

Microheterogeneity of the major grass group 6 allergen Phl p 6: Analysis by mass spectrometry

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The precise structural characterization of allergens is a basic requirement to improve diagnostics and to find therapeutic strategies against allergic disorders. Natural grass pollen allergens exhibit a wide variety of isoforms and it is still unknown whether this microheterogeneity is essential for the allergic reaction or has a functional effect on sensitization. Well-defined recombinant allergens are considered to replace natural allergens for clinical trials. For the major timothy grass pollen allergen Phl p 6 (~12 kDa) and a recombinant rPhl p 6 we determined the structural microheterogeneity by two-dimensional electrophoresis (2-DE), high-resolution electrospray ionization-Fourier transform-mass spectrometry (ESI-FT-MS) of the intact molecules, and by tryptic peptide mass fingerprinting using matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). Natural Phl p 6 is a mixture of mainly two isoforms that differ by two amino acids leading to a mass difference of 5 Da. For each of this two isoforms six variants were identified with modifications at the C- and/or N-terminus. The recombinant Phl p 6 comprises the same structure as one of the main isoforms indicating that it represents a major part of the natural Phl p 6.

Keywords: Electrospray ionization-Fourier transform-mass spectrometry / Grass pollen allergen / Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry / Two-dimensional gel electrophoresis

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1 Introduction

Allergic disorders, e.g., hayfever and asthma bronchiale, result from sensitization by specific molecules from allergen sources. About 20% of the population in industrialized countries reveal immunoglobulin E (IgE) antibodies against innocent components [1]. Grass pollen allergy is one of the most common allergies, up to 40% of patients are sensitive towards grass pollen allergens [2]. Whilst pollen extracts contain about 1000 different components [3], only a few elicit allergic reactions and are thus allergens. Timothy grass pollen (*Phleum pratense*) is one of the major allergen sources in Europe, containing about ten different groups of allergens [4]. Each allergen group consists of proteins translated by different genes. This causes structural microheterogeneity resulting from single amino acid exchanges. Furthermore, post-translational modifications, e.g., glycosylation, disulfide bridges, etc., increase

the number of isoforms. The occurrence of microheterogeneity with a wide variety of isoforms is typical for grass pollen allergens and whether it has a functional effect on sensitization is still unknown. The aim of an improved therapy is the preparation of an individual composition of the components causing an IgE-mediated allergic response [5]. The condition for this kind of therapy is the component-resolved diagnosis restricted to only those components which show IgE reactivity [6]. For this reason, the standardization and characterization of the particular allergens is a basic requirement. The production of recombinant allergens can be a suitable tool to reach this goal. About 75% of sensitive patients produce IgE antibodies against Phl p 6 [7] indicating that Phl p 6 is a major allergen. Concerning recombinant allergens, Petersen *et al.* [8] and Vrtala *et al.* [7] published different isoforms.

The aim of this study was to characterize the structure of natural Phl p 6 (nPhl p 6) and to compare it with recombinant rPhl p 6 which has been expressed in *Escherichia coli*. nPhl p 6 was isolated from timothy grass pollen by a chromatographic procedure. Both preparations were analyzed by 2-DE and high resolution ESI-FT-MS. Tryptic digest and peptide mass fingerprinting by MALDI-TOF-MS were performed in order to obtain additional structural details.

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Abbreviation: IgE, immunoglobulin E

2 Materials and methods

2.1 Purification of nPhl p 6

nPhl p 6 was purified from timothy grass pollen (ARTU Biologicals, Lelystad, The Netherlands) as described by Suck *et al.* [9]. Briefly, the purification was performed by metal chelate chromatography using a chelating Sepharose charged with Ni^{2+} ions, followed by hydrophobic interaction chromatography using a Phenyl Sepharose High Performance matrix and size exclusion with Superdex 75 (Amersham Biosciences, Uppsala, Sweden). After desalting and lyophilization nPhl p 6 was stored at -20°C until use.

