

Epitope analysis of isoforms of the major allergen *Phl p V* by fingerprinting and microsequencing

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Summary

The major allergen of timothy grass pollen (*Phleum pratense*), designated as *Phl p V*, consists of isoallergenic components of 38 and 32 kDa with pI values of 5.2–7.5 and 4.8–5.9, respectively. The different-sized proteins reveal similarities in IgE reactivity, N-terminal sequence and protein staining. For epitope analysis of these allergens a combination of enzymatic cleavage of electrophoretically separated proteins and immunoblotting techniques with subsequent N-terminal sequencing was performed. After isolation of the components from two-dimensional PAGE gels, proteins were enzymatically cleaved and separated by SDS-PAGE. By endoproteinase Glu-C cleavage six IgE-reactive fragments of each 32 kDa protein and three of each 38 kDa allergen were obtained. Microsequencing of the fragments revealed internal sequences that did not show any similarities between the different-sized allergens. Therefore, we assume only slight structural variations among allergens of similar sizes, whereas the 32 and 38 kDa proteins reveal great differences.

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Introduction

Allergic reactions of the immediate type, such as hay fever, occur shortly after contact of a specific allergen with its corresponding IgE antibodies which are bound to mast cells. Clinical symptoms are mainly caused by release of several mast cell mediators. Thus the IgE-binding epitopes of the allergens play an important role and must be elucidated for the understanding of this mechanism and to improve the diagnosis and therapy of allergic disease.

Major allergenic components in grass pollen belong to the grass groups I and V. The group I allergens have already been intensively studied, and these allergens have partial sequence homology with the allergens of grass groups II and III [1–4]. Additionally, groups II and III are crossreactive [5]. No immunochemical relationship was identified with grass group V, which was first identified in timothy grass pollen [6]. It is suggested that this allergen group is responsible for 90% of the allergenic activity found in timothy pollen-sensitized patients [7]. Matthiesen and Löwenstein [8] demonstrated the IgE prevalence of group V allergens in a wide variety of grass species

by cross-radioimmuno-electrophoresis. After affinity chromatography of timothy grass pollen by copper-chelate they [9] identified two IgE-reactive components of 33 and 29 kDa and termed them *Phl p Va* and *Phl p Vb*, respectively. Moreover, they determined a pI value of 4.7 by isoelectric focusing. Silvanovich *et al.* [10] investigated three cDNA clones from Kentucky Bluegrass pollen (*Poa pratensis*), a species closely related to timothy grass, that coded for major allergens of 28–38 kDa with pI values of 9.6–10.2. They classified these basic components into the new allergen grass group IX.

We [11] previously reported major allergens of 38 and 32 kDa in timothy grass pollen that showed similar reactivities and N-terminal sequences to the allergens *Phl p Va* and *Phl p Vb* published by Matthiesen and Löwenstein [9]. In order to analyse these IgE-reactive components, we performed enzymatic cleavage of the 38 and 32 kDa proteins with subsequent immunological characterization and internal microsequencing of the fragments.

Materials and methods

Preparation of timothy pollen extract

Timothy pollen (1g) (ARTU Biologicals, Lelystad, The Netherlands) was suspended in 55 ml of 0.1 mol/l

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NH₄HCO₃ buffer (pH 8.0) and incubated under agitation at room temperature for 30 min. After centrifugation for 30 min at $17\,000 \times g$ (4°C) the supernatant was dialysed against double-distilled water. After lyophilization the extract was stored at 4°C until use.

Patient sera and monoclonal antibodies

For the identification of IgE-reactive proteins we used a mixture of sera from three grass pollen-sensitive patients [Pharmacia CAP System, CAP score VI (radio allergosorbent test for the quantification of specific IgE, scores according to manufacturer's manual; Pharmacia Diagnostics AB, Uppsala, Sweden)]. The monoclonal antibodies Bo 1, BG 6 and Bo 9 were raised against crude timothy pollen extract. The monoclonal antibody 'grass 4B1' [12] was kindly provided by R. van Ree (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).

