

RESEARCH ARTICLE

Characterization of *Phleum pratense* pollen extracts by 2-D DIGE and allergen immunoreactivity

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The allergen content of standardized pollen material is crucial for an effective diagnosis and treatment. However, variations in IgE reactivities of allergic patients to different preparations of *Phleum pratense* pollen have been reported. In order to define and directly compare the allergen composition of pollen preparations provided by different suppliers, a comprehensive proteome analysis of three different timothy grass pollen extracts was performed. More than 140 proteins were annotated comprising the pollen proteome/allergome in a global 2-D map. With regard to the individual pollen preparations, several major differences in the overall protein composition were detected that also affected known *Phleum* allergens and their isoforms. Importantly, these differences were also reflected at the level of antibody reactivities in 1-D and 2-D immunoblots. As a consequence, it is suggested that the observed differences should be taken into consideration aiming for a standardized diagnosis and therapy of grass pollen allergies as recommended by international medical agencies.

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1 Introduction

In industrialized countries, about 10% of the population suffers from (IgE-mediated) allergies against grass pollen that manifest in symptoms of rhinitis, conjunctivitis or bronchial asthma. Timothy grass (*Phleum pratense*) pollens are one of the most common seasonal allergen sources in Europe and the US. They are composed of at least ten structurally distinct allergens, classified into the so-called 'allergen groups' [1]. The allergenic elicitors are components that are quickly released from the pollen after they get into contact with the humid mucosa, e.g. of the respiratory tract.

Although many attempts have been made to alleviate the symptoms, the only curative strategy is an allergen-specific immunotherapy by administration of allergenic extracts in increasing concentrations over time. Although the mechanism is not completely understood, improvement is observed concerning rescue medication score and life quality of the treated grass pollen allergic patients [2]. Although different species of grass pollen share a high degree of homology, they come as heterogeneous mixtures of allergenic and nonallergenic proteins, polysaccharides, irritants and adjuvant factors that vary between species.

Due to the overlapping geographical distribution and similar pollination calendars and because of similar pollen morphology associated with the presence of highly homologous proteins, serum IgE reactivity or skin prick testing often do not allow the identification of individual grass species that cause disease in individual patients [3]. None-

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Abbreviation: ABC, ammonium bicarbonate

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theless, it is still a matter of controversy whether single allergens, defined pollen extracts of a single species or rather pollen mixtures would be more suitable to desensitize allergic patients. Thus, a mixture of pollens from five Pooideae (*Poa pratensis*, *Anthoxanthum odoratum*, *Dactylis glomerata*, *Lolium perenne* and *P. pratense*) has been recently used for allergen-specific immunotherapy for the treatment of common grass pollen allergy [4].

At present, whole pollen extracts are widely employed for diagnosis and therapy. During the last two decades, many individual pollen allergens have been immunologically and biochemically characterized and are available in recombinant form [1]. However, there are several drawbacks for using these recombinant proteins for diagnosis or therapy, since they may differ from their natural counterparts in conformation or structure. Also, posttranslational modifications such as disulfide bonds and glycosylation in the natural proteins or environmental modifications like nitrosylation might influence IgE binding and allergenicity. Moreover, most natural allergens occur in pollen as isoallergens that are proteins of an allergen group with minor structural differences but potential major variances in their reactivity. Also, adjuvant factors such as lipopolysaccharides, bioactive lipid mediators and arabinogallactans add allergenic properties to the complex of natural pollen extracts [5–7]. Although individual components have not routinely been quantified and validated, pollen extracts are less expensive and the presence of most pollen components in a given extract might be beneficial compared to recombinant allergens.

In order to improve diagnosis and immunotherapy, and to determine the biological potency of allergenic pollen extracts, a detailed qualitative and quantitative characterization of their constituents is apparently mandatory.

For the production of allergenic extracts, companies mostly use their individual in-house standards to determine the concentration of one of the major allergens and skin tests as proof of the biological potency of the extract [8]. Sander *et al.* compared grass pollen preparations for skin tests and immunotherapy more in detail [9]. For standardization and comparison of different extract preparations, however, it is necessary to determine not only the concentration of individual allergenic components but also the ratios of the different constituents to account for potential dose-effect relationships. Detailed knowledge of their components and their composition is also requested by a guideline of the European Medicines Agency that has recently come into force (EMA/CHMP/BWP/304831/2007).

We used high-resolution 2-D DIGE to analyze and directly compare three batches of timothy grass pollen from different worldwide suppliers, which form the basis for the production of pharmaceutical extracts for diagnosis and therapy. We compared the different extracts regarding the quantitative and qualitative composition of individual allergens. Moreover, we identified individual allergens and isoforms thereof by mAbs, and tested allergen recognition with patients' immune sera in 1-D and 2-D Western blots.