2.2 Recombinant Phl p 6

According to Petersen *et al.* [8] a cDNA library of timothy grass pollen was screened for Phl p 6 isoforms and a full-length cDNA was isolated with a sequence identical to that described by Vrtala *et al.* [7]. The cDNA was subcloned into a pMAL-c vector (New England BioLabs, Beverly, MA, USA) and transformed in *E. coli* JM 109 cells (Promega, Madison, WI, USA). The expression of the fusion protein was induced with 0.3 mM IPTG at OD_{600} 0.6–0.8. After 3 h incubation the cells were harvested, resuspended in 20 mM Tris, 200 mM NaCl, and 1 mM EDTA, pH 7.4, and lysed by FrenchPress with addition of Benzonase (Merck, Darmstadt, Germany). After centrifugation the supernatant was applied onto an amylose column (Amersham Biosciences) and the maltose-binding protein (MBP)-rPhl p 6 fusion protein was eluted by 20 mM Tris, 200 mM NaCl, 1 mM EDTA, and 10 mM maltose, pH 7.4. The MBP was cleaved from rPhl p 6 by protease Xa digestion (New England BioLabs) in a dilution of 1:250 for 72 h at 4°C . Further purification of rPhl p 6 was performed by metal chelate chromatography using a chelating Sepharose charged with Ni^{2+} ions and size exclusion with Superdex 75 (Amersham Biosciences). After desalting and lyophilization rPhl p 6 was stored at -20°C until use.

2.3 2-DE and blotting

2-DE was performed using home-made gels as previously described [10, 11]. Polyacrylamide gels (4%T, 4%C, size $240 \times 80 \times 0.5$ mm) with an immobilized pH gradient of 4–10 were used for separation in the first dimension (IEF) [12]. 3 mm immobilized pH gradient strips were rehydrated overnight in 8 M urea, 10 mM DTT, 0.5% Nonidet P-40 (Sigma-Aldrich, St. Louis, MO, USA), 0.4% Servalyt 3–10, and 0.1% Servalyt 4–6 (Serva, Heidelberg, Germany). 7.5 μg of protein in 10 μL double-distilled water

was applied by cup-loading and separated with 3000 V and 2 mA for 7 h. SDS-PAGE was carried out in the second dimension using gradient gels (5–20%, size $240 \times 80 \times 0.5$ mm). Molecular masses and pI were determined by comparison with low range SDS-PAGE standard (14.4–97.4 kDa; Bio-Rad, Richmond, CA, USA) and IEF Marker 3–10, Liquid Mix (Serva). Proteins were detected by silver staining [13]. For the identification of allergens the proteins were transferred onto a nitrocellulose membrane by thermoblotting [14]. The blots were incubated with patient serum in a dilution of 1:20. After incubation with alkaline phosphatase-conjugated goat anti-human IgE (Allergopharma, Reinbek, Germany), the binding pattern was visualized by a substrate solution consisting of 0.033% w/v nitro-blue tetrazolium and 0.017% w/v 5-bromo-4-chloro-3-indolyl phosphate potassium salt (Sigma) in TBS (pH 9.5) [15].

2.4 ESI FT-MS

The masses of nPhl p 6 and rPhl p 6 were analyzed using a high-resolution ESI-FT ion cyclotron resonance (FTICR) mass spectrometer (APEX II, Bruker Daltonics, Billerica, MA, USA) equipped with a 7 Tesla actively shielded magnet and an Apollo ion source. The lyophilized proteins were dissolved in a 50:50:0.001 v/v/v mixture of methanol, water, and acetic acid and sprayed with a flow rate of 2 $\mu\text{L}/\text{min}$. For a straightforward interpretation of the heterogeneous samples the obtained positive ion mass spectra were charge-deconvoluted and mass numbers refer to neutral molecules.