Two-dimensional PAGE

Two-dimensional PAGE was performed to Görg *et al.* [13]. In the first dimension IEF was performed on gel strips (T=4%, C=4%; size $240 \times 8 \times 0.5$ mm³) containing an immobilized pH gradient from 3.6 to 9.3. Timothy pollen extract was dissolved in double-distilled water, diluted to a concentration of 10 µg/µl and 100 µg applied per lane. The separation was performed at 3000 V and 2 mA for 7 h. For the second dimension, SDS-PAGE gels (size $240 \times 8 \times 0.5$ mm³) were prepared with a stacking gel (T=5%, C=4%) and a separating gel (T=8–12%, C=4%). After equilibration for 15 min in a buffer containing 0.5 mol/l Tris, 6 mol/l urea, 30% (v/v) glycerol, 2% (w/v) SDS and bromphenol blue as marker, the gel strips were alkylated for another 15 min in the same buffer, which also contained 260 mmol/l iodoacetamide. The separation by SDS-PAGE was carried out at 200 V and 30 mA in the stacking gel and at 800 V in the separating gel. After the two-dimensional PAGE the gel was stained by Coomassie R-250 (Serva, Heidelberg, Germany) or the proteins were immediately blotted onto a transfer membrane. The determination of pI and molecular weight was achieved by the use of marker protein test mix 9 (Serva, Heidelberg, Germany) and low range SDS-PAGE standards (Biorad, Richmond, USA), respectively.

Peptide SDS-PAGE

The protein fragmentation was done according to the procedure of Cleveland *et al.* [14] using a fingerprinting kit (Promega, Heidelberg, Germany). Protein spots under

investigation were excised from two-dimensional PAGE gels and washed twice in double-distilled water for 30 min. Gel pieces containing 2.5 µg protein per cm (roughly estimated) were applied to a vertical SDS gel (stacking gel T=5%, C=4%; running gel T=7.5–20%, C=4%) and overlaid with solutions of different endoproteases (Glu-C: protein=1:20; Lys-C: protein=1:20; alkaline protease: protein=1:1000). The run was performed at 100 V. Just before the dye-front reached the running gel, electrophoresis was stopped for 30 min. After this the separation was completed at 200 V followed by electroblotting.

Electroblotting

For the transfer of the proteins from the SDS gels to blotting membranes we utilized the semi-dry blotting technique [15]. The proteins were transferred onto nitrocellulose membrane (Schleicher & Schüll, Kassel, Germany) for immunological detections or onto ProBlott membrane (Applied Biosystems, Weiterstadt, Germany) for subsequent amino acid sequencing. Blotting was done at 0.8 mA/cm² for 30 min using 10 mmol/l CAPS (pH 11.0) [16].

Protein staining and immunological detections

After blotting, the nitrocellulose membrane was stained by 0.1% (v/v) India ink (Pelikan AG, Hannover, Germany) [17].

For the detection of allergens, the nitrocellulose membrane was incubated in pooled patients' sera or in supernatants of monoclonal antibodies. The IgE reactivity was identified by mouse-anti-human IgE (Medac, Hamburg, Germany). Subsequently alkaline phosphatase-conjugated goat-anti-mouse IgG and IgM (Dianova, Hamburg, Germany) was added. 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 mol/l Tris-buffered saline (pH 9.5) [18].

Amino acid sequence analysis

The ProBlott membrane was washed in double-distilled water after blotting for 30 min. Afterwards the membrane was stained by 0.1% (w/v) Coomassie R-250 (Serva, Heidelberg, Germany) in 50% methanol for 10 min, destained in 50% methanol and air-dried.

Protein spots containing the presumed allergen were cut out. Microsequencing was performed by Edman degradation using a 473 A protein sequencer with on-line PTH amino acid analyser (Applied Biosystems, Weiterstadt, Germany).

