

Group V allergens in grass pollens. I. Purification and characterization of the group V allergen from *Phleum pratense* pollen, *Phl p V*

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Summary

An allergen from *Phleum pratense* (timothy) pollen, *Phl p V*, has been isolated by a combination of copper chelate affinity chromatography and ion exchange chromatography. *Phl p V* binds IgE from serum of grass-sensitized donors as revealed in immunoelectrophoretic techniques and in SDS–PAGE immunoblot, and luminescence immunoassay (LIA) inhibition experiments indicate that the allergen represents a significant part of the IgE binding capacity of the extract. In immunoelectrophoresis, *Phl p V* is revealed as a single precipitate. However, molecular weight studies show that *Phl p V* consists of at least two isoforms with similar immunochemical properties, but with different molecular size. After SDS–PAGE treatment purified *Phl p V* is identified as two IgE-binding components, *Phl p Va* and *Phl p Vb*, with molecular weights 33 and 29 kD. After HPLC gel filtration, *Phl p Va* and *Phl p Vb* are identified in the major 30-kD eluate. After Sephadex G75 gel filtration of whole pollen extract, *Phl p V* is identified in fractions corresponding to molecular weights 47 and 25 kD. The 47-kD fraction corresponds to *Phl p Va/Phl p Vb* as seen in SDS–PAGE, while the 25-kD component presumably corresponds to a degradation product present in whole pollen extract. The NH₂-terminal sequence of *Phl p V*, corresponding to approximately 10% of the molecule, has been determined. The sequence shows minor variations in some residues and contains besides many alanine residues also hydroxyproline; the sequence reveals no homologies to any known NH₂ terminal sequence of other proteins. The amino acid composition, revealing 26 mole % alanine and no cysteine, does not show any similarities to other known amino acid compositions of allergens. From the amino acid composition determination and an immunoelectrophoretic comparison, *Phl p V* is estimated to represent 6% (w/w) of the whole pollen extract.

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Introduction

When grass pollen extracts are investigated in crossed radioimmunoelectrophoresis (CRIE), several IgE-binding components are visible [1–6]. Some of these components strongly bind IgE from almost all investigated sera, and undoubtedly they are of major significance in grass pollen allergy.

The most dominant IgE-binding precipitate in extracts from *Phleum pratense* (timothy) pollen as seen in CRIE,

antigen 25 (Ag25), has been isolated and investigated by Løwenstein using immunoelectrophoretic techniques [7]. Ag25 appeared to be the major fraction of antigen B (AgB) which was isolated and described by Malley *et al.* in earlier studies of *Phleum pratense* pollen [8]. At present, no immunochemical similarities between Ag25 and the allergens purified before from *Lolium perenne* pollens, *Lol p I*, *Lol p II*, *Lol p III* and *Lol p IV* [9] have been detected [10; and unpublished observations]. Therefore the allergen was named *Phl p V* according to the allergen nomenclature subcommittee established under the auspices of the World Health Organization [11].

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Here we describe a new isolation procedure of *Phl p V*, using affinity chromatography, and present a physicochemical characterization.

Materials and methods

Pollen extracts

Three batches of pollen from *Phleum pratense* (A, B and C) were obtained from Allergon (Uppsala, Sweden) as dried defatted material. According to the manufacturer's certificates, each pollen sample contained approximately 95% pure grass pollen grains and approximately 5% other plant parts from the flowers of the same grass. Detectable foreign pollens constituted less than 0.5%.

The pollens were stored dry at -20°C until use. They were extracted within 4 years of collection. The extraction was performed at 5°C for 20 hr with a carbonate buffer solution (1/10 w/v in 0.125 M NH_4HCO_3 and 0.015 M NaN_3), centrifuged (approximately 23 000 g for 1 hr at 0°C), dialysed (twice against 0.005 M NH_4HCO_3 , once against water, 24 hr each, 5°C) using Visking dialysis tubing (Union Carbide, Danbury, CN) with limiting pore size corresponding to approximately <10 kD, and lyophilized. The prepared extracts (*Phlp-A*, *Phlp-B* and *Phlp-C*) were stored dry at -20°C until use.

Solutions of the extracts (10 mg/ml) were always freshly prepared in 0.1 M NaHCO_3 and 0.015 M NaN_3 solution for immunization of rabbits (*Phlp-A*), or in 0.15 M NaCl and 50% (v/v) glycerol for use in analysis (*Phlp-B* and *Phlp-C*).

Antibodies

Polyspecific rabbit anti-*P. pratense* pollen antibodies (anti-*Phlp*) were raised on *Phlp-A* (three rabbits each receiving a monthly injection of 5 μg pollen extract in Freund's incomplete adjuvant). The immunization, monthly bleeding and subsequent isolation of the immunoglobulins by ammonium sulphate fractionation were performed according to Harboe and Ingild [12]. The antibodies, approximately seven times concentrated relative to their concentration in serum, were stored at 5°C in a solution containing 0.015 M NaN_3 and 1% aprotinin (Novo Nordisk, Bagsværd, Denmark) to avoid proteolytic degradation of the immunoglobulins [13]. A pool of antibodies from 143 bleedings was used.

Monospecific anti-*Phl p V* antibodies were raised on *Phl p V* (two rabbits each receiving a monthly injection of 1 μg purified allergen in Freund's incomplete adjuvant) and purified after five bleedings as described above.