2.5 Tryptic digest and MALDI-TOF-MS

50 μg of nPhl p 6 and rPhl p 6 were digested overnight at 37°C with 2.5 μg trypsin, sequencing grade modified (Roche Diagnostics, Mannheim, Germany) in 200 μL of 50 mM NH_4HCO_3 buffer (pH 8.0) containing 5 mM CaCl_2 . The peptides were separated by RP-HPLC using a HyPurity Elite C18 5 μ column (250×4.6 mm; Thermo Hypersil-Keystone, Runcorn, UK) and ÄKTA purifier (Amersham Biosciences). The separation was started for 5 min using solvent A (0.1% TFA in water) at a flow rate of 1 mL/min and followed by a gradient run up to 100% solvent B (80% ACN, 0.08% TFA in water) in 10 min. Peptide-containing fractions were pooled and dried in a SpeedVac SPD12P concentrator (Thermo Savant, Holbrook, NY, USA). The samples were solved in 2 μL of a 2:1 mixture of 0.1% TFA and acetonitrile. 0.5 μL sample was spotted on a MALDI target plate and mixed with 0.5 μL saturated matrix solution (3,5-dimethoxy-4-hydroxy cinnamic acid, SA, in a 2:1 mixture of 0.1% trifluoroacetic acid and acetonitrile). MALDI-TOF analyses of the peptides were per-

formed using a Reflex III (Bruker-Daltonics) in the linear and reflector TOF configuration applying an acceleration voltage of 20 kV. External mass scale calibration was performed with an appropriate mixture of peptides of known structure. Tryptic digests were analyzed applying Biotools 2.0 (Bruker Daltonics).

3 Results and discussion

3.1 2-DE and immunoblotting

2-DE analysis and immunological studies were performed to characterize the recombinant Phl p 6 and the isoforms of chromatographical separated nPhl p 6. As shown in Fig. 1A, nPhl p 6 consists of about seven isoforms in a *pI* range of 5.2–6.5 and masses of about 12 kDa. The prominent spots were detected with *pI* of 5.5 and 6.0. No other proteins were detected indicating the high purity of isolated nPhl p 6. The silver-stained 2-DE gel of nPhl p 6 shows an identical spot pattern to the proteins of the 2-DE blot detected by patient serum (Fig. 1B). This confirms that all isolated isoforms of nPhl p 6 were IgE-reactive. Furthermore, the spot pattern of isolated nPhl p 6 is comparable to the pattern of Phl p 6 detected in timothy grass pollen extract by mAb Bo 12 (data not shown). Thus, all isoforms of nPhl p 6 were comprised.

The recombinant Phl p 6 was detected in a silver-stained 2-DE gel as a single spot with a *pI* of 6.0 and a molecular mass of about 12 kDa (Fig. 1C). The additional spot at *pI* 5.5 might be caused by different post-translational modifications, e.g., a deletion of *N*- and/or *C*-terminal amino acids. A contamination with other bacterial proteins could

be excluded since no proteins of other size than those in Figs. 1C, D were determined. The predicted *pI* of 5.49 differs from the *pI* of 6.0 measured by 2-DE whereas the mass of rPhl p 6 is in agreement with the predicted data. Compared with nPhl p 6, the spot of rPhl p 6 is congruent with one spot in the pattern of nPhl p 6 indicating that the rPhl p 6 isoform also occurs among the isoforms of natural Phl p 6. The immunoblot of rPhl p 6 stained with patient serum (Fig. 1D) shows that the recombinant allergen is also IgE-reactive which is a prerequisite for allergic reactions.

3.2 Characterization of intact nPhl p 6 and rPhl p 6 by MS

High-resolution ESI-FT-MS was applied to analyze the isolated intact molecules for two reasons. nPhl p 6 consists of several isoforms. To detect all isoforms, we performed ESI-FT-MS since it is more sensitive and efficient than 2-DE even at more narrow *pH* gradients. Furthermore, different isoforms may not be detected by peptide mass fingerprinting after tryptic digest due to incomplete coverage of the tryptic fragments. The charge-deconvoluted spectrum obtained in the positive ion mode (Fig. 2A) shows seven peaks in a mass range between 11 264 Da to 11 975 Da, whereas that of rPhl p 6 (Fig. 2B) comprises only one prominent peak with the highest abundant peak at *m/z* 11 785.02 which is in excellent agreement with the calculated mass (*m/z* 11 785.03) of the sequence predicted by Vrtala *et al.* [7]. Peaks of low intensity which are not further labeled represent most probably sodium adduct ions.