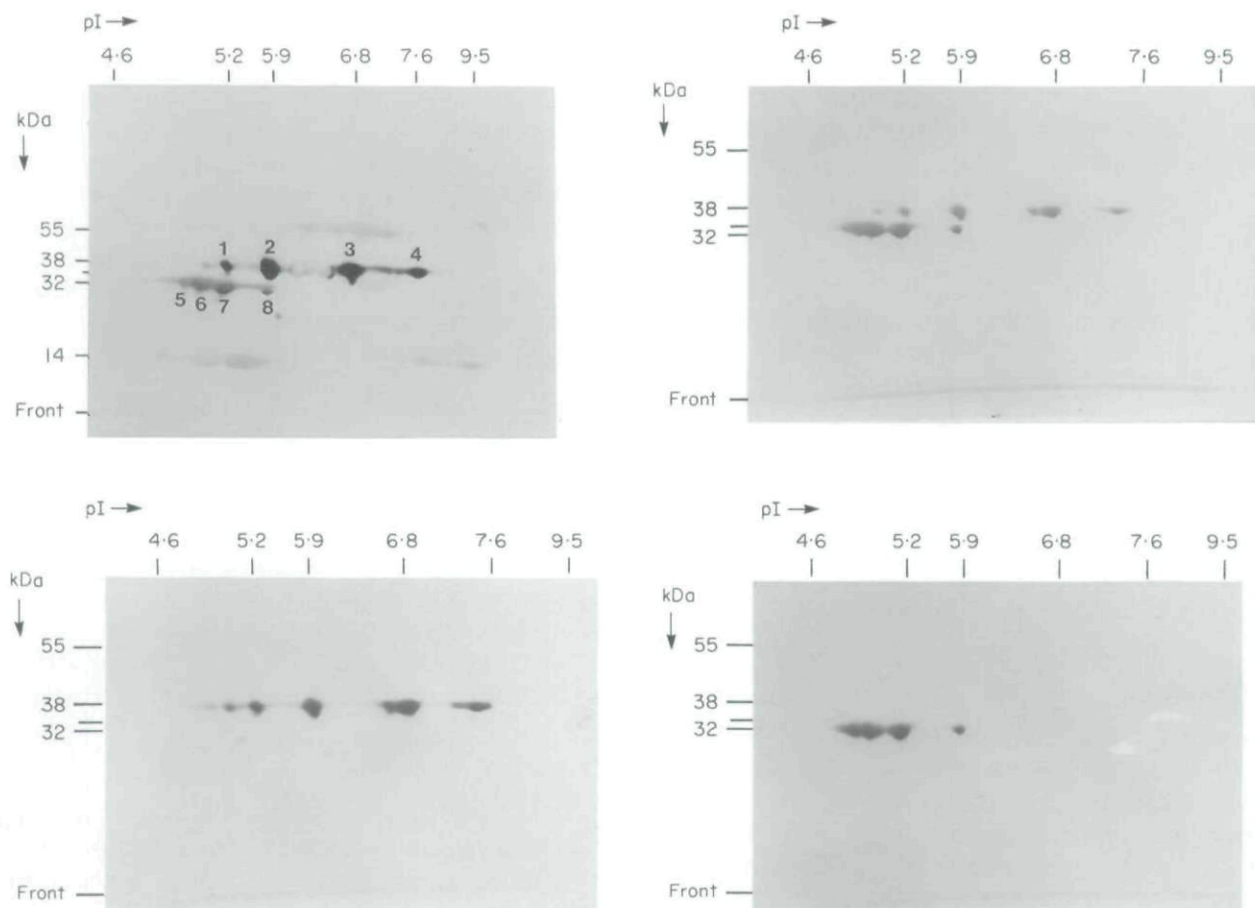


Fig. 1. Two-dimensional immunoblotting of timothy pollen extract incubated with patients' pool serum (a) and group V specific supernatants of monoclonal antibodies (Bo 1 (b), BG 6 (c) and Bo 9 (d)).

Results

Detection and differentiation of group V allergens

The separation and identification of grass group V allergens (*Phl p V*) from crude timothy pollen extract was performed by two-dimensional PAGE followed by immunoblotting with pooled serum from grass pollen-sensitized patients and with monoclonal antibodies.

