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Phl p 3: Structural and immunological characterization of a major allergen of timothy grass pollen

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Clinical and
Experimental
Allergy

Summary

Background The relevant importance of individual allergens for allergic sensitization is only partially understood. More detailed information on allergen structure and how it influences immunological responses can lead to better diagnosis of disease and improved preparations for allergen-specific immunotherapy. Grass pollen contains several different allergens, and although the group 3 allergens have been classified long ago, their structure and allergenicity have been poorly investigated.

Objective To characterize Phl p 3 from timothy grass pollen and compare it with Phl p 2 with respect to biochemical structure and allergenicity.

Methods Natural Phl p 2 and Phl p 3 were separated from a pollen extract by chromatography and characterized by 2D electrophoresis and protein sequencing. The complete sequences were determined by DNA cloning and detected in natural pollen extracts by mass spectrometry. Further comparisons of the allergens were made for IgE-binding and cross-reactivity, allergenicity was determined by basophil CD203c activation and skin prick test and 3D structures were compared by molecular modelling.

Results Phl p 3 reveals molecular masses of 10.958 and 10.973 kDa and *pI*s of 8.9 and 9.3, respectively, Phl p 2 a molecular mass of 10.816 kDa and a *pI* of 4.6. The sequence identity is 58%. In spite of these differences in the primary structures, both allergens reveal similar conformational structures, resulting in similar immunological and allergological moieties.

Conclusions The group 3 and group 2 allergens are major allergens with similar 3D structures. Although they differ considerably in their protein sequences and their *pI*s, they show only a slightly higher immunological reactivity for Phl p 3 on the B-cell level (conformational epitopes). But distinct differences between the sequences may influence reactivity at the T cell level.

Keywords DNA cloning, grass pollen allergen, IgE reactivity, mass spectrometry, molecular modelling

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Introduction

The prevalence of allergic airway disease attributable to inhalant allergens such as pollen has been increasing steadily, and a recent population-based survey in Europe shows a prevalence of 23%, with values as high as 29% in some countries [1]. The only potentially curative and

specific treatment of a type I allergy is hyposensitization therapy, which is performed using whole allergen extracts that are standardized in terms of total allergenic activity and possibly one or two individual allergens. The development of better defined therapeutics will be essential in order to improve immunotherapy and to derive beneficial structure–effect relationships. Additionally, a component-

resolved diagnosis [2] covering all important allergenic structures seems to be promising to determine the individual sensitization profiles.

Concerning grass pollen allergy, 11 different so-called 'allergen groups' have been identified [3]. Some allergen groups show partial sequence similarity and IgE-cross reactivity e.g. groups 1–3 and groups 5 and 6 [4–7]. Greater knowledge and understanding of structural and immunological similarities between different allergens and isoforms will contribute to improved diagnosis and better therapeutics. The most important allergenic components can be included, but those that bear only cross-reactive structures may be omitted. However, cross-reactivity has to be given careful consideration as its importance can vary greatly, e.g. shrimp tropomyosin bears a high allergenic potential in contrast to chicken tropomyosin [8]. Homologues of the birch pollen allergen Bet v 1 are distributed in various species of fruit, but the benefit of a birch pollen hyposensitization therapy differs individually [9]. Therefore, structural analysis of the allergens is urgently needed.

Ansari et al. [4, 10] were the first to define the protein sequences of the ryegrass pollen (*Lolium perenne*) allergens Lol p 2 and Lol p 3. As recombinant strategies were not available at that time, they analysed the complete allergens by Edman degradation and determined a sequence identity of 59%. The *pIs* differed considerably with 5.3 for Lol p 2 and 9.4 for Lol p 3 [4, 10]. On the T cell level, Baskar et al. [11] identified one common epitope on Lol p 1, 2 and 3 and a further T cell epitope exclusive to Lol p 3. Besides the HLA-DR3 association found for all three allergens, Lol p 3 additionally corresponded to DR5 and showed a higher immunoreactivity than Lol p 1 [12]. Although these results clearly indicate immunological differences between the three allergens, the relationship has not yet been studied in detail.

In Northern and Central Europe, timothy grass (*Phleum pratense*) pollen is an important allergen source. Screening a cDNA library of timothy pollen using patients' IgE yielded an allergen coding clone designated 'Phl p 2/3' according to sequence similarity. Later, it was clearly defined as Phl p 2 [13]. A Phl p 3-producing clone has not been isolated so far.

We have now developed an efficient isolation procedure to separate the low-molecular mass allergens Phl p 2 and Phl p 3. Based on the different biochemical features, we determined the protein structure of Phl p 3 by DNA cloning and studied the IgE-reactivity and allergenicity of Phl p 3 in comparison with Phl p 2.

Materials and methods

Allergen extracts and patients' sera

Five grams of various grass pollen species (Allergon, Angelholm, Sweden) were incubated in 20 mL of 20 mM

Tris/HCl buffer (pH 8.0). Afterwards, the soluble fraction was isolated by centrifugation ($17\,000 \times g$, 30 min), and the extract was stored at -20°C .

Sera were drawn from grass pollen-allergic individuals with their consent (CAP classes > 3 ; kindly provided by Dr. F. Eberhardt, Medical Clinic, Borstel).