2 Materials and methods

2.1 Sample preparation

Pollen grains from *P. pratense* were purchased from three manufacturers (Allergon, Ängelholm, Sweden; ARTU Biologicals, Lelystad, The Netherlands; Greer Laboratories, Lenoir, NC, USA; made pseudoanonymous). The supplied batch-specific information regarding collection, processing and contaminants is given in Supporting Information Table S1. Pollen extracts were uniformly prepared by a standardized basic extraction. In brief, 1 g of the supplied grass pollen was incubated in 50 mL of 0.1 M NH_4HCO_3 buffer (pH 8.0) for 30 min simulating the natural mucosal release. Afterward, the soluble fraction was isolated by centrifugation at $17\,000 \times g$ for 30 min and dialyzed against double distilled water overnight. The extract was lyophilized and stored at 4°C until use.

2.2 Sera and mAbs

Sera were drawn from individuals suffering from grass pollinosis with CAP classes > 3 with their informed consent (kindly provided by Dr. Frank Eberhardt, Medical Clinic, Borstel, Germany). Sera from healthy volunteers served as controls. A panel of nine mAbs had been raised against timothy grass pollen extract or a mixture of grass pollen species of the temperate climate in our laboratory [10]. These mAbs are directed against defined grass allergen groups. The mAb 3C4 recognizes group 4 allergens and was kindly provided by Professor Helmut Fiebig (Allergopharma, Reinbek, Germany).

2.3 1-D electrophoresis and Western blot analysis

About 10 µg of protein extract was separated by SDS-PAGE on a 12.5% gel. Following transfer to NC membranes (GE Healthcare, Munich, Germany) by tank-blotting (25 mM Tris, 192 mM Glycine, 20% v/v methanol and 0.015% w/v SDS), blots were developed using mouse mAbs against Phl p 1 (HB7), Phl p 5 (Bo1), Phl p 13 (AF6), Phl p 4 (3C4) and Phl p 6 (Bo12), or anti-Phl p 2/3 rabbit serum with respective HRP-conjugated anti-mouse or anti-rabbit secondary antibodies and ECL detection reagents using ECL Hyper Film (GE Healthcare).

2.4 2-D electrophoresis and Western blot analysis

For 2-D PAGE, 60 µg of lyophilized pollen extract were dissolved in 120 µL 2-D buffer for 20 min (7 M urea, 2 M thiourea and 4% w/v CHAPS). Before separation on 18-cm IPG strips (pH 3–11NL, GE Healthcare), 1% w/v DTT and 1% v/v ampholytes were added. Samples were applied by

cup-loading and isoelectrically focused on 18-cm pH 3-11NL IPG strips using an IPGphor 3 unit (GE Healthcare) at 20°C to a total of 22 kVh, with a maximum voltage of 8000 V (100 V for 6 h, 300 V for 5 h, 500 V for 5 h, gradient from 500 V to 4000 V in 2.5 h, gradient from 4000 V to 8000 V in 1.5 h and 8000 V for 15 min). Subsequent SDS-PAGE separation was performed on 12.5% gels using a Protean II cell (Bio-Rad, Munich, Germany). 2-D gels were transferred to 0.2 µm NC membranes (GE Healthcare) by tank blotting (using the above mentioned buffer). Proteins were stained using Sypro Ruby Blot Stain (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. For Western blot matching, reference markers were placed on the membranes before fluorescent detection. After blocking with 5% w/v BSA in TBS +0.05% Tween, membranes were probed with human control sera (C1 and C2) and reblotted with individual or mixed (1:1) patient's sera (P1 and P2). For reblotting, membranes were incubated in stripping solution (100 mM 2-mercaptoethanol, 2% SDS, 60 mM Tris, pH 6.8) for 30 min at 56°C. To overlay the Western blot signals, total protein stain images were loaded into Adobe's Photoshop (version 7.01), digitally recolored and used as background. Grey scale Western blot images were transformed into RGB colors, normalized and stained in red or green. Blot stain and Western blot images were then combined in a new image using reference markers on both image layers where the mode for the foreground layer was set to multiplication.

2.5 Fluorescent labeling and 2-D DIGE

For 2-DE, 100 µg of lyophilized pollen extracts were dissolved on ice with 50 µL DIGE-buffer (pH 8.5) containing 7 M urea, 2 M thiourea, 30 mM Tris and 4% w/v CHAPS. The labeling with CyDyes and the subsequent 2-D separation were described elsewhere [11]. Briefly, 50 µg of protein *per* sample were labeled with equal amounts of Cy5 or Cy3 (CyDyes, GE Healthcare) and spiked with 50 µg of unlabeled protein. Mixed samples were applied by cup loading on 24 cm IPG-strips rehydrated at 20°C in 450 µL DeStreak solution (GE Healthcare) with 0.5% v/v IPG buffer. IEF was carried out for a total of 60 kVh for pH 3-11NL at 20°C with gradual voltage increase (150 V for 3 h, 300 V for 3 h, 500 V for 3 h, gradient from 500 to 1000 V in 6 h, gradient from 1000 to 10 000 V for 4 h and 10 000 V until total kVh were reached). After reduction and alkylation of the gel strips, the second dimension was performed on 12.5% polyacrylamide gels using Ettan Dalt 6 equipment (GE Healthcare) under continuous cooling at 20°C.