A serum pool prepared for standard standardization studies consisting of sera from at least 500 non-treated

grass-pollen-allergic individuals, was used in IgE binding experiments. A serum pool from 1000 normal Danish adults was used as a control serum.

Immuno-electrophoresis

Analyses using crossed immuno-electrophoresis (CIE) and fused rocket immuno-electrophoresis (FRIE) were performed at pH 8.6 using 1% agarose gels (type HLA; Litex, Copenhagen, Denmark) as earlier described [14–16].

Crossed radioimmuno-electrophoresis (CRIE) was performed as earlier described [17]. Briefly, the CRIE plates were prepared as follows: cold-dried, not blue-stained CIE plates were incubated overnight at room temperature with 250 μl serum diluted in 8 ml 0.9% wt/vol NaCl, followed by three washings in 0.9% (w/v) NaCl. Hereafter they were incubated at 20°C overnight with 56 nCi (125 000 c.p.m.) ^{125}I -labelled anti-IgE (ALK, Hørsholm, Denmark), followed by three washings (0.9% w/v, NaCl). The dried plates were autoradiographed on X-ray films (Kodak X-omat L) at -80°C for 5 days.

Fused rocket radioimmuno-electrophoresis (FRRIE) was used for testing of fractions in the first purification procedures. Briefly, FRIE plates were incubated with 1 ml of serum in 25 ml 0.9% (w/v) NaCl and 225 nCi ^{125}I -labelled anti-IgE. Otherwise they were treated as CRIE plates. Further details on the electrophoreses are given in Results.

SDS-PAGE and immunoblotting

Electrophoresis in polyacrylamide gels was performed using a 7% (w/v) acrylamide stacking gel (in 0.015 M Tris/HCl and 0.1 M taurine, adjusted to pH 7.25, with 5% (w/v) $\text{N,N}'$ -diallyltartardiamide as crosslinker), a 16% (w/v) acrylamide separation gel (in 0.828 M Tris/HCl, adjusted to pH 8.8, with 2% w/w, bisacrylamide as crosslinker) and basic electrode buffer (0.964 M Tris/HCl, 0.466 M taurine and 0.15%, w/v, SDS, adjusted to pH 8.8).

Before electrophoresis the samples were pre-treated for 2–5 min at 100°C in application buffer (0.150 M Tris/HCl, 0.324 M dithiothreitol and 1%, w/v, SDS, adjusted to pH 7.25, with bromophenol blue as marker). The electrophoresis was performed on the reduced samples at 80 mA in the stacking gel and at 160 mA in the separation gel. The electrophoresis was terminated when bromophenol blue was approximately 1 cm from the bottom of the gel (after 180–210 min). Molecular weight markers were the low range SDS-PAGE standards from BioRad (Richmond, CA) (rabbit muscle phosphorylase b subunit, 97 kD; bovine serum albumin, 66 kD; ovalbumin, 43 kD; bovine erythrocyte carbonic anhydrase, 29 kD; soybean trypsin inhibitor, 20 kD; chicken egg lysozyme; 14.4 kD).

Electroblotting of SDS-PAGE gels to nitrocellulose paper (GSWP 0.22 μm , Millipore, Milford, MA) was performed semidry at approximately 2.4 mA/cm² for 90 min as described earlier by Kyhse-Andersen [18]. The nitrocellulose papers were immediately blocked for 2 min in Tween buffer (2% v/v Tween 2 in 0.05 M Tris and 0.15 M NaCl, adjusted to pH 10.3) and washed in 1/9 diluted Tween buffer.

Detection of proteins on electroblots were done by gold staining. After incubation in citrate buffer (0.05 M sodium citrate, adjusted to pH 3.0), the electroblot was incubated overnight with a gold chloride solution freshly prepared as 3 ml/sample gold chloride stock solution mixed with 1 ml/sample citrate buffer (pH 3.0) and 0.2 ml/sample Tween 20 (4% v/v). The gold chloride stock solution was pre-made as follows: chloroauric acid (Johnson Matthey Materials, Boyston, U.K.) was gently boiled with 1% sodium citrate (0.3 ml/mg chloroauric acid) for 30 min and stored cold and dark.

To obtain IgE immunoblots the nitrocellulose strips were incubated overnight with 40 μl /sample human serum diluted 1/100 (v/v) in incubation buffer (0.05% Tween 20 in 0.05 M Tris and 0.15 M NaCl, adjusted to pH 10.3), followed by three washings in incubation buffer, followed by overnight incubation with 10 nCi/sample ¹²⁵I-anti-IgE (ALK) in incubation buffer.

Purification of Phl p V

Several batches of *Phl p V* were purified from *Phlp-B* by a combination of copper chelate chromatography and ion exchange chromatography.

Pollen extract (approximately 200 mg *Phlp-B*) was applied on a chelating Sepharose column (Pharmacia, Uppsala, Sweden; gel volume 25 ml) which was formerly activated with Cu⁺⁺ ions (0.1 M Cu₂SO₄, 5 H₂O) as described by the manufacturer. Elution of extract was performed using a pH gradient (pH changed from 7.5 to 4.0 in steps of 0.5 using phosphate and acetate buffers with I=0.05, elution volume equal to 2 column volumes in each step) followed by elution with EDTA (0.05 M in 0.4 M NaCl). Fractions of 2 ml were collected and tested in FRIE and FRRIE. Fractions corresponding to pH 5.5 and 5.0 were pooled, dialysed and lyophilized.