Figure 1a demonstrates the IgE-reactive proteins in timothy pollen. There are eight allergens showing intense reactivity: four spots of 38 kDa with pI ranging from 5.2 to 7.5 and four of 32 kDa with pI 4.8–5.9. Each of the different-sized proteins consists of four components with different pI. The eight proteins were also identified by the monoclonal antibody Bo 1 (Fig. 1b) and by the allergen grass group V-specific monoclonal antibody grass 4B1 (not shown) indicating group V a as 38 kDa and group V b as 32 kDa. The monoclonal antibody BG 6 (Fig. 1c) only identifies the 38 kDa components while the antibody

Bo 9 (Fig. 1d) reacts with the 32 kDa proteins. Therefore, both proteins reveal at least one identical epitope (Bo 1) and two different epitopes (BG 6 and Bo 9).

Comparing the binding patterns of the monoclonal antibodies with those of patients' IgE (Fig. 1a) it is clear that all of the eight proteins (32 and 38 kDa) are allergens.

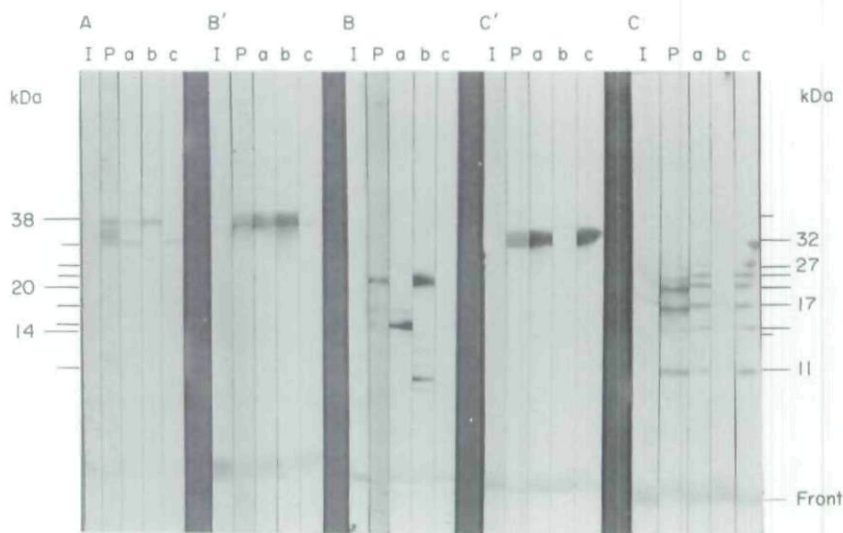
Fingerprinting of the 38 and 32 kDa isoallergens

In order to discover whether the allergens of the same molecular weights show identical structures and to elucidate the differences between the 32 and 38 kDa allergens, we performed fingerprinting of the eight allergens of group V. Up to 400 µg of crude pollen extract were separated by two-dimensional PAGE and the gel was stained with Coomassie blue.

Although there were many proteins detectable with molecular weights of 20–40 kDa, the eight proteins belonging to *Phl p V* were detectable by intense spots with

Fig. 2. Demonstration of the IgE-reactive fragments after Glu-C digestion and peptide SDS-PAGE blot.

Untreated crude timothy pollen extract (A), 38 kDa allergens untreated (B') and digested by Glu-C (B), 32 kDa allergens untreated (C') and digested by Glu-C (C). I. Protein staining by India ink. P. Patients' IgE. a. Monoclonal antibody Bo 1. b. Monoclonal antibody BG 6. c. Monoclonal antibody Bo 9.



a light blue stain in contrast to other proteins. Therefore, a reliable identification of the group V allergens was obtained, although the protein spots showed different sizes.

The excised proteins 1–8 were treated with the endoproteinases Lys-C, alkaline protease and Glu-C after they were loaded onto the peptide SDS gel. After separation the fragments were transferred onto nitrocellulose membrane for the immunological detection of antigenic and allergenic epitopes. IgE-reactive fragments were obtained only after cleavage by Glu-C (Fig. 2). The binding patterns of the patients' IgE as well as the monoclonal antibodies revealed no differences between the allergenic proteins of the same size (data not shown). Digestion with the endoproteinases Lys-C and alkaline protease did not succeed in producing fragments that were IgE-reactive or reactive to the monoclonal antibodies, even after the use of different concentrations of the enzymes.