An enlargement of the respective ion regions of nPhl p 6 and rPhl p 6 around 11 785 Da showed a difference of 3 mass units between the most abundant peaks (Figs. 3A and B) and a more complex pattern of isotopic peaks indicating that nPhl p 6 represents a mixture of different components. In literature, there are two sequences published [7, 8] differing by the exchange of two amino acids at the positions 14 (V → I) and 95 (R → H), respectively, leading to a mass difference of only 5 mass units. The theoretical calculated isotopic distribution of both isoforms is shown in Fig. 3C. The sum of both isotopic distributions resembles that of nPhl p 6 measured by high-resolution MS (Fig. 3A), thus suggesting that both isoforms exist in the mixture of natural material. Both isoforms consist of six additional *C*- and *N*-terminal variants, since all peaks of nPhl p 6 are composed of two proteins differing by 5 mass units (not shown in detail).

The mass difference of 186 Da is in agreement with the protein sequence data of Petersen *et al.* [16], who identified two additional amino acids EG ($\Delta m = 186$) at the

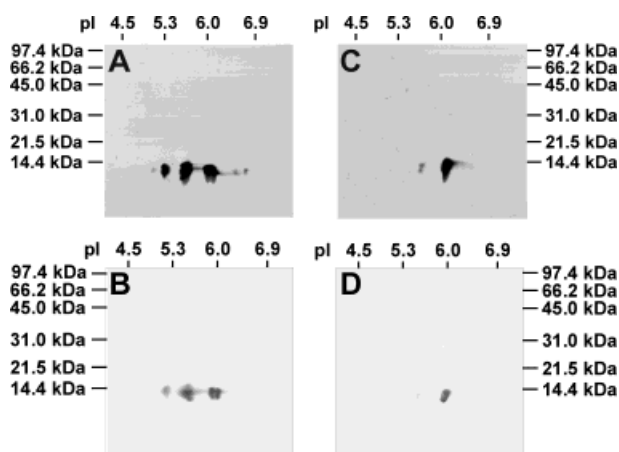


Figure 1. 2-DE and 2-DE immunoblotting of nPhl p 6 and rPhl p 6. (A) Silver-stained gel of nPhl p 6. (B) Detection of IgE-reactive proteins of nPhl p 6 using patient serum. (C) Silver-stained gel of rPhl p 6. (D) Detection of IgE-reactive proteins of rPhl p 6 using patient serum.