The obtained 10–15 mg *Phl p V*-enriched fraction was dissolved in acetate buffer (pH 4.0, I=0.05) and applied on a carboxymethyl (CM) Sepharose column (Pharmacia; gel volume 10 ml). Elution of extract was performed using a pH gradient (pH changed from 4.0 to 7.5 in steps of 0.25 as described above). Fractions of 1 ml were collected and tested in FRIE and FRRIE. Fractions corresponding to pH 5.50 and 5.75 were pooled, dialysed and lyophilized. Approximately 5 mg of purified *Phl p V*

were obtained in this way and used for immunizing rabbits, inhibition experiments and physicochemical analysis.

LIA inhibition experiment

Test of allergenicity of *Phl p V* was done using a luminescence immunoassay (LIA) for specific IgE (Ciba Corning Diagnostics, Boston, MA), described earlier in principle [19,20].

Briefly, extract *Phlp-C* was covalently coupled to microscopic paramagnetic particles (PMP) and used as solid phase in an inhibition experiment. Serum pool (25 μl , 1/4 dilution in water) was inhibited with *Phl p-C* (25 μl , concentrations ranging from 1 to 10⁻⁵ mg/ml) or with equal amounts of purified *Phl p V* (as measured in CIE using anti-*Phl p V*) and incubated with *Phlp-C*-coated PMP. Free and bound IgE were magnetically separated, and non-inhibited IgE was detected in a luminometer (Magic Lite Analyzer; Ciba Corning Diagnostics) using a monoclonal anti-IgE (ALK) coupled to the chemiluminescent reagent acridinium ester [21].

HPLC gel filtration experiment

Gel filtration of the purified *Phl p V* was performed on a Superose 12 column in 0.125 M NH₄HCO₃ (flow rate 0.75 ml/min) on a HPLC system (Pharmacia). Approximately 10 μg of the purified fraction were applied on the column and the absorption spectrum (200–370 nm) was monitored using a diode array detector (Pharmacia; Rapid Spectral Detector 2140). Molecular weight of the purified protein was determined from interpolation of molecular weight standards (bovine serum albumin, 66 kD; ovalbumin, 43 kD; carbonic anhydrase, 29 kD; bovine alpha-chymotrypsinogen A, 26 kD; horse muscle myoglobin, 17.5 kD; horse heart cytochrome C, 12.4 kD; pancreas aprotinin, 6.5 kD; all from Sigma, St Louis, MO).

Sephadex G75 gel filtration and preparative isoelectric focusing

Molecular weight determinations of *Phl p V* were performed in a gel filtration experiment on a Sephadex G75 column, as described earlier [6]. A solution of pollen extract (55 mg *Phlp-C* in 1 ml buffer) was fractionated on the column, and a gel filtration profile was obtained by means of FRIE, FRRIE and SDS-PAGE IgE immunoblot. *Phl p V* was identified using FRIE with anti-*Phl p V* in the intermediate gel. Molecular weights were interpolated from molecular weight markers (as described in the HPLC studies, but excluding aprotinin and carbonic anhydrase).

The isoelectric point (pI) of *Phl p V* was determined in a

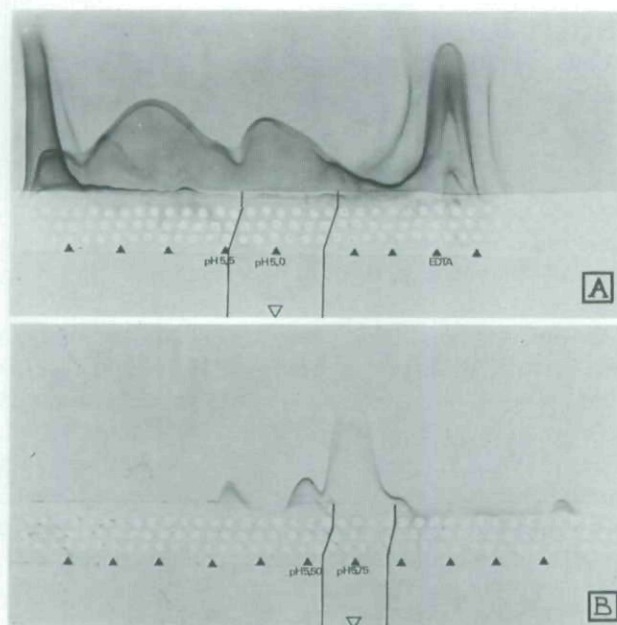


Fig. 1. Two step isolation of *Phl p V*. (A) FRIE analysis of 100 fractions from first-step column (copper chelate Sepharose); (B) FRIE analysis of 115 fractions from second-step column (CM Sepharose). Black triangles indicate change of elution buffers. Fractions between black lines were pooled (as indicated in the text). In both cases 14 μ l of the each fraction were applied in wells against 8.3 μ l/cm² of anti-*Phlp*. Electrophoresis for 16 hr at 2 V/cm after allowing the fractions to fuse for 1 hr.

preparative isoelectric focusing experiment on an Ampholine 8101 column (Pharmacia) as earlier described [6]. A solution of pollen extract (30 mg *Phlp*-B in 1 ml buffer) was fractionated on the column, and investigated in FRIE and FRRIE. *Phlp* were identified using FRIE with anti-*Phl p V* in the intermediate gel.