Figure 2 shows the protein and binding patterns of a 38 kDa protein (spot 2) and a 32 kDa protein (spot 7) without and after treatment with endoproteinase Glu-C. Identical binding patterns were obtained among the 38 kDa isoforms [1–4] and among the 32 kDa isoforms [5–8], respectively (data not shown). After cleavage, the 38 kDa proteins demonstrated three IgE-reactive fragments of 20, 16 and 14 kDa. The 32 kDa proteins exhibited IgE-binding cleavage products of 27, 25, 20, 17, 15 and 11 kDa. The exact separation between the 38 and 32 kDa proteins is demonstrated by immunodetection in Fig. 2: the 38 kDa protein only reacted with the monoclonal antibodies Bo 1 and BG 6, while the 32 kDa component was exclusively identified by the antibodies Bo 1 and Bo 9.

Microsequencing of the IgE-reactive fragments

In order to determine the sequences of the epitopes on the 38 and 32 kDa allergens we performed microsequencing of those IgE-reactive fragments which existed in sufficient amounts. The experiments were repeated with higher concentrations of the 38 and 32 kDa proteins and the pieces containing the desired IgE-reactive fragments were excised from the ProBlott membranes for microsequencing.

Table 1 demonstrates some sequences of the 38 and 32 kDa allergens after enzymatic digestion. The sequence data of the 14 kDa fragment reveal an internal part of the 38 kDa protein, while the 20 kDa allergen shows the same *N*-terminus as determined for the complete 38 kDa protein. After cleavage of the 32 kDa protein, five fragments were detected. The 17 kDa fragment revealed the same sequence as the complete 32 kDa allergen and represented the *N*-terminal part of that protein. The 25, 20 and 15 kDa fragments showed identical sequences to each other as regards their *N*-termini. For the 11 kDa cleavage product we could not determine a definite sequence as it seemed to be a mixture of two different proteins of identical molecular weights.

Discussion

For epitope mapping of the 38 and 32 kDa allergens in timothy grass pollen we performed a fingerprinting technique. This fingerprinting is a generally useful and reliable technique for the comparison of proteins. We obtained fragments of the allergens and compared their immunological reactivities and amino acid sequences.

Table 1. Microsequencing of the IgE-reactive fragments of the 38 and 32 kDa allergens after cleavage with endoproteinase Glu-C. (N-terminal sequences of the original 38 and 32 kDa proteins were already published [11].) P'-hydroxyproline.

Protein/fragment	1	5	10	15	20																
38 kDa	A	D	L	G	Y	A	P'	A	T	P'	A	A	P'	A	A	G	Y	T	P'	A	
20 kDa	A	D	L	G	Y	A	P'	A	T	P'	A	A	P'	A	A	G	Y	T	P'	A	
14 kDa	V	K	V	I	P	A	G	E	L	Q	V	I	E	K	V	D	A	A	F		
32 kDa	A	D	A	G	Y	A	P'	A	T	P'	A	A	A	G	A	E	A	G	K	A	
25 kDa	A	Q	F	T	S	A	P'														
20 kDa	A	Q	F	T	S	A	P'	K	A	Q	T	A	K	A	P	G	L	V	P	K	L
17 kDa	A	D	A	G	Y	A	P'	A	T	P'	A	A	A	G							
15 kDa	A	Q	F	T	S	A	P'	K	A	Q	T	A	K	A	P	G	L	V	P	K	L
11 kDa	V	T	A	V	K	P	V	*	T	K	P	I	M	A	L	I	A	A	G		
	A	Q	F	G	Y	F	D	*	A	L	A	G	P'	L	K	V	P		V		

* No assignment.