2.6 Image analysis and spot picking

The in-gel fluorescence was scanned on a Typhoon Trio (GE Healthcare) at appropriate wavelengths to detect Cy3 and Cy5-specific emissions corresponding to the protein

concentration in every single spot. The intra-gel spot detection of the multiplexed gel images was performed using differential in-gel analysis included in the DeCyder 6.5 software (GE Healthcare). Images for each gel were merged; spot boundaries were detected followed by a normalization of the spot volumes. For spot picking, gels were stained with Flamingo Pink (Bio-Rad) following the manufacturer's instructions. Image Master 6.0 software was used for spot selection. Selected spots were picked automatically on an Ettan Spotpicker (GE Healthcare) using a 2-mm picking head. The picked gels were again scanned to document the locations of the punched spots.

2.7 In-gel tryptic cleavage and sample processing

Isolated spots were washed three times with HPLC grade water, dehydrated in a first step with 25 mM ammonium bicarbonate (ABC) in 50% ACN and shrunk in pure ACN as a second step, according to Schmidt *et al.* [12]. The dry gel pieces were then rehydrated with 100 ng sequencing-grade porcine trypsin (Serva, Heidelberg, Germany) in 10 µL of 5 mM ABC. After rehydration, 10 µL of 5 mM ABC was added. For tryptic in-gel digestion, samples were incubated at 37°C overnight. To extract the peptides, 20 µL of 0.3% TFA in ACN was added and the samples were sonicated in a water bath (Sonorex Super RK100; Bandelin, Berlin, Germany) for 15 min. Liquid phases were collected, lyophilized, redissolved in 0.8 µL MALDI matrix solution (3.2 mg/mL CHCA (LaserBio Labs, Sophia-Antipolis, France) in 65% ACN/0.1% TFA), spotted on 192 well stainless steel MALDI plates and air-dried.

2.8 MS and database analysis

Mass spectrometric measurements of tryptic peptides were performed on a 4700 Proteomics analyzer MALDI-TOF/TOF instrument (Applied Biosystems, Framingham, MA, USA) as basically described in Schmidt *et al.* [13]. Briefly, MS spectra were acquired by accumulation of 2000 laser shots. A maximum of five precursor ions were fragmented by MS/MS. Precursor ions were selected with 0.2 Da mass tolerance. MS spectra were calibrated internally using autolytic fragments of porcine trypsin with 50 ppm mass tolerance. For MS/MS measurements, the instrument default calibration was updated prior to the run. Expected tryptic autolysis product peaks were excluded from the selection. Spectral data were processed by the GPS Explorer 3.6 software, submitted to MASCOT 2.0 (Matrix Science, London, UK) and searched against the in-house NCBI nonredundant database (NCBI nr, date 02/23/2009) confined to proteins from viridiplantae (7 894 593 protein entries). The obligate modification on cysteine residues by carbamidomethylation, potential modification of methionine by oxidation, a maximum of one missed tryptic cleavage site in

a peptide fragment. The mass tolerance of precursor was set to 50 ppm, and the fragment ion mass tolerance was set to 0.2 Da (MS/MS). A protein was accepted to be identified with a probability greater than 95% according to MASCOT's total protein score (≥ 70).

For greater stringency of the database search results, the false-positive rate was determined, *i.e.* the searches were repeated using the same search parameters against the same database that was previously randomized keeping the accessions of each entry using the decoy.pl script (http://www.matrixscience.com/help/decoy_help.html). Using all analyzable spectral data the false-positive rate was calculated to be 5.6%.

3 Results

3.1 Pollen extracts differ in their overall protein composition

In order to define their constituents and to address the variability of individual pollen preparations, timothy grass (*P. pratense*) pollens from three different suppliers were prepared by standard basic extraction. The characteristics of the individual pollen preparations with regard to their origin, collection mode, processing and potential contaminants are listed in the Supporting Information Table S1. When equal

amounts of the three extracts were compared by 1-D gel electrophoresis, we noticed differences in the overall protein distribution (Fig. 1A) that were also reflected at the level of IgE-reactivities using sera from individuals suffering from grass pollinosis (not shown). Based on this, we decided to analyze and to compare the pollen extracts at a higher resolution and applied them to 2-DE (Fig. 1B–D). Here, although the overall spot patterns were very similar, we noticed one major difference in extract 2 that lacked characteristic sets of proteins with molecular masses around 30 and 50–70 kDa. In order to define the observed differences more precisely, the individual pollen extracts were labeled pairwise with Cy5 or Cy3, respectively, and compared by 2-D difference gel electrophoresis. To exclude dye-specific labeling, additional replicates were performed in dye-swap experiments. Moreover, a mixture of equal amounts of the three extracts were labeled with Cy2 and used as an internal standard (not shown). On average, in each of six separate gels 1090 spots were detected (Table 1). The statistical analysis of the performed DIGE experiments allowed a pairwise comparison of the protein patterns (Fig. 2D–F) and revealed that extracts 1 and 3 only differed in 9% of the included spots. In contrast, extract 2 contained about 25% differentially abundant proteins when compared to extracts 1 or 3.