NH₂ terminal sequence and amino acid composition

The *NH₂* terminal amino acid sequence was determined on a 477 Pulsed Liquid Phase Protein/Peptide Sequencer in combination with a 120A HPLC on-line PTH-amino acid analyser (Applied Biosystems, Foster City, CA) as described [22]. Sequences on two different batches of purified *Phl p V* were determined.

The amino acid composition was determined after derivatization with *o*-phthalaldehyde (OPA) as described by Barkholt and Jensen [23]; prevention of reduction of cysteine during hydrolysis was done by derivatization with 3,3'-dithiodipropionic acid. The content of tryptophane was not determined.

Results

In a two-step procedure using copper chelate affinity chromatography and ion exchange chromatography *Phl*

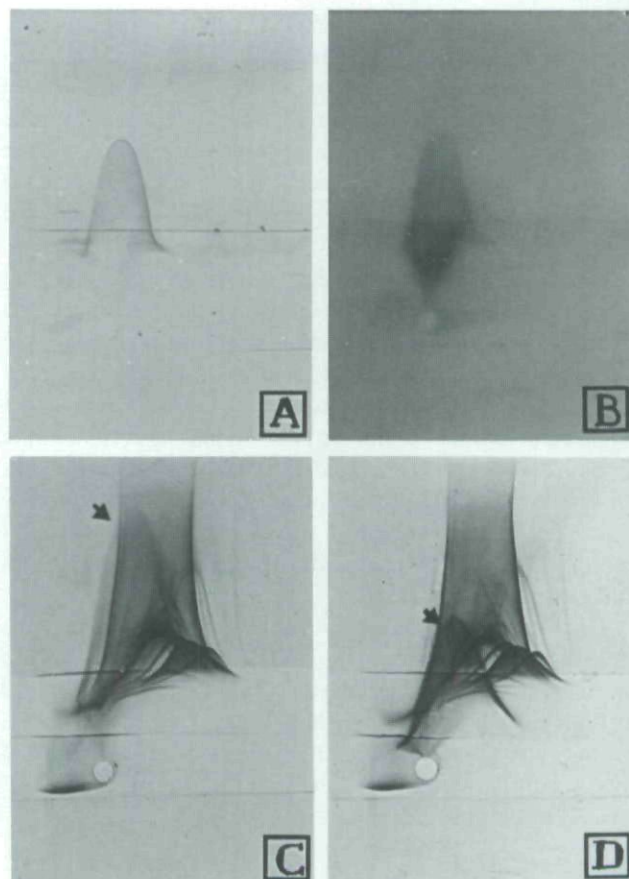


Fig. 2. *Phl p V* examined by immunoelectrophoretic techniques. (A) CIE with 5 μ g *Phl p V* against 12.8 μ l/cm² anti-*Phl p*; (B) CRIE: autoradiograph of (A); (C) CIE with 75 μ g *Phl p*-B against 12.8 μ l/cm² anti-*Phl p*; (D) CIE as in (C), but with 2 μ l/cm² anti-*Phl p V* in the intermediate gel. In all cases first dimension electrophoresis for 30 min at 10 V/cm, second dimension electrophoresis overnight at 2 V/cm.

p V was isolated from *P. pratense* pollen extract (Fig. 1). The purity of the isolated *Phl p V* was examined by immunoelectrophoretic techniques (Fig. 2) by SDS-PAGE electroblotting (Fig. 3) and by HPLC (Fig. 4).

In CIE and CRIE using anti-*Phlp*, *Phl p V* is revealed as a single highly dominant Coomassie blue stainable and IgE-binding precipitate which is situated in the anodic gel (Fig. 2A and B). A very weak Coomassie blue-stained precipitate which does not bind IgE is visible to the right of the *Phl p V* precipitate, and a weak cathodic IgE-binding precipitate (not stained in CIE), possibly representing an impurity, is visible to the right of the application slit.

In SDS-PAGE electroblots, *Phl p V* is revealed as two IgE-binding components, *Phl p Va* and *Phl p Vb*, with molecular weight of 33 kD and 29 kD (Fig. 3). In gold-stained blot minor impurities with 12 kD, 55 kD, 80 kD

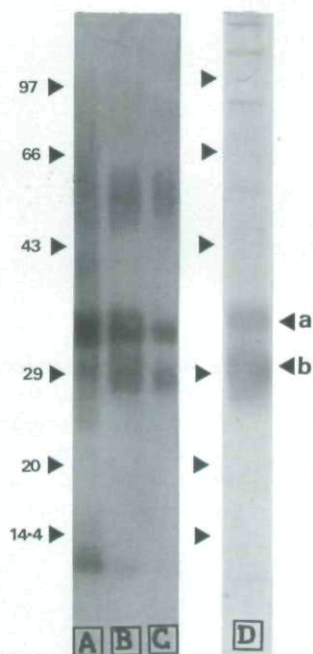


Fig. 3. *Phl p V* examined in SDS-PAGE: Lane A–C, IgE immunoblots. (A) 5 µg of pollen extract; (B) 5 µg of *Phl p V*-enriched fraction from copper chelate column; (C) 1 µg of *Phl p V*; (D) gold-stained electroblot, else as (C) (*Phl p Va* and *Phl p Vb* are indicated with arrows).

and 110 kD are present; the 55-kD band seems to correspond to a diffuse band which bind IgE in the immunoblot.