Usually, proteins under investigation are eluted from the gel and treated with enzymes followed by reverse-phase HPLC [19]. This chromatographical separation is especially useful for very small peptides as each cleavage product can be detected. In our case we performed the fingerprinting technique with subsequent detection by patients' sera in order to identify fragments containing the undestroyed IgE-reactive epitopes. The immunological detection procedure restricted our investigation to a number of relevant fragments that bear epitopes for human IgE and/or the monoclonal antibodies. For our investigations this method has proved to be more suitable than the chromatographical separation. The 38 and 32 kDa allergens were characterized in previous studies [11,20]. Similarities between the two different-sized molecules were demonstrated with respect to the protein staining, the occurrence of hydroxyproline residues and the absence of a detectable carbohydrate content. Apart from the molecular weights they differ in pI; while the 38 kDa components tend to have more basic iso-electric points (pI 5.2–7.5), the 32 kDa components have acidic isoelectric points (pI 4.8–5.9). Further differences were identified by microsequencing and amino acid analysis, while there were no differences between the four isoallergens of the 38 kDa and the four isoallergens of the 32 kDa. Iso-allergens among grasses were first documented by Johnson and Marsh [2] for two allergens in rye grass. The proteins were identical with respect to immunological reactivity and physical and chemical properties, but revealed two distinct proteins after starch gel electropho-

resis. Perez *et al.* [4] deduced two forms of the major allergen of rye grass *Lol p I* from their cDNA sequence data: they demonstrated four amino acid exchanges between the different isoforms. Such slight variations between proteins might cause detectable differences in pI, but these differences would not be recognized with the fingerprinting method which separated peptides by molecular weight.

After two-dimensional PAGE separation combined with Glu-C digestion and subsequent fragment separation we again did not find any differences in the immunological reactivity between the allergenic components of identical molecular weights. However, we demonstrated clear distinctions in the binding patterns between the 38 and 32 kDa allergens in timothy grass pollen; the Glu-C cleavage of the 38 kDa protein resulted in three IgE-reactive fragments, while the same treatment generated six IgE-reactive proteins from the 32 kDa allergen. This demonstrates that the 38 and 32 kDa allergens show clear differences in structure. The difference in size cannot be simply explained by the existence of a putative 38 kDa precursor protein, which is post-translationally modified by a 32 kDa protein by cleavage. Therefore, the question arises, whether or not the two proteins in spite of some similarities, e.g. identical antigenic epitopes for the antibody Bo 1, bear identical IgE-binding epitopes.

For further characterization of the major allergenic components of 32 and 38 kDa we performed microsequencing of the fragments and obtained internal

sequences of both proteins. The 14 kDa splitting product of the 38 kDa allergen reveals sequence identities to the three precursor proteins of Kentucky bluegrass (*Poa pratensis*) KBG 31, KBG 41 and KBG 60 classified by grass group IX [10,21]. Only two of the 19 amino acids determined are different. These slight differences might be due to phylogenetic variations between the grass species. Concerning the cleavage products of the 32 kDa allergens, we identified three fragments of identical N-terminal sequences (25, 20 and 15 kDa proteins). Using the European and American protein data banks (Swiss-prot and PIR) no sequence identities > 50% to any known protein were found.

The classification of the major allergens at 38 and 32 kDa according to the accepted allergen nomenclature is difficult. As previously demonstrated [11] the 32 and 38 kDa allergens revealed 60% sequence identities concerning the 20 N-terminal amino acid residues and are in good agreement with the N-terminal sequence data for the whole *Phl p V* mixture [9]. Matthiesen *et al.* [22] compared N-terminal sequences of *Phl p V*, *Poa p IX* and *Lol p Ib* and demonstrated a high homology between these proteins. Thus the identity of the 14 kDa fragment of timothy grass pollen and *Poa p IX* is not surprising. Further epitope analysis is necessary to decide whether the difference in pI is an adequate criterion for classifying the allergens into different grass groups. At the least, the allergen grass groups V and IX are very similar. In order to improve allergen classification it is necessary to identify the IgE-binding epitopes of the proteins and to classify the allergens according to them.

For the investigation of the IgE-mediated mechanism and for hyposensitization therapy high amounts of the pure allergens are necessary. We are in the process of cloning and expressing recombinant 38 kDa and 32 kDa allergen components of timothy grass pollen from a cDNA library. The internal sequence is, therefore, very useful, since it is more unique than the N-terminus of the 32 kDa allergen.

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