Chromatographic isolation of Phl p 2 and Phl p 3

The first steps of the isolation procedure were performed according to Suck et al. [14]. Briefly, lyophilized pollen extract was reconstituted in starting buffer (1 M $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris/HCl, 1 mM EDTA, pH 8.0) and the solution was separated by hydrophobic interaction chromatography (Phenyl Sepharose High Performance; GE Healthcare, Munich, Germany). The flow-through fraction (Phl p 13, Phl p 1, Phl p 2, Phl p 3 and cytochrome c (Phl p 10)) was separated into three fractions by gel filtration (Superdex 75; GE Healthcare). The low molecular allergens Phl p 2, Phl p 3 and Phl p 10 comprised one peak, which makes about 1–2% of the crude pollen extract, and were further separated by cation exchange chromatography (Source 15 S; GE Healthcare) and sodium phosphate (pH 7.0) as running buffer. Elution was conducted by a gradient of 0–1 M NaCl. Phl p 2 was isolated in the effluent, while Phl p 3 and Phl p 10 were eluted and differentiated according to absorbance at 420 nm indicating cytochrome c (Phl p 10). Phl p 2 and Phl p 3 share 0.3% and 0.5%, respectively.

Western blotting

SDS-PAGE was performed as described by Laemmli [15] on homogenous gels ($T = 15\%$, $C = 2.6\%$) under reducing conditions. 5 μg protein per cm was loaded onto the gel. After SDS-PAGE, the proteins were subsequently transferred on to a nitrocellulose membrane (0.45 μm pore size, Schleicher & Schüll, Dassel, Germany) by semi-dry blotting for 30 min at 0.8 mA/cm^2 [16]. After blotting, the nitrocellulose membranes were treated with 0.1 M Tris-buffered saline, pH 7.4, containing 0.05% (v/v) Tween 20 (TBST) to block free protein-binding sites. For detection of the IgE-reactive proteins, the membrane was cut into strips and incubated with patients' sera (1 : 20 in TBST). After washing, incubation was performed with the secondary antibody, alkaline phosphatase-conjugated monoclonal anti-human IgE (1 : 2000) (Allergopharma, Reinbek, Germany). Binding patterns were visualized by substrate solution containing nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate, potassium salt (BCIP) in 0.1 M Tris-buffered saline, pH 9.5 [17].

2D-PAGE and immunoblotting

2DE was performed using gels as described previously [18]. Briefly, immobilized pH gradient strips in a pH range

of 3–10 ($T = 4\%$, $C = 4\%$) were used for separation in the first dimension (IEF) with 3000 V and 2 mA for 7 h. SDS-PAGE was carried out at 200 V and 30 mA in the second dimension using gradient gels ($T = 5\text{--}20\%$, $C = 4\%$). Molecular masses and pI s were determined by comparison with Multi Mark coloured Standard (4–250.000 Da, Invitrogen, Karlsruhe, Germany) and IEF Marker 3-10, Liquid Mix (Serva, Heidelberg, Germany). Afterwards, the gels were silver stained.

For the identification of allergens, proteins were transferred onto nitrocellulose membranes and immunostained.

Identification of cross-reactive structures

The identification of similar immunological reactivity between allergens was performed according to the absorption method [19] with modifications. The allergens were blotted separately onto nitrocellulose membranes, excised, blocked with TBST and incubated overnight with a patients' pooled serum (three allergic individuals). After washing with TBST to discard unbound serum components, bound antibodies were eluted with 0.1 M glycine-HCl buffer containing 0.5% bovine serum albumin (BSA) (pH 2.5). The solutions were subsequently adjusted to neutral pH and used for immunostaining.

Protein sequencing and mass spectrometry

Samples of chromatographically purified Phl p 2 and Phl p 3 allergens were determined by Edman degradation and by mass spectrometric analyses.

Microsequencing was performed using a Procise protein sequencer with an on-line PTH amino acid analyzer (PE Biosystems, Weiterstadt, Germany).

The molecular masses and biological heterogeneity were determined using a high-resolution Electrospray Ionization Fourier Transform Ion Cyclotron Resonance mass spectrometer (FT-ICR MS; APEX II, Bruker Daltonics, Billerica, MA, USA) equipped with a 7 T actively shielded magnet and an Apollo ion source as described elsewhere [20]. Briefly, the proteins were dissolved in a mixture of methanol, water and acetic acid (50 : 50 : 0.001 (v/v)) and sprayed at a flow rate of 2 μ L/min. The resulting positive ion mass spectra were charge deconvoluted and mass numbers refer to neutral molecules.

Circular dichroism measurements

Samples were adjusted to a concentration of 50 μ mol/L. Far UV circular dichroism spectra (190–260 nm) were recorded in a 1 mm quartz cuvette on a Jasco J-715 A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) equipped with a temperature controller. The samples were heated by raising the temperature linearly at a

rate of 30 $^{\circ}$ C/h to 100 $^{\circ}$ C and also recorded after cooling down to 20 $^{\circ}$ C.