To pinpoint the differences between individual extracts, we punched out the respective spots for subsequent mass

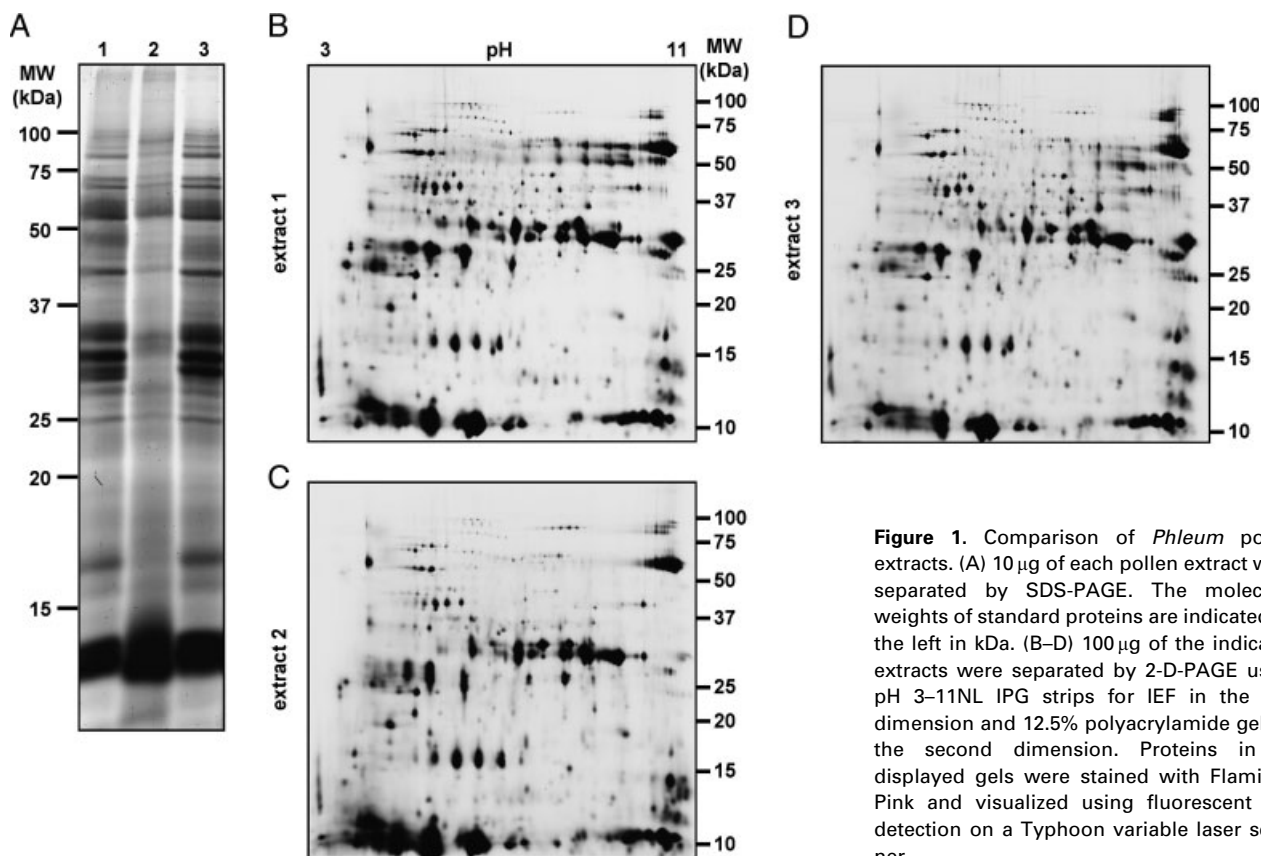


Figure 1. Comparison of *Phleum* pollen extracts. (A) 10 μ g of each pollen extract were separated by SDS-PAGE. The molecular weights of standard proteins are indicated on the left in kDa. (B–D) 100 μ g of the indicated extracts were separated by 2-D-PAGE using pH 3–11NL IPG strips for IEF in the first dimension and 12.5% polyacrylamide gels in the second dimension. Proteins in all displayed gels were stained with Flamingo Pink and visualized using fluorescent dye detection on a Typhoon variable laser scanner.

spectrometrical analysis. We identified 144 protein spots, 74 of which were annotated as timothy grass allergens (Supporting Information Table S2). The allergen spectrum covered nine of ten known *Phleum* allergens (Supporting Information Table S3). For verification, individual proteins were identified at corresponding gel locations in up to six independent experiments (*e.g.* spot #62 in Supporting Information Table S2).

3.2 Different abundances of major allergens mostly concern individual isoforms

For the detailed analysis of the allergen composition of a given extract, individual protein spots were identified by MS and also by immunoreactivity. Phl p 1 represents one of the major grass pollen allergens. Different isoforms/fragments of Phl p 1 were identified in 21 distinct spots with molecular masses between 20 and 33 kDa in a fairly broad *pI* range of 5.8–8.5. Interestingly, the distribution of individual isoproteins or fragments was not equal in all extracts. For example, the Phl p 1 spot #82 was present at comparable levels, while the isoform in spot #113 (at a slightly lower molecular weight) was only detectable in extract 2. This observation was verified in four technical replicates with a calculated difference of up to 70-fold (Fig. 3). Phl p 5 also represents a dominant allergen of *P. pratense* with an IgE prevalence of >90%. It consists of two major subgroups with apparent molecular masses of 33 kDa (Phl p 5a) and 27 kDa (Phl p 5b). Each isoform, however, is split into at least four isoallergens, that again showed differences in the three extracts as revealed in four separate experiments. Phl p 5 spot #87 is increased by 7.5-fold in extract 2, whereas Phl p 5 spot #104 is reduced in extract 2 by up to 54-fold.