After HPLC gel filtration, the absorption profile indicates that *Phl p V* mainly consists of a 30-kD component, with possible minor impurities at 15 kD and 60 kD (Fig. 4). The major component corresponds to *Phl p Va* and *Phl p Vb* as seen in SDS-PAGE (data not shown).

The allergenicity of *Phl p V* was tested in a LIA inhibition experiment (Fig. 5). The inhibition capacity of isolated *Phl p V* was compared with the inhibition capacity of the initial pollen extract containing the same amount of purified allergen (as seen in CIE using anti-*Phl p V*). In high concentrations the isolated fraction was able to inhibit more than 75% of the *P. pratense*-specific IgE in the serum pool.

Rabbit anti-*Phl p V* identify one anodic precipitating antigen, formerly identified as Ag25 (Fig. 2C and D). The same precipitate was identified using an old stock of rabbit anti-Ag25 antibodies (data not shown). In FRIE of Sephadex G75 gel filtration fractions of whole pollen extract *Phlp-C*, anti-*Phl p V* identify an IgE-binding precipitate corresponding to two molecular weights of 47 kD and 25 kD (Fig. 6). The 47-kD component was

identified as *Phl p Va* and *Phl p Vb* in SDS-PAGE IgE immunoblot (Fig. 7), while the 25-kD component seems to correspond to a low molecular weight band, not identified in the isolated *Phl p V*. In FRIE on fractions from preparative isoelectric focusing, anti-*Phl p V* reveal an IgE binding precipitate corresponding to pI 4.7 (data not shown).

The NH₂ terminal sequence of *Phl p V* was determined in two batches of purified protein. The first 25 residues were detected; in some of these positions (including the first position) more than one significant amino acid was identified (Table 1). Among the 25 residues, nine were identified as alanine, and six as hydroxyproline (Hyp). Hyp is eluted close to alanine and usually with a trace close to histidine; Hyp is present in a sequence of 10 amino acids, –Gly–Tyr–Ala/Thr–Hyp–Ala–Ala–Hyp–Ala–Thr–Hyp–, which along the 25 residues is repeated twice with only one non-significant difference.

The determined NH₂ terminal sequence neither reveal any homology to other reported sequences of grass pollen allergens [24–28], nor to other proteins which sequences have been stored in the Genepro databank (Version 4.20, Riverside Scientific Interprises, Seattle, WA).

The amino acid composition of *Phl p V* reveals a high alanine content of 26 mole%, less than 2 mole% hydroxyproline and no cysteine (Table 2). The amino acid composition is not similar to other reported compositions of grass pollen allergens. From the amino acid composition determination and comparisons of *Phl p V* and *Phl p C* in CIE the amount of *Phl p V* in whole pollen extract was estimated to be 6% on weight-to-weight basis.

Discussion

Characterization of *Phl p V*

An IgE-binding component of *P. pratense* (timothy) pollen, *Phl p V* (earlier Ag25) [7], has been isolated using a combination of copper chelate chromatography and ion exchange chromatography. The purity of the isolated *Phl p V* was examined using immunoelectrophoresis, SDS-PAGE and HPLC gel filtration.

In CIE and CRIE, *Phl p V* is presented as one dominant IgE-binding precipitate, but in SDS-PAGE *Phl p V* is presented as two IgE-binding components, *Phl p Va* and *Phl p Vb*, with slightly different molecular weights of 33 kD and 29 kD. After HPLC gel filtration these components are identified in the 30-kD fraction. The isolated *Phl p V* is highly dominant in the preparations, as seen by all three techniques. However, some impurities with different molecular weights were present in low amounts. An IgE-binding impurity of 55–60 kD was present (SDS-PAGE and HPLC), possibly representing traces of *Phl p IV* (earlier Ag30 or *Phl p VII*), a basic protein character-

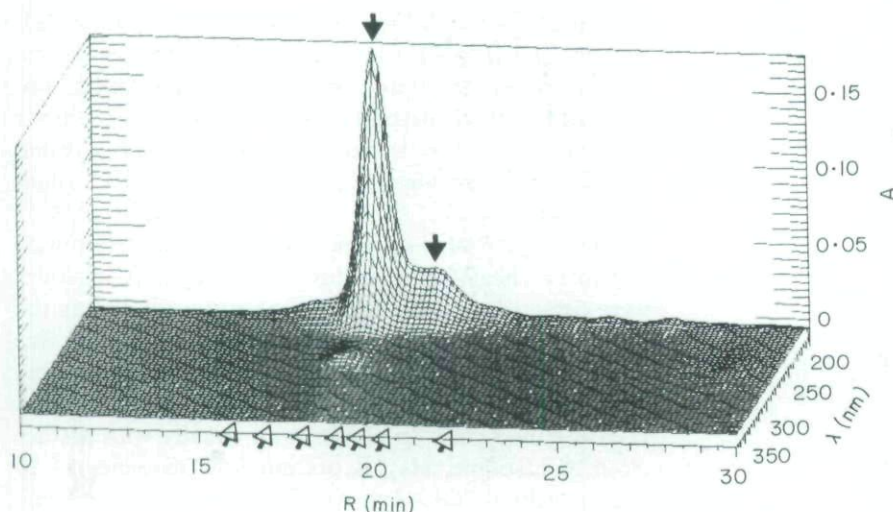


Fig. 4. *Phl p V* examined in HPLC. Gel filtration profile of 5 µg *Phl p V* (diode array detector topogram). R, retention time (min) with molecular weight markers indicated by arrows, wavelength (nm) and absorbance (A).