Cloning of Phl p 2 and Phl p 3

DNA was prepared from timothy grass spikes using the Plant DNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was used as a template to amplify PCR products using the following primers:

pPhl p 2s: 5'-GTCCCGAAGGTGACGTT-3';
pPhl p 2as: 5'-CTACTCTTCTGGCGCGT-3';
pPhl p 3s: 5'-GCSGTGMAGGTGACSTT-3';
pPhl p 3as1: 5'-CTACTCTCTGGGGTGT-3';
pPhl p 3as2: 5'-CYAGTTGACTCTGGGGTG-3'.

The following PCR program was run: 95 $^{\circ}$ C for 2 min, 30 cycles consisting of a denaturation step at 95 $^{\circ}$ C for 30 s, an annealing step 52 $^{\circ}$ C for 30 s, and an elongation step at 72 $^{\circ}$ C for 2 min.

For amplification of Phl p 2, the specific primers pPhl p 2s and pPhl p 2as were used (according to the Phl p 2-DNA [13]). To amplify Phl p 3, we combined the degenerated pPhl p 3s with pPhl p 3as1 (constructed according to the Dac g 3-DNA [21]), Phl p 3as2 (deduced according to the Lol p 3 protein sequence [4]) and pPhl p 2as, respectively. The PCR products were ligated by TA cloning into pGEM-T (Promega, Madison, WI, USA). The vectors were transformed into *Escherichia coli* XL-1 Blue (Stratagene, Heidelberg, Germany) by electroporation, and plasmids from positive clones were subjected to sequence analysis.

CD203c basophil activation assay

Aliquots of 100 μ L heparinized whole blood of grass pollen-allergic patients were stimulated with the purified allergens in 10-fold serial dilutions starting with 10 nm/L. Non-stimulated cells and stimulation with anti-IgE (Allergopharma) served as controls. Samples were incubated for 20 min at 37 $^{\circ}$ C. Afterwards, 5 μ L phycoerythrin-conjugated anti-CD203c (Immunotech, Marseille, France) was added to each sample and incubated for 20 min at 25 $^{\circ}$ C. To lyse the red blood cells, 2 mL of Quicklysis (Medac, Hamburg, Germany) was added, mixed and incubated for a further 10 min. After centrifugation at 640 g for 6 min, the cells were resuspended in 400 μ L PBS and measurements were performed on a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA). For the CD203c activation assay, at least 100 000 events from leucocytes were collected containing 600–1200 basophils (owing to the forward/side scatter pattern and the expression of CD203c). In a previous test, it was confirmed that non-allergic donors showed no basophil activation (< 200 basophils under the experimental conditions).

Skin prick test

Skin prick test (SPT) was performed following Guidelines for clinical diagnosis of allergic diseases [22]. Purified natural allergens were dissolved in physiologic saline and applied starting with 50 µg/mL in a standard SPT. 1.7 mg/mL histamine-dihydrochloride and 5000 BU timothy grass pollen extract (Allergopharma) served as controls. Wheal areas and flaring were measured and compared by calculation of surface in diameter.

Protein modelling

Model building, refinement and analysis were performed using SWISS-MODEL and the program Deep View. The atomic coordinates of the pollen allergen Phl p 2 (PDB code 1BMW) were used to build the three-dimensional model of Phl p 3. The programs TURBO-FRODO (Bio-Graphics, Marseille, France) and LSQKAB [23] were used to draw the Ramachandran plot and to perform the superposition of the models. PROCHECK [24] was used to assess the geometric quality of the model.

Results

Isolation and biochemical characterization of Phl p 3 and Phl p 2 from timothy pollen extract

The allergens Phl p 3 and Phl p 2 were isolated from crude timothy pollen extract by the sequential fractionation steps hydrophobic interaction chromatography, gel filtration of the unbound hydrophilic fraction and cation exchange chromatography of the low-molecular mass components. The last chromatographic step resulted in three peaks: one effluent fraction (Phl p 2) and two fractions eluting at different saline concentrations (Phl p 3 and Phl p 10). Phl p 10, which is a cytochrome c molecule, could be detected and identified by its absorption at 410 nm. (None of the available sera from grass pollen-allergic subjects showed IgE antibody reactivity and therefore the molecule was not studied further.) The purity of the allergen preparations was investigated by 2D PAGE and by protein sequencing.

As shown in Fig. 1, the silver-stained gels demonstrate spots of about 11 kDa as estimated by the marker proteins. Phl p 3 (Fig. 1a) reveals one diffuse spot in the alkaline region spanning *pI* 7.8–9.0. Investigation of a narrower pH range in the alkaline region did not achieve a higher resolution of single spots (data not shown). Phl p 2 (Fig. 1b) shows at least five protein spots in the acidic region of *pI* 4.8–5.9. All detected protein spots were allergenic isoforms, as they bound IgE antibodies on the blotted samples (data not shown).