Another difference between the extracts concerns isoallergens of the high molecular mass allergen group Phl p 13.

This putative polygalacturonase was identified in at least seven separate spots with an average molecular mass of 50 kDa and *pI* of 8.0. As demonstrated in Fig. 3, Phl p 13 was markedly reduced in extract 3 and was not detected in extract 2. Interestingly, polygalacturonase was also identified in spot #69 at a different location (MW 35 kDa, *pI* 6.5). The three given examples not only highlight the reproducibility of the used 2-D approach but also stress the potency to qualitatively and quantitatively characterize individual allergens and isoallergens in a given extract. For more information on different volume ratios of individual spots, please refer to Supporting Information Table S2.

3.3 Verification of DIGE results by Western blotting

We next addressed the question whether the differential distribution of individual allergens or isoallergens was also reflected at the level of antibody reactivities. Here we focused on antibodies against the seven major pollen allergens (Phl p 1, 2, 3, 4, 5, 6 and 13) that usually show an IgE prevalence of more than 50% (Supporting Information Table S3). In a first series of 1-D Western blots, antibodies against Phl p 1, 2/3, 4 and 6 did not reveal major differences in immunostaining of the individual extracts (Fig. 4). In contrast, differences in Phl p 5 and Phl p 13 immunostaining became apparent that also indicated reactivities to proteins at different molecular masses, *e.g.* comparing extract 2 to extracts 1 or 3 (Fig. 4).

For the subsequent 2-D Western blots, we therefore focused on Phl p 5 and Phl p 13. We used the Phl p 5-specific antibody Bo1 that recognizes both subgroups 5a and 5b. In 2-D immunoblots, antibody reactivities revealed three spots with similar molecular masses but different *pI* for both subgroups (Fig. 5). In extract 1, however, the antibody Bo1 presumably recognized additional forms of

Table 1. Experimental design for comparison of different standard pollen extracts

Sample 1	Sample 2	Gel no.	2 SD	Included	Decreased in sample 2		Increased in sample 2	
					Total	In %	Total	In %
Extract 2	Extract 3	Dige 83, 33773	3.936	1052	96	9.1	158	15.0
		Dige 97, 72992	4.091	1095	128	11.7	160	14.6
		Mean			112	10.4	159	14.8
Extract 2	Extract 1	Dige 83, 33772	4.336	991	109	11.0	131	13.2
		Dige 97, 72991	4.429	1138	131	11.5	117	10.3
		Mean			120	11.3	124	11.8
Extract 3	Extract 1	Dige 83, 33774	2.427	1085	60	5.5	31	2.9
		Dige 97, 72993	2.483	1181	64	5.4	51	4.3
		Mean			62	5.5	41	3.6

Samples were labeled as indicated with fluorescent dyes. The 2 SD (2-fold square deviation) indicates differences in the spot volume ratio for the raw data of all included spots in a given gel. In a normally distributed data set, 95% of the spots fall within this value. In other words, a low number indicates a higher similarity between the extracts. A given protein in a differential spot is defined to be increased or decreased when its relative spot volume is beyond a threshold of 2.5.

Phl p 5a (spot #108) and Phl p 5b (spot #89) that were also identified as differentially abundant spots in 2-D DIGE experiments. Of note, the 2-D-Western blot with Bo1 confirmed that Phl p 5 isoforms in extract 2 display a slightly reduced molecular mass (see also Fig. 4).

In case of Phl p 13, the antibody AF6 was used (Fig. 5D–F). Besides the seven Phl p 13 proteins annotated in the 2-D map, four additional spots displayed immunoreactivity in extract 1. Among them, spots #44 and #45 were identified as “predicted protein” of *Populus trichocarpa* (gi:222849203), which according to the Gene Ontology database also represents a polygalacturonase. Surprisingly, the antibody AF6 also stained a group of 12 additional spots below 45 kDa in extract 3 (Fig. 5F), which was also detected as a prominent band in 1-D blots before (Fig. 4). In addition, spot #69 (35 kDa, pI 6.6) was verified by AF6-reactivity as a Phl p 13 fragment. The comparison of the Phl p 13 spot patterns indicates a complete loss of high molecular mass proteins in extract 2 that is associated with the appearance of relatively minor Phl p 13 spots at lower molecular masses.