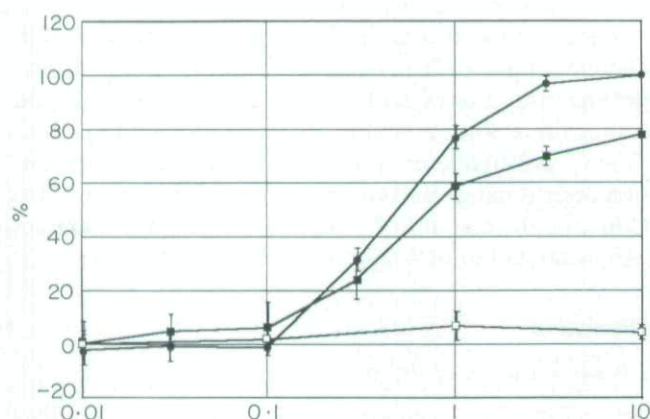


Fig. 5. Examination of the allergenic activity of *Phl p V*. LIA inhibition experiment using *Phl p-C* as solid phase. Serum pool inhibited with (●) whole pollen extract *Phl p-C*; (■) purified *Phl p V*; and (□) *Dermatophagoides pteronyssinus* (dust mite) extract as a negative control. Extract concentrations in µg/ml. *Phl p V* concentration was equivalent to pollen extract as described in text (1 µg *Phl p-C* corresponds to 60 ng *Phl p V*). Each point corresponds to the mean of a triple determination; s.d. indicated.

ized by Löwenstein [7] and Haavik *et al.* [29]. *Phl p IV* impurities might also explain the weak cathodic IgE-binding precipitate seen in CRIE. High molecular components of 80 kD, 110 kD and 150 kD are also seen in the preparation but only in SDS-PAGE gold-stained blots; it is assumed that these components correspond to aggre-

gates of the low molecular weight components. Traces of a low molecular weight component with approximately 12–15 kD is also seen (gold-stained blot and HPLC); this component does not bind IgE.

In whole pollen extract, anti-*Phl p V* identifies a 47-kD component as seen in the Sephadex G75 gel filtration experiment (Fig. 6). This component corresponds to *Phl p Va* and *Phl p Vb* (Fig. 7). However, anti-*Phl p V* also identifies a 25-kD component which is immunochemically similar to the high molecular weight component. It is assumed that in whole pollen extract degradation products of *Phl p V* are present, and this has also been indicated by recent studies using monoclonal antibodies [30].

The allergenic importance of *Phl p V* has been studied by Löwenstein [7] in CRIE and by Østerballe *et al.* [31] in a hyposensitization study, and it is also the topic of an accompanying paper [32]. Here, the allergenic activity of the purified allergen was investigated in a LIA with whole pollen extract as solid phase. Compared with the pollen extract, *Phl p V* could inhibit approximately 75% of the *P. pratense*-specific IgE found in a grass serum pool. However, the importance of the isolated allergen might be overestimated due to the presence of impurities in the preparation (which will affect the activity determination in high concentrations), and the inhibition experiment only indicates that *Phl p V* possesses a significant allergenic activity.

Phl p V is identified as two isoforms, both binding IgE. The existence of such isoforms have also been observed

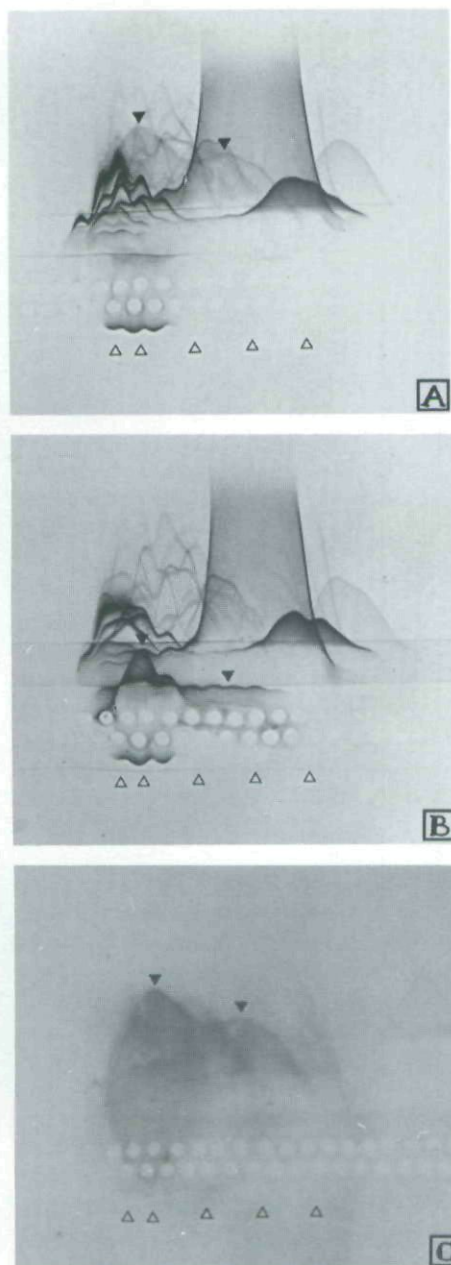


Fig. 6. Examination of *Phl p V* in whole pollen extract. FRIE analysis of 42 fractions (nos. 20 to 61) from a Sephadex G75 Superfine separation of *Phl p*-C. FRIE as in Fig. 1. (A) Intermediate gel is empty; (B) intermediate gel contains 20 µl/cm² anti-*Phl p V*; (C) autoradiograph corresponding to (A).