Further information on the fractions was obtained by Edman degradation. We determined 25 N-terminal amino

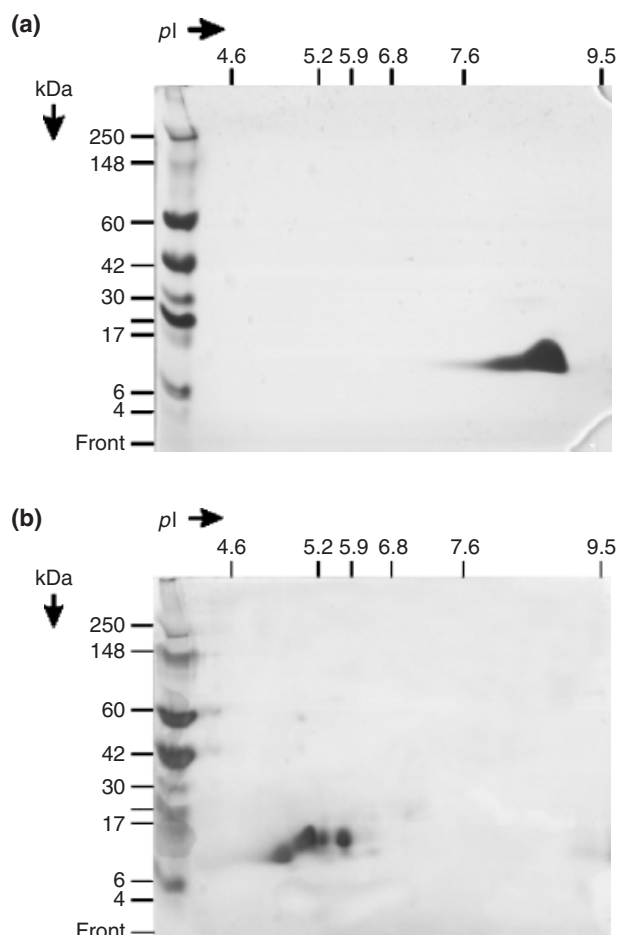


Fig. 1. 2D PAGE for the identification of natural Phl p 3 (a) and Phl p 2 (b) isolated from timothy grass pollen extract. Detection was performed by silver staining.

acid residues (Table 1). The Phl p 3 N-terminal revealed an 84% and 40% sequence identity with Dac g 3 [21] and Lol p 3 [4], respectively. The protein sequence of the Phl p 2 sample was the same as published by Dolecek et al. [13].

Cloning of Phl p 3 and identification of the isoforms in the natural extracts

The complete Phl p 3 sequence was determined with PCR using genomic DNA as a template. A 5'-degenerated primer was constructed from the N-terminal protein sequence. Because of the lack of C-terminal sequence information, we designed three different antisense primers deduced from the Lol p 3 protein sequence as degenerated primer and specific primers from pPhl p 2 and pDac g 3 DNA data, respectively. The three PCR attempts resulted in different products. Using the pLol p 3 as-primer we obtained no PCR product. Using the pPhl p 2 and pDac g 3-based primers, we obtained products of 400 and 300 bp, respectively (Fig. 2a). The pPhl p 2as-primer bound ~100 bp downstream in the non-coding region (3'). In

Table 1. Physicochemical characteristics of Phl p 3 and Phl p 2

Allergen	2D PAGE		Protein sequencing	Calculated		ESI
	MW	pI	N-terminal	MW	pI	MW
Phl p 3	11	7.8–9.0	AVQVTFTVQKGS DPKKLVLDIKYTR	10957.7 10972.7	8.9 9.3	10972.9 10957.9
Phl p 2	11	4.8–6.0	VPKVTFTVEKGSNEKHLAVLVKYE G	10815.3	4.6	10815.0

ESI, Electrospray Ionization.