3.4 Serum reactivities to different standard pollen extracts

To test the actual relevance of the differential extract composition regarding patients' immunoreactivities, we applied two sera from grass pollen allergic patients and from two healthy control subjects to 2-D immunoblots

(Fig. 5A–C). The gels were loaded with equal amounts of protein and processed under internally standardized conditions concerning incubation and exposure times. The control sera stained up to four immunoreactive spots that, however, were similar in all three extracts. In contrast, the patients' sera revealed the clear differences in immunoreactive proteins present in the individual preparations. Thus, the pooled patients' sera detected 16 spots in extract 1, 7 in extract 3 and only 5 in extract 2. According to the 2-D map established in this study, individual reactivities could be assigned to the major allergens Phl p 1, Phl p 4 and Phl p 5. In addition immunoreactivity to Phl p 13 was detected after prolonged exposure times in extracts 1 and 3. Notably, the aforementioned differences in Phl p 13 patterns and the two different Phl p 5 isoallergens were also seen at the level of immunoreactivities (Supporting Information Fig. S2).

4 Discussion

The direct comparison of grass pollen extracts from three different suppliers by 1-D electrophoresis indicated variations in the protein content that was also reflected at the level of individual major allergens. This was verified by quantitative 2-D PAGE, SDS-PAGE, and Western blotting using specific mAbs and immune sera of pollen allergic patients.

In the present study, we mainly focused on *Phleum* proteins that displayed significant differences in their

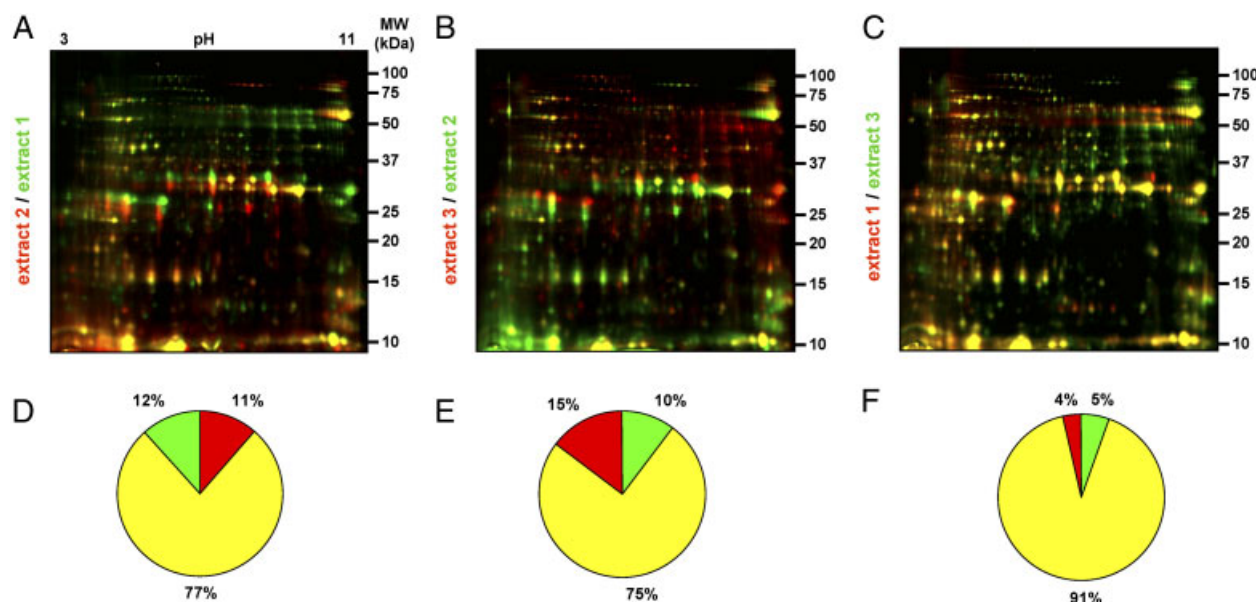


Figure 2. 2-D DIGE analysis of *Phleum* pollen extracts. 150 µg of pollen extract were applied on a 2-D PAGE using pH 3–11NL IPG strips and 12.5% polyacrylamide gels. 50 µg of each pollen lysate were labeled with CyDyes as follows: (A) extract 2 Cy5 (red), extract 1 Cy3 (green); (B) extract 3 Cy5, extract 2 Cy3; (C) extract 1 Cy5, extract 3 Cy3. Proteins were visualized using fluorescent dye detection at variable wavelength (Cy3: 580 BP30 nm, Cy5: 670 BP30 nm) on a Typhoon laser scanner. Spot detection and data interpretation using differential in-gel analysis software resulted in spots with different volume ratios displayed in corresponding charts (D–F) using the following color coding: yellow = similar; red = increased in Cy5 channel; green = increased in Cy3 channel when setting the threshold to 2.5.