for other grass pollen allergens [9,33] and might be due to both variation in isoelectric properties or molecular weight probably caused by post-translational modifications of the primary sequence (e.g. amidations or glycosylations). Despite the presence of isoforms of *Phl p V*, it has been possible to obtain a reproducible NH₂ terminal

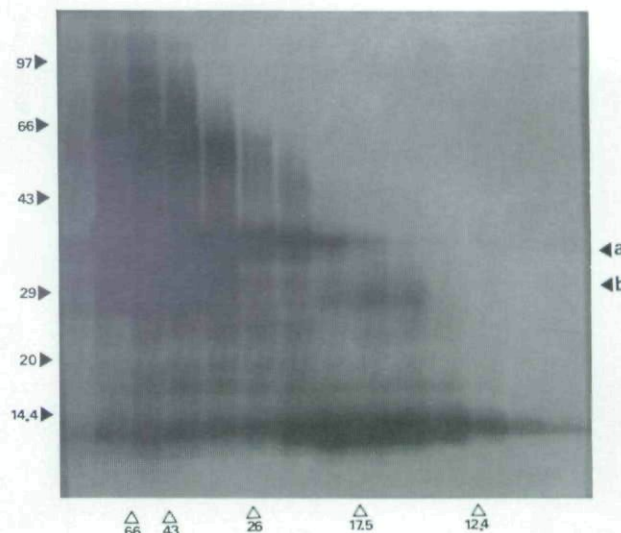


Fig. 7. Examination of *Phl p* in whole pollen extract. SDS-PAGE IgE immunoblot profile of 18 fractions from Fig. 6 (nos. 28, 30, 32–56). In each well 5 µl of the fractions were applied. Bottom, molecular weight markers in G75 Sephadex gel filtration, left, molecular weight markers in SDS-PAGE, right, *Phl p V* components: a, *Phl p Va*; b, *Phl p Vb*; *, proposed degradation product of *Phl p V*.

sequence of the protein. The sequence was obtained from a preparation containing both *Phl p Va* and *Phl p Vb*. Therefore, these proteins must be homologous, considering their NH₂ terminal sequences, despite the differences in molecular weights. The proposed impurities seem not to have disturbed the sequencing, possibly because of the low stoichiometric amounts in the preparation. In residues nos. 1, 3, 6 and 16 more than one amino acid was dominant; such amino acid residue variations have also been seen in other pollen allergens, i.e. *Lol p II* and *Lol p III* [27,28]. The observed variation can be due to differences in the sequences of *Phl p Va* and *Phl p Vb*, but they might also be a consequence of grass subspecies variation (which has not been taken into consideration during pollen collection), or a result of the presence of slightly different proteins from closely related genes in the same genome. Except for one residue (residue no. 3), all variations in the determined amino acid sequence of *Phl p V* could be caused by a single base shift.

The NH₂ terminal sequence is rather unusual, since it contains a high amount of alanine and a modified amino acid, Hyp. During the sequence experiments Hyp is eluted close to alanine with a minor top close to histidine. However, Hyp was also detected after the amino acid determinations although in relatively low amounts.

The existence of Hyp in plant cell proteins is rather well established; Hyp-containing proteins include extensin, a high molecular weight structural element of plant cell

Table 1. The NH₂-terminal amino acid sequence of *Phl p V* as determined in two sequence analyses. More than one significant residue was found in position nos. 1, 3, 6 and 16. Other known NH₂-terminal sequences of grass pollen allergens are shown for comparison. In the *Cyn d I* sequence, Xaa indicates an unknown amino acid, presumably asparagine (glycosylation site)

	NH ₂ terminal sequence		Reference
<i>Phl p V</i>	Ala-Asp-Leu-Gly-Tyr-Ala-Hyp-Ala-Thr-Hyp-Ala-Ala-Hyp-Gly-Ala-Gly-Tyr-Thr-Hyp-Ala-Thr-Hyp-Ala-Ala-Hyp-Ser-Ala-Gly		
<i>Lol p I</i>	Ile-Ala-Lys-Val-Pro-Gly-Pro-Gly-Pro-Asn-Ile-Thr-Ala-Glu-Tyr-Gly-Asp-Lys-Trp-Leu-Asp-Ala-Lys-Ser-Thr-Trp-Tyr-Gly-Lys-Pro-Thr-Ala-Met-Gly-Asp-Lys-Pro-Gly-Pro-Gly-Pro-Xaa-Ile-Thr-Ala-Thr-Tyr-Gly-Asp-Lys-Trp-Leu-Asp-Ala-Lys-Ala-Thr-Phe-Tyr-Gly-Ala-Ala-Pro-Val-Glu-Phe-Thr-Val-Glu-Lys-Gly-Ser-Asp-Glu-Lys-Arg-Leu-Ala-Leu-Ser-Ile-Lys-Tyr-Arg-Lys-Glu-Gly-Asp-Thr-Lys-Val-Asp-Leu-Thr-Val-Glu-Lys-Gly-Ser-Asp-Ala-Lys-Thr-Leu-Asn-Ile-Lys-Tyr-Thr-Arg-Pro-Gly-Asp		[26, 27] [30] [28] [29]