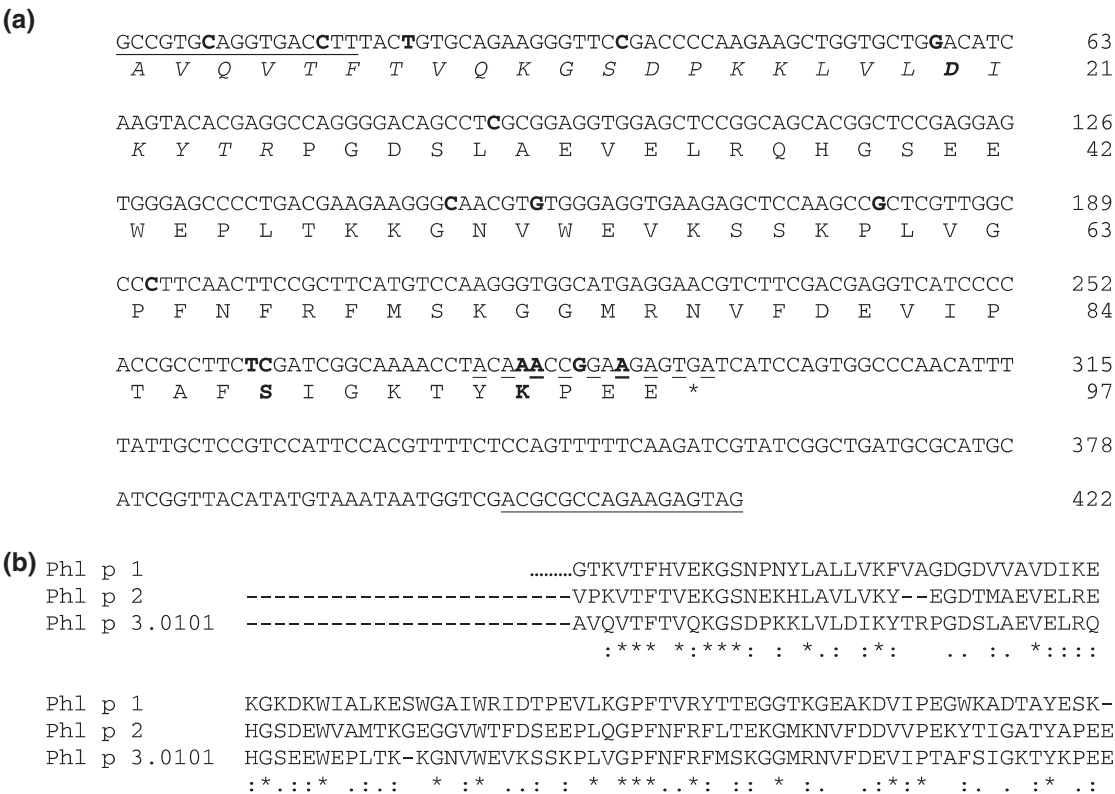


Fig. 2. (a) Nucleotide and deduced amino acid sequence of the clone pPhlp3.0101 isolated from genomic timothy grass pollen DNA. Binding regions of the primers are underlined. Structural differences to the clone pPHLP3.0102 are indicated in bold letters (D20N, S88K, K94T). The N-terminus determined by protein sequencing is printed in italics. (b) Sequence alignment from Phl p 1 [25], Phl p 2 [13] and Phl p 3.0101 performed by CLUSTAL W. Positions of identical and similar amino acids are marked.

the coding region, the DNA sequences differed by 14 nucleotides (Fig. 2a). The deduced protein sequences coded for two isoforms (Phl p 3.0101 and Phl p 3.0102) consisting of 97 amino acids that differed by three amino acid residues (positions D20N, S88K, K94T). The protein sequences contained no cysteine residues and no N-glycosylation motifs. Owing to the binding at the 3' end of the coding region, the PCR product obtained using the pDac g 3as-primer showed four nucleotide differences and an amino acid exchange at position 94. Therefore, the isoform designated Phl p 3.0101 was submitted to the NCBI Database (accession number AY421969).

For structural comparison, we aligned the protein sequences of Phl p 3, Phl p 2 and Phl p 1 (Fig. 2b). Phl p 2 and Phl p 3.0101 were 57% identical and 77% similar. Additionally, both allergens showed sequence similarities to the C-terminal part of Phl p 1 with a Phl p 2:Phl p 1 identity of 41% (similarity 57%) and a Phl p 3:Phl p 1 identity of 29% (similarity 52%). The chromatographically isolated fractions were analyzed by high-resolution ESI FT-ICR MS to identify the corresponding allergens and isoforms in the pollen extract. Figure 3a demonstrates the charge deconvoluted spectrum of Phl p 3 showing principal peaks at *m/z* 10972.9 Da and 10957.7 Da that are in excellent

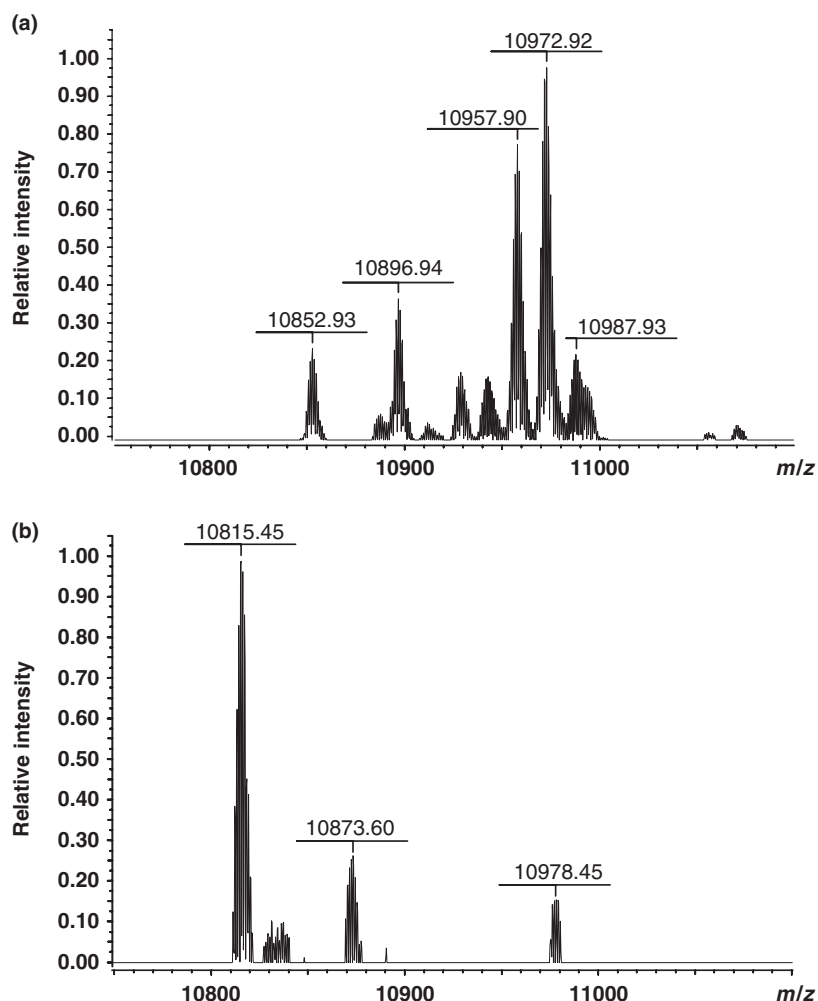


Fig. 3. High-resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectra (ESI FT-ICR MS) of natural Phl p 3 (a) and Phl p 2 (b). Mass numbers given refer to the highest isotopic peak.