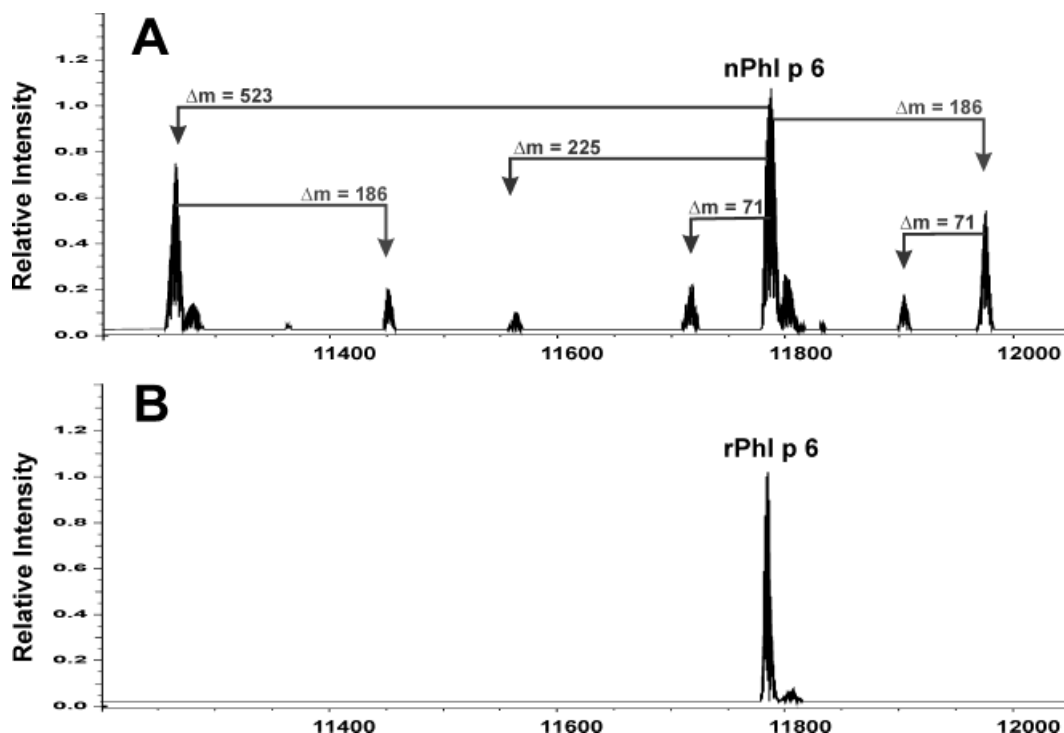


Figure 2. Charge deconvoluted ESI FT-MS mass spectrum of positive ions of (A) natural and (B) recombinant Phl p 6. The mass differences between the prominent peaks are indicated by grey arrows.

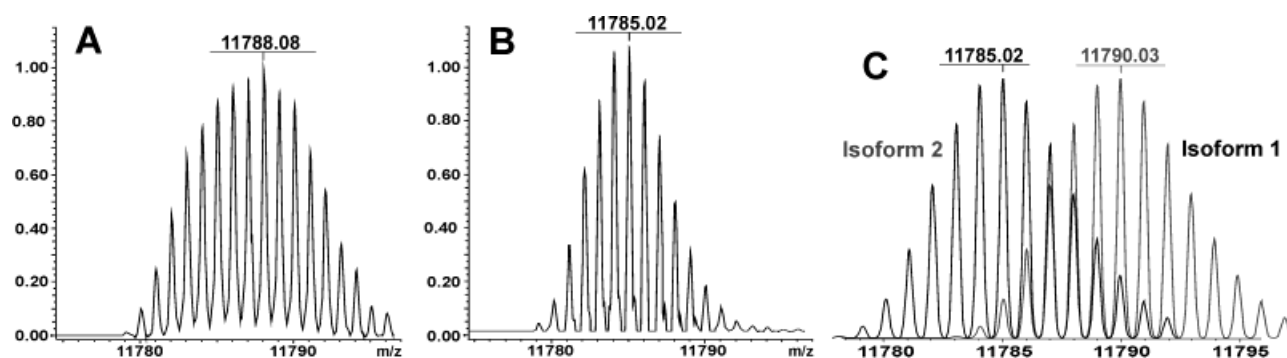


Figure 3. Enlargement of the respective molecular ion regions around 11 785 Da. (A) nPhl p 6. (B) rPhl p 6. (C) Calculated isotopic distribution under consideration of the amino acid sequence depicted in Fig. 4.

N-terminus of nPhl p 6. This dipeptide is a part of the signal sequence and is usually split off when the signal peptide is released. The other mass differences ($\Delta m = 71$, 225, and 523) correlated to putative *C*-terminal deletions of one (A), three (PGA) and six (AVKPGA) amino acid residues, respectively. These differences in the *C*-terminal sequence might be caused by post-translational processing of nPhl p 6 molecules. It should be further emphasized that obviously Phl p 6 is not glycosylated although there is a possible *N*-glycosylation site.

3.3 Tryptic digestion of nPhl p 6 and rPhl p 6

A tryptic fingerprint of rPhl p 6 and nPhl p 6 was performed in order to support the results of the mass spectrometric characterization of the intact isoforms. The coverage of nPhl p 6 and rPhl p 6 was 87.4% based on the sequences of the two isoforms (Fig. 4). The tryptic fragments of nPhl p 6 detected by MALDI-TOF are shown in Table 1. The fragments of aa 10 to 19 of both isoforms differing by only one amino acid (position 14: V→I) were

Sequence of Phl p 6, Isoform 1

GKATTEEQKL IED**V**NASFRA AMATTANVPP ADKYKTFEAA
FTVSSKRNLDAVSKAPQLV PKLDEVYNAA YNAADHAAPE
DKYEAFVLHF SEAL**R**IIAGT PEVHAVKPGA

Sequence of Phl p 6, Isoform 2

GKATTEEQKL IED**I**NASFRA AMATTANVPP ADKYKTFEAA
FTVSSKRNLDAVSKAPQLV PKLDEVYNAA YNAADHAAPE
DKYEAFVLHF SEAL**H**IIAGT PEVHAVKPGA

Figure 4. Sequences of the two isoforms of Phl p 6 described by Petersen *et al.* [8] and Vrtala *et al.* [7]. The two differing amino acids at position 14 and 95 are in bold.