Table 2. Amino acid composition of *Phl p V*

Amino acid	mole%
Hyp	1.4
Asp/Asn	5.4
Thr	7.6
Ser	5.1
Glu/Gln	10.2
Pro	4.5
Gly	6.4
Ala	25.7
Cys	0.0
Val	6.6
Met	0.7
Ile	3.6
Leu	4.7
Tyr	3.5
Phe	4.1
His	0.8
Lys	8.8
Arg	1.0
Trp	ND

ND, not determined.

walls [34,35], and water-soluble arabinogalactan proteins (AGPs) which have been isolated from a number of plant tissues, including stigma surfaces and which functionally seem to be involved in cell adhesion [36,37]. Hyp is usually modified with arabinose (extensins) or AGPs through acid-labile glycosidic bindings.

The existence of AGPs in allergenic fractions from *P. pratense* pollen was observed by Haavik *et al.* [38], and supports the presented observations on *Phl p V*. However, the amount of Hyp detected in the amino acid analysis does not account for the amount of Hyp in the determined NH₂ terminal sequence. This might be due to partial degradation of Hyp to proline or it might be due to the proposed glycosylations of Hyp which might intervene with the OPA derivatization; this is a matter of further investigation.

Comparison with other grass allergens

Phl p V does not reveal characteristics that indicate structural similarities to other grass pollen allergens.

Group I allergens possess similar molecular weights in SDS-PAGE as *Phl p V*, and on this basis *Phl p V* could be confounded with the proposed group I component of *P. pratense*, *Phl p I*. However, the NH₂ terminal sequence of *Phl p V* does not resemble the known NH₂ terminal sequences of group I allergens, *Lol p I* and *Cyn d I*

[24,25,28] (Table 1). Moreover, in SDS-PAGE an IgE-binding component of 34 kD, presumably representing *Phl p I*, is identified in the pollen extract (Fig. 3A and Fig. 7), but it has not been identified in the purified *Phl p V*. Finally, monospecific antibodies raised against *Phl p V* and *Lol p I* precipitate different components in all investigated grasses, as shown elsewhere [30,32].

Group II and group III allergens have been described in *Lolium perenne* and do not show any sequence homologies to *Phl p V* [26,27]. The CIE precipitation pattern excludes the possibility that *Phl p V* might represent the earlier-described, high molecular weight and heat-stable *P. pratense* allergen, *Phl p IV* (earlier Ag30 and *Phl p VII*) [7,29]. *Phl p IV* is revealed as a very dense, cathodic precipitate in CIE which has not been found in the purified *Phl p V*, and *Phl p IV* is not affected by anti-*Phl p V* in immunoelectrophoretic studies (Fig. 2D). However, traces of *Phl p IV* might be present in the preparation in concordance with the CRIE pattern of the *Phl p V* preparation (Fig. 2B).

Phl p VI (earlier Ag19) [7] is a low molecular weight allergen that makes dense precipitates in CIE and FRIE, and which also shows a high affinity for copper-chelate Sepharose. No *Phl p VI* precipitate has been seen in the purified *Phl p V* in CIE, but the prepared anti-*Phl p V* seems to have a very weak effect on the *Phl p VI* precipitate (Fig. 5). Possibly this is due to a minor *Phl p VI* impurity in the *Phl p V* preparation used for immunization.

The existence of similar allergens in pollen extracts from different grass species have been verified in immunochemical studies with monoclonal antibodies [30,39–45]. Usually monoclonal antibodies have been raised against whole pollen extracts, and it seems that in several cases antibodies might have been raised against allergenic components similar to *Phl p V*. The clone FMC-A7 which was raised on a *L. perenne* pollen extract by Smart *et al.* [39] and studied by Singh and Knox [40] is possibly raised against a group V component, as seen in SDS-PAGE. Other monoclonal antibodies, also raised on a *L. perenne* pollen extract and recently described by van Ree *et al.* [33], is specific to group V allergens and have been used in purification of a proposed group V component in *Dactylis glomerata* (as revealed with anti-*Phl p V* in our laboratory). Also, monoclonal antibodies raised by Müller *et al.* [45] against a *P. pratense* pollen extract bind *Phl p V* (as revealed with anti-*Phl p V* in our laboratory). Moreover, in several published immunoblot studies, dominant IgE-binding bands with molecular weights slightly under the group I allergens are seen, e.g. R8 and R9 in a study on *L. perenne* by Moran *et al.* [46] and fractions nos. 9 and 10 in a study on *D. glomerata* by Ford *et al.* [47].

In a following investigation the existence of *Phl p V*,

similar components in pollens from other grasses have been studied [32]. Why the group V allergens have generally not been recognized as distinct allergens might be explained by their presentation in SDS-PAGE: group I and V allergens possess very similar molecular weights after an SDS-PAGE treatment, and group V allergens might have been overlooked because of this.

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