agreement (ppm level) with the calculated masses predicted from the DNA sequences of the two clones (Table 1). Phl p 2 (Fig. 3b) showed a major peak at 10 815.5 Da, which agrees with the calculated mass of the recombinant form. The identical molecular masses indicate that the molecules exist as main constituents in the natural allergen extract and that they are not post-translationally modified. These results were further confirmed by tryptic peptide mass finger printing, where the identified masses covered all the masses of the calculated fragments (data not shown).

Immunoreactivity and allergenicity of Phl p 3, Phl p 2 and Phl p 1

To determine the immunological importance of the allergens, we studied the IgE prevalence to Phl p 3, Phl p 2 and Phl p 1. Seventy sera from grass pollen-allergic individuals (CAP score > 3 to timothy grass pollen and IgE reactivity to crude timothy pollen extract in Western blot)

were screened by Western blotting. Forty sera (57%) bound to all three allergens, while 28 (40%) bound only to Phl p 1. (Two of the 70 sera did not bind to Phl p 1, Phl p 2 or Phl p 3, but reacted with Phl p 5 and/or Phl p 13.) These results demonstrate that Phl p 1, Phl p 2 and Phl p 3 are major allergens and that, interestingly, no IgE antibodies react exclusively with Phl p 2 or Phl p 3. Sera of three healthy non-pollen-allergic persons served as negative controls.

To determine whether the IgE-reactivity is caused by independent or cross-reactive epitopes, a patient's pooled serum was affinity-purified by absorption to Phl p 1, Phl p 2 and Phl p 3, respectively. The eluted antibodies were used later for Western blotting (Fig. 4a).

The Phl p 1-affinity-purified IgE antibodies bound Phl p 1 exclusively. The Phl p 2 and Phl p 3-purified IgE antibodies showed cross-reactivity between each other, although the binding to the Phl p 3 allergen seemed to be more intense. No cross-reactivity of IgE antibodies was detectable between Phl p 1 and Phl p 2/Phl p 3.

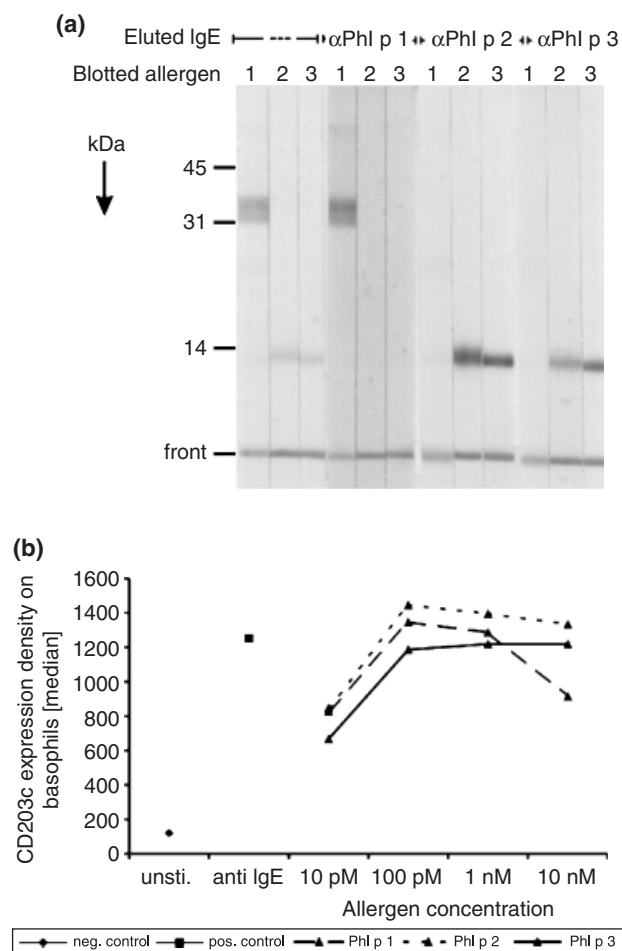


Fig. 4. Comparison of IgE reactivity and allergenicity of Phl p 1, Phl p 2 and Phl p 3. (a) Studies on the IgE cross-reactivity using a patients' pooled serum without pre-absorption (–) or affinity-purified by absorption to immobilized Phl p 1, Phl p 2 or Phl p 3. The bound antibodies were eluted and studied for their binding to the pollen extract and to the isolated allergens Phl p 1–Phl p 3 (1–3). (b) Determination of basophil activation by up-regulation of CD203c. Whole blood of a grass pollen allergic donor was incubated with serial dilutions of the purified natural allergens Phl p 1, Phl p 2 and Phl p 3 and determined for CD203c-expression by FACS analysis. Anti-IgE and an unstimulated sample served as controls.