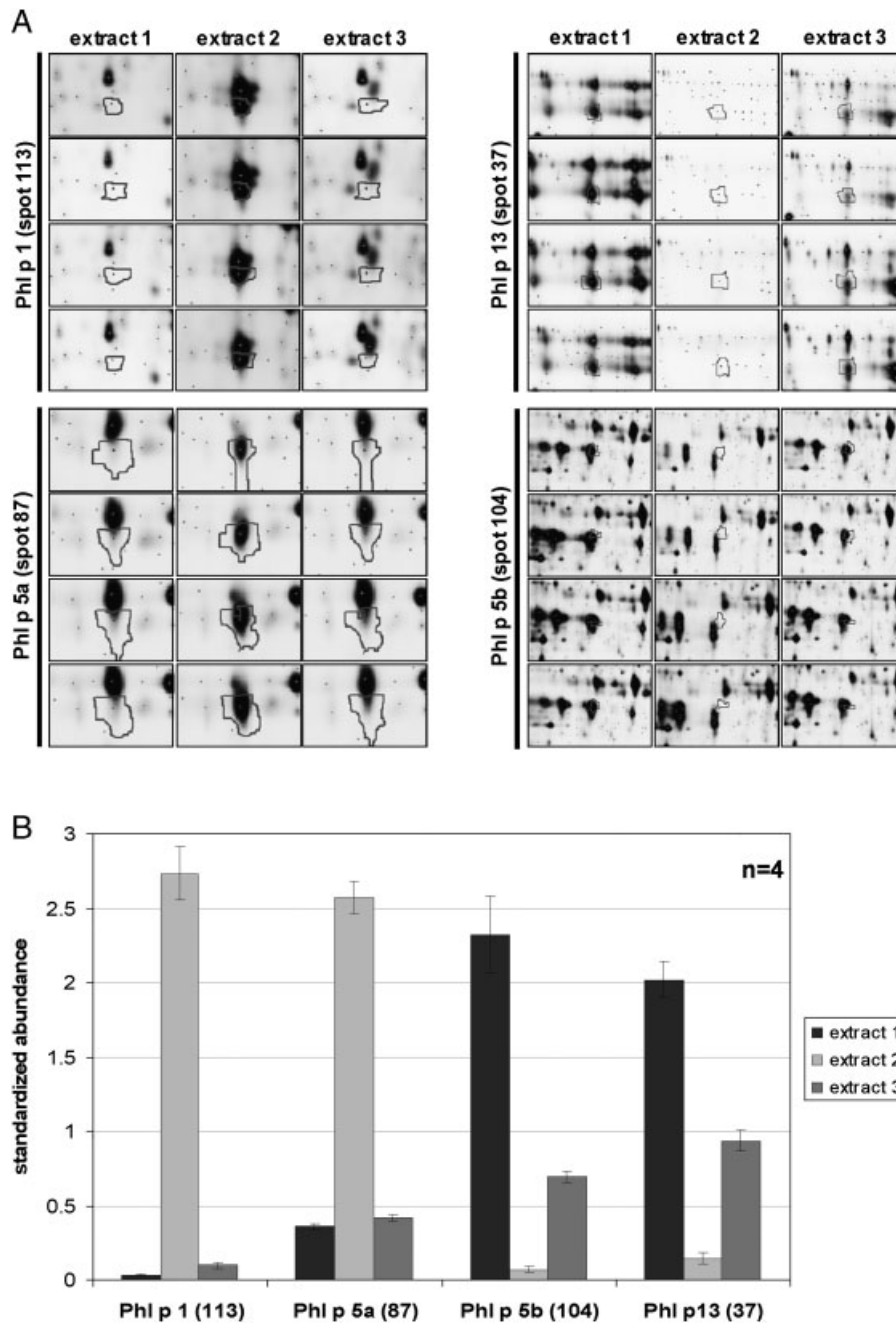


Figure 3. Variations in selected spot volumes. Spot patterns of four individual gels (see Table 1) were matched using biological variance analysis software. Protein spots of four characteristic allergens (A; see also Supporting Information Fig. S1) were selected for statistical evaluation (B).

relative or absolute abundances in individual grass pollen preparations. Therefore, MS was primarily used to identify protein spots in 2-D DIGE gels that were altered by at least 2.5-fold with regard to their determined volume ratios. Since mass spectrometric analyses require comprehensive databases, we had to extend the incomplete database available for *P. pratense* (with only 152 entries) by searching for the obtained peptide masses in a database restricted to green plants. Although this strategy yielded high identification rates in some instances, the data interpretation has to take into account that identified proteins were occasionally

annotated to other plant species (see Supporting Information Table S2).

Nine of ten allergen groups known for timothy grass pollen were identified (Supporting Information Table S3). Only Phl p 7, a small allergen with a low IgE prevalence (Supporting Information Table S3), could not be annotated, since the separation conditions were optimized for 10–100 kDa. Notably, Phl p 4, one of the major pollen allergens characterized by a *pI* around 9.0 and an apparent mass of 55 kDa, was present in comparable amounts in all three extracts (identified in spots #27–32). Its major location

