p I on the cDNA and protein level. *J Allergy Clin Immunol* 1995; 95:987–94.
- 6 Perez M, Ishioka GY, Walker LE, Chesnut RW. cDNA cloning and immunological characterization of rye grass allergen Lol p I. *J Biol Chem* 1990; 265:16210–5.
- 7 Griffith IJ, Smith PM, Pollock J et al. Cloning and sequencing of Lol p I, the major allergenic protein of ryegrass pollen. *FEBS Lett* 1991; 279:210–5.
- 8 Laffer S, Vrtala S, Duchene M et al. IgE-binding capacity of recombinant timothy grass (*Phleum pratense*) pollen allergens. *J Allergy Clin Immunol* 1994; 94:88–94.
- 9 Schramm G, Bufe A, Petersen A et al. Mapping of IgE binding epitopes on the recombinant major group I allergen of velvet grass pollen, rHol 1.1. *J Allergy Clin Immunol* 1997; 99:781–7.
- 10 Suphioglu C, Singh MB. Cloning, sequencing and expression in *Escherichia coli* of Pha a 1 and four isoforms of Pha a 5, the major allergens of canary grass pollen. *Clin Exp Allergy* 1995; 25:853–65.
- 11 Ball T, Vrtala S, Sperr WR et al. Isolation of an immunodominant IgE hapten from an epitope expression cDNA library. *J Biol Chem* 1994; 269:28323–8.
- 12 Petersen A, Becker WM, Moll H, Blümke M, Schlaak M. Studies on the carbohydrate moieties of the timothy grass pollen allergen Phl p I. *Electrophoresis* 1995; 16:869–75.
- 13 Petersen A, Vieths S, Aulepp H, Schlaak M, Becker WM. Ubiquitous structures responsible for IgE cross-reactivity between tomato fruit and grass pollen allergens. *J Allergy Clin Immunol* 1996; 98:805–15.
- 14 Petersen A, Becker WM, Schlaak M. Characterization of grass group I allergens of timothy grass pollen. *J Allergy Clin Immunol* 1993; 92:789–96.
- 15 Geysen HM, Rodda SJ, Mason TJ, Tribbick G, Schoofs PG. Strategies for epitope analysis using peptide synthesis. *J Immunol Meth* 1987; 102:259–74.
- 16 Elsayed S, Holen E, Haugstad MB. Antigenic and allergenic determinants of ovalbumin. *Scand J Immunol* 1988; 27:587–91.
- 17 Elsayed S, Holen E, Dybendal T. Synthetic allergenic epitopes from the amino-terminal regions of the major allergens of hazel and birch pollen. *Int Arch Allergy Appl Immunol* 1989; 89:410–5.
- 18 Jameson BA, Wolf H. The antigenic index; a novel algorithm for predicting antigenic determinants. *CABIOS* 1988; 4:181–6.
- 19 Demeulemester C, Peltre G, Laurent M, Pauheleux D, David B. Cyanogen bromide-activated nitrocellulose membranes: a new tool for immunoprint techniques. *Electrophoresis* 1987; 8:71–3.
- 20 Leary JJ, Brigati DJ, Ward DC. Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots. *Proc Natl Acad Sci USA* 1983; 80:4045–9.
- 21 van Regenmortel MHV. Structural and functional approaches to the study of protein antigenicity. *Immunol Today* 1989; 10:266–72.
- 22 Becker WM, Schaubschläger W, Westphal W, Schlaak M. Purification of grass pollen allergens by means of immobilized monoclonal antibodies. In: Chmiel H, Hammes WP, Baily JE, eds *Biochemical engineering*. Stuttgart: Gustav Fischer Verlag, 1988:440–2.
- 23 Gershoni JM, Palade GE. Protein blotting: Principles and applications. *Anal Biochem* 1983; 131:1–15.
- 24 Carter JM. Epitope mapping of a protein using the Geysen (PEPSCAN) procedure. In: Dunn BM and Pennington MW, eds *Peptide Analysis Protocols*. Meths Mol Biol, Vol. 36, Totowa, New Jersey: Humana Press, 1994:207–23.
- 25 van Ree R, Hoffman DR, van Dijk W et al. Lol p XI, a new major grass pollen allergen, is a member of a family of soybean trypsin inhibitor related proteins. *J Allergy Clin Immunol* 1995; 95:970–8.
- 26 Hopp TP, Woods KR. Prediction of protein antigenic determinants from amino acid sequences. *Proc Natl Acad Sci USA* 1981; 78:3824–8.
- 27 Pellequer JL, Westhof E, van Regenmortel MHV. Predicting locations of continuous epitopes in proteins from their primary structure. *Meth Enzymol* 1991; 203:176–201.