The allergenicity of the three allergens was investigated by the CD203c activation assay. Whole blood from three allergic donors (who had already been studied by the screening) was incubated with serial dilutions of the purified allergens.

Figure 4b shows a representative experiment. Phl p 1, Phl p 2 and Phl p 3 caused comparable strong activations of CD203c on basophils.

The allergenicity of the pollen allergens was further studied by SPT. Compared with a healthy control, the three grass pollen-allergic patients showed a strong wheal and flare reaction (7 mm in diameter) at concentrations of 10^{-6} – 10^{-8} M with the particular purified allergens Phl p

1, Phl p 2 and Phl p 3. No significant difference was observed in the intensity of the skin reaction.

Modelling the 3D structure of Phl p 3

Because of the discrepancy in the sequence data between Phl p 3 and Phl p 2, on the one hand, and the similarity in immunoreactivity, on the other, we determined the structural details. Therefore, we performed CD spectroscopy of both allergens.

Figure 5 shows the molar ellipticity of both purified fractions of Phl p 3 and Phl p 2 in the range between 200 and 250 nm. The spectra are quite similar, with a maximum at 205 nm and a minimum at about 210 nm, but Phl p 2 has a smaller positive and negative peak ellipticity than Phl p 3. This can be interpreted as a slightly higher degree of randomly orientated secondary structure for Phl p 2.

Using the crystal structure of Phl p 2 [26] as a template, we aligned the Phl p 3 sequence in order to gain an impression of the 3D structure. As depicted in Fig. 6, Phl p 3 fits well with the Phl p 2 molecule. The predicted structure shows the typical all β -fold with eight β -strands packed into a sandwich of two anti-parallel β -sheets.

After superimposing the two structures, the average rms (root mean square) deviation was 0.47 Å. The largest deviations (approx. 3–3.5 Å) were located in the short loops between the β -strands—amino acid residues 23–28, 48–51 and 72–73. Thus, the global protein structures appear similar. We performed epitope mapping of Phl p 3 and Phl p 2 with overlapping hexadecamers and an increment of amino acids, but were not able to identify any IgE-reactive epitopes (data not shown). Thus, the differences in the short loop regions did not cause sequential epitopes, but may contribute to one or more conformational IgE-binding epitopes.

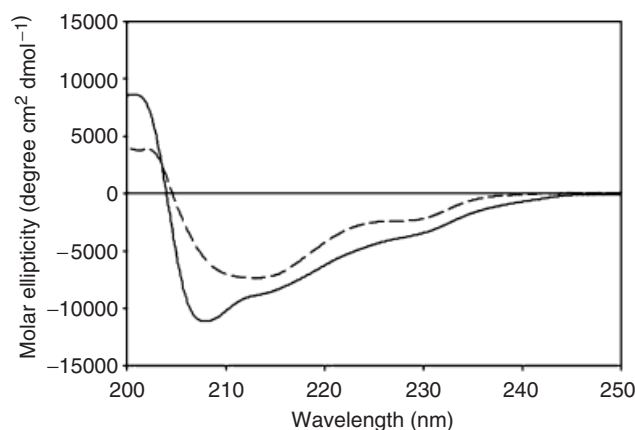


Fig. 5. Far-UV spectra of natural Phl p 3 (broken line) and Phl p 2 allergen (solid line). The spectra were recorded in 10 mmol NaH₂PO₄/Na₂HPO₄, pH 8.1, at T = 30 °C with a protein concentration of 50 μ mol.

Discussion

The aim of this study was to investigate the group 3 allergen of timothy grass pollen and to determine its biochemical structure and its allergenic importance. Our separation strategy for the low-molecular mass allergens proved to be very effective and yielded pure Phl p 3 and Phl p 2 allergens for further structural and immunological comparison. The major allergen Phl p 3 could be completely separated from cytochrome c. In contrast to the studies of Ekramodoullah et al. [27], who investigated the corresponding component in ryegrass and termed it grass group 10, but in agreement with the findings of Ansari et al. [28], IgE reactivity could not be confirmed with our serum collective. While Phl p 2 has already been characterized in detail [13], we concentrated on Phl p 3. This major allergen was cloned and two isoforms are now available as recombinant allergens and as standards.

Phl p 2 and Phl p 3 reveal a sequence identity of 57% and show identities of 41% and 29% to the C-terminal half of Phl p 1. Group 1 allergens exist in all grass species, suggesting a profound importance for plant physiology, and this widespread distribution also seems to be true for groups 2 and 3. Identical DNA sequences for group 2 allergens among various grass species from temperate climatic zones (*P. pratense* [13], *Lolium italicum* [AJ13139], *Dactylis glomerata* [AJ13134], *Holcus lanatus* [AJ131336], *Poa pratensis* [AJ131337]) have been reported. We isolated a Phl p 2 clone with the same sequence as published by Dolecek et al. [13]. At least two isoforms have been investigated by biochemical methods and the corresponding N-terminal sequences are available [29]. We now report the identification of two Phl p 3 isoforms, whereas there have been previous reports of group 3 allergens in a few temperate grasses (*L. perenne* [A33422], *D. glomerata* [AAB42200], *Triticum aestivum* [Z50867]) and recently also in the tropical grass *Zea mays* [AY331720]. In contrast to group 2, particular allergens of group 3 reveal a considerable sequence variation. For example, whereas Phl p 3 and Dac g 3 show 90% identity, and Phl p 3 and Lol p 3 78%, there is only 35% identity between Phl p 3 and Zea m 3.