Table 1. MALDI-TOF analysis of nPhl p 6 digested by trypsin

Peptide	MH ⁺ (calculated)	MH ⁺ (observed)
1–2	204.13	Out of range
3–9	806.39	806.49
10–19 (14 = V)	1163.61	1163.60
11–19 (14 = I)	1177.62	1177.61
20–33	1357.68	1357.61
34–35	310.18	Out of range
20–35	1648.84	1648.69
36–46	1187.59	1187.56
47	175.12	Out of range
48–55	817.44	817.56
56–62	752.47	752.57
63–82	2176.98	2176.88
83–95 (95 = R)	1581.81	Not detected
96–110	1459.83	Not detected
83–110 (95 = H)	3003.57	Not detected
C- and N- terminal variants of nPhl p 6		
EG + 1–2	390.20	Out of range
83–109 (95 = H)	2932.54	Not detected
83–107 (95 = H)	2778.46	Not detected
83–104 (95 = H)	2480.26	Not detected
96–109	1388.79	Not detected
96–107	1234.72	Not detected
96–104	936.51	936.57

detected but not the C-terminal fragments containing the second amino acid substitution (position 95: R→H). The isoform with an arginine residue at position 95 has an additional tryptic cleavage site. From the C-terminal variants only one fragment of the isoform with an arginine at position 95 was detectable. This fragment showed a deletion of the six C-terminal amino acids. The other fragments were not be detected by MALDI-TOF-MS. The tryptic digest of rPhl p 6 showed also a coverage rate of 87.4%. The detected fragments are shown in Table 2 and are the same as those of the natural protein with identical sequence. These data are consistent to the results obtained by the mass analysis of the intact nPhl p 6.

Table 2. MALDI-TOF analysis of rPhl p 6 digested by trypsin

Peptide	MH ⁺ (calculated)	MH ⁺ (observed)
1–2	204.13	Out of range
3–9	806.39	806.43
11–19	1177.62	1177.51
20–33	1357.68	1357.54
34–35	310.18	Out of range
20–35	1648.84	1648.61
36–46	1187.59	1187.46
47	175.12	Out of range
48–55	817.44	817.48
56–62	752.47	752.50
63–82	2176.98	2176.75
83–110	3003.57	Not detected

4 Concluding remarks

Based on cDNA library screening, two different amino acid sequences have been described resulting from the exchange of two amino acids [7, 8]. It was yet not proven if both isoforms, which differ only by 5 mass units, are realized in natural isolated Phl p 6. The data presented indicate clearly that nPhl p 6 consists of both described isoforms in nearly equal amounts. Further six N- and C-terminal variants of these two isoforms could be detected reflecting different post-translational processing. The rPhl p 6 is identical in pI and mass to isoform 2 of nPhl p 6 (sequence given in Fig. 4). These results demonstrate that high-resolution mass spectrometric analysis of intact allergens can supply valuable information on the micro-heterogeneity. In contrast to the identification of isoforms on the cDNA level, these methods further may allow the determination of post-translational modifications, e.g., glycosylation, different processing, disulfide bridges, *etc.*

A detailed knowledge of the structure of allergens is a basic requirement for the understanding of the pathomechanism of allergic disorders and it is important for the improvement of diagnosis and therapy of allergic disorders. Post-translational modifications might have an influence on the IgE binding so that the knowledge of the structure of allergens is important for the improvement of diagnostic and therapy of allergic disorders. On the basis of these structural data the IgE-reactive structures can be analyzed and effective vaccines can be designed. The IgE reactivity of the allergens can be decreased by selective mutations and these mutants can be used as moderated components in specific therapy (hyposensitization) [17, 18].

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