The sequence homology of the three allergen groups suggests a common origin. Phl p 1, Phl p 2 and Phl p 3 may have been derived from the same precursor molecule, e.g. Phl p 2/3 may have been generated by partial gene duplication of the C-terminal part of the group 1 molecule. Phl p 2 and Phl p 3 most probably segregated later, as their structural homology is much higher. The three molecules subsequently evolved separately, leading to isoform variations by point mutation. The major pollen allergens Phl p 5 and Phl p 6 also appear to have a common origin [30].

Cosgrove [31] assumed a cellulose-binding activity as the physiologic function of group 1 (C-terminal part), 2

and 3 allergens, as the protein sequences show some similarity with family-45 endoglucanases. In this case, it would appear that group 1 and 2/3 allergens are able to render the enzymatic activity over a wide pH range, as Phl p 2 has an acidic, Phl p 1 a neutral and Phl p 3 an alkaline pI. By homology modelling of Lol p 1 and Phl p 2 (and some other expansins), Barre and Rougé [32] predicted similar secondary and conformational structures which – according to our studies (Fig. 6) – are probably also true for the Phl p 3 molecule.

The group 2 and 3 allergens show different immune responses at the T cell level [11] and thus should also reveal differences in sequential B cell epitopes. But we did not identify any IgE-reactive epitope by epitope mapping of Phl p 3 and Phl p 2 with overlapping peptides. This is consistent with the data of Dolecek et al. [13], who also could not identify a sequential IgE-binding epitope on Phl p 2. The IgE-binding epitopes in pollen allergens are mainly of conformational nature. Vrtala et al. [9], for example, showed that a cleavage of Bet v 1 into two fragments of similar size completely destroyed the IgE reactivity of the birch pollen allergen. We assume that probably the loop regions that show the greatest differences in the 3D structures of Phl p 2 and Phl p 3 might cause the different affinities. As affinity-purified anti-Phl p 2 antiserum bound stronger to Phl p 3 than to Phl p 2, the Phl p 3 allergen is probably immunodominant.

Even though the sequence data for Phl p 2 and Phl p 3 isoforms gave no indications for post-translational modifications as the calculated masses exactly matched their natural counterparts in the purified samples, we performed further studies with the natural isolated allergens in order to detect probable differences in the protein folding structure. According to our Western blotting experiments with the affinity-selected antibody fractions (Fig. 4a), we observed no difference between Phl p 2 and Phl p 3, except for a slightly stronger binding to Phl p 3. However, basophil activation assay and SPT did not reveal significant differences either, which does not contradict a cross-reactivity between the allergens.

However, it is not clear whether or not allergens with extensive similarities might have different potentials to induce allergic sensitization. During sensitization,

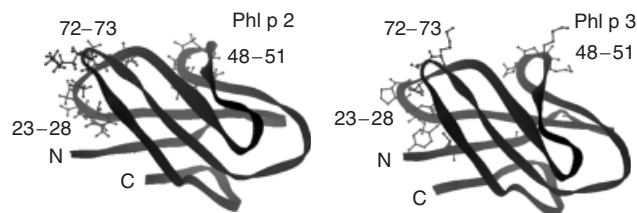


Fig. 6. Predicted Phl p 3 structure as modelled on the basis of the crystal structure of Phl p 2 (1WHO 6041; 1WHP 6040, 1997; 1BMW 9425, 1998). The diagram shows similarities in the eight β -strands, but differences in the three loops.

allergens are processed by proteolytic digestion in antigen presenting cells and the resulting peptide fragments are presented to T cells. The processing as well as the presentation might be variable with respect to pH changes and sites of proteolytic cleavage, thus having an impact on the stimulatory effect. On the basis of inhibition studies using human sera reactive to Lol p 1, 2 and 3, Ansari *et al.* [28] demonstrated substantial cross-reactivity between Lol p 2 and Lol p 3. We have confirmed these results in the case of Phl p 2 and Phl p 3; in terms of similar IgE-prevalence, basophil activation and SPT results were obtained. Furthermore, our immunoblotting studies with the affinity-purified *Phleum* allergens also show that there is no substantial cross-reactivity between Phl p 1 and either Phl p 2 or Phl p 3, again echoing the findings of Ansari *et al.* [28] with *Lolium* allergens.

Despite the extensive similarities between Phl p 2 and Phl p 3, there are clear differences that determine their relative immunological reactivities. The cloning and sequencing of Phl p 3 will provide a basis for obtaining a deeper understanding of the relationship between structure, sensitizing potential and immunological cross-reactivity. The benefits of allergen-specific immunotherapy can differ substantially from patient to patient and a better understanding of individual allergen structures should help to decide which molecules are the most immunologically important for the success of treatment.

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