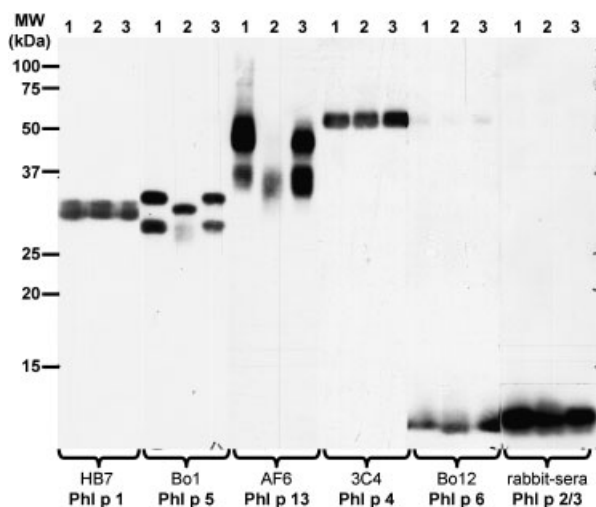


Figure 4. Western blot analysis of major allergens in different pollen extracts. 10 µg of each pollen extract (1–3) were separated on 12.5% polyacrylamide gels and transferred to a NC membrane. The membrane was split and incubated with six specific antibodies against Phl p 1 (HB7), Phl p 5 (Bo1), Phl p 13 (AF6), Phl p 6 (Bo12) and Phl p 2/3, followed by HRP-conjugated second step antibodies and ECL detection.

matched published 2-D Western blots [14]. Also the similar immunoreactivity of patients' sera supported the assumption that Phl p 4 was not significantly altered in the individual preparations. The identification of two spots at 90 kDa (#27,28) as Phl p 4 in extracts 2 and 3 pointed to a putative dimerization or isoforms that differ by their carbohydrate content. In this context, it has been reported that post-translationally modified Phl p 4 might contain up to 10–15% carbohydrates and exhibits thermal stability and protease resistance [15, 16].

The most abundant allergen spots represent group 1, 5 and 13 allergens. In accordance with published data (reviewed in [1]), Phl p 1 isoforms displayed an average molecular weight mass of about 30 kDa. Interestingly, six additional spots (#111–116) were identified as Phl p 1 by peptide mass fingerprinting at a molecular mass region below 28 kDa in extract 2. These fragments might represent processed variants of Phl p 1 that were, however, not recognized by Phl p 1-specific mAbs (Fig. 4).

The two subgroups of Phl p 5 (Phl p 5a and Phl p 5b) consist of at least four isoallergens that migrate in the range of 27–33 kDa in SDS-PAGE [17]. In line with published data [18], we clearly discriminated the two subgroups. They do not only differ in their primary amino acid sequence (~30%), but also in molecular characteristics with a slightly higher mass and a more basic *pI* in the case of Phl p 5a. Applying 2-D DIGE in combination with Phl p 5-specific mAbs, we detected additional variants of Phl p 5a (#89) and 5b (#108) in extract 1 (Supporting Information Fig. S2). Importantly, these variants were recognized in immunoblots by sera of pollen allergic patients indicating that they

in fact serve as relevant allergens. Of note, although the extracts 1 and 3 were similar in their overall protein composition, the additional Phl p 5 variants were not detected in extract 3. Thus, the observed immunoreactivity stresses the question of the importance of individual isoallergens for diagnosis and therapy [19].

Phl p 13 proteins form the most recently described group of grass pollen allergens that migrate as two major bands around 55 kDa in 1-D electrophoresis [20]. In accordance with previous results of our group [21], we detected four individual spots as components of the upper band in extract 1 and at least six individual spots forming the lower band using the Phl p 13-specific antibody AF6 (Fig. 5D). Surprisingly, this monoclonal antibody also reacted with several other spots at lower molecular masses in extracts 2 and 3, indicating that these extracts might contain processed Phl p 13 compounds (Fig. 5E and F). Previous observations indicated that purified Phl p 13 tended to degrade at 4°C whereas it appeared stable in extracts [21]. However, besides partial degradation, also seasonal or local variations of the overall protein content of *Phleum* grass pollen might alter the total allergen composition [22].

The applied 2-D DIGE approach did not only reveal major differences in protein distribution, but also indicated small changes in spot abundancies in individual extracts. Furthermore, combining high-resolution 2-D electrophoresis and immunoblotting, the reactivity-spectra of allergic patients can be displayed at the level of single allergen isoforms as demonstrated in this paper (Supporting Information Fig. S2). This also allows a comparison of recombinant allergens with their natural counterparts in a given extract and might help to discriminate between 'allergenic' and 'immunogenic' isoforms, which in turn might be more suitable to be used for immunotherapy [23]. Moreover, as recently demonstrated by Fenaille *et al.* for group 1 grass pollen allergens, MS might eventually lead to the detailed analysis of posttranslational modifications (*e.g.* proline hydroxylation, *N*-glycosylation) of candidate allergens [24].

A further advantage of 2-D DIGE/immunoblotting compared to antibody-based detection systems (*e.g.* ELISA [25]) is the fact that more isoallergens or variants can be detected, discriminated and quantified. This detailed discrimination proved to be important with regard to the immunoreactivity of Phl p 13 mAbs toward intact and degraded proteins in extract 1 and 3. Here, the patients' sera only recognized the intact form of Phl p 13 in extract 1. Consequently, it is important to define the exact composition of commercially available pollen extracts, since these extracts eventually might serve as starting material for products that will be used for diagnosis, therapy monitoring and even for the treatment of pollen allergy. The immunological consequence of underrepresented or even missing allergens would be an incomplete induction of protective immunity or even a new sensitization against further extract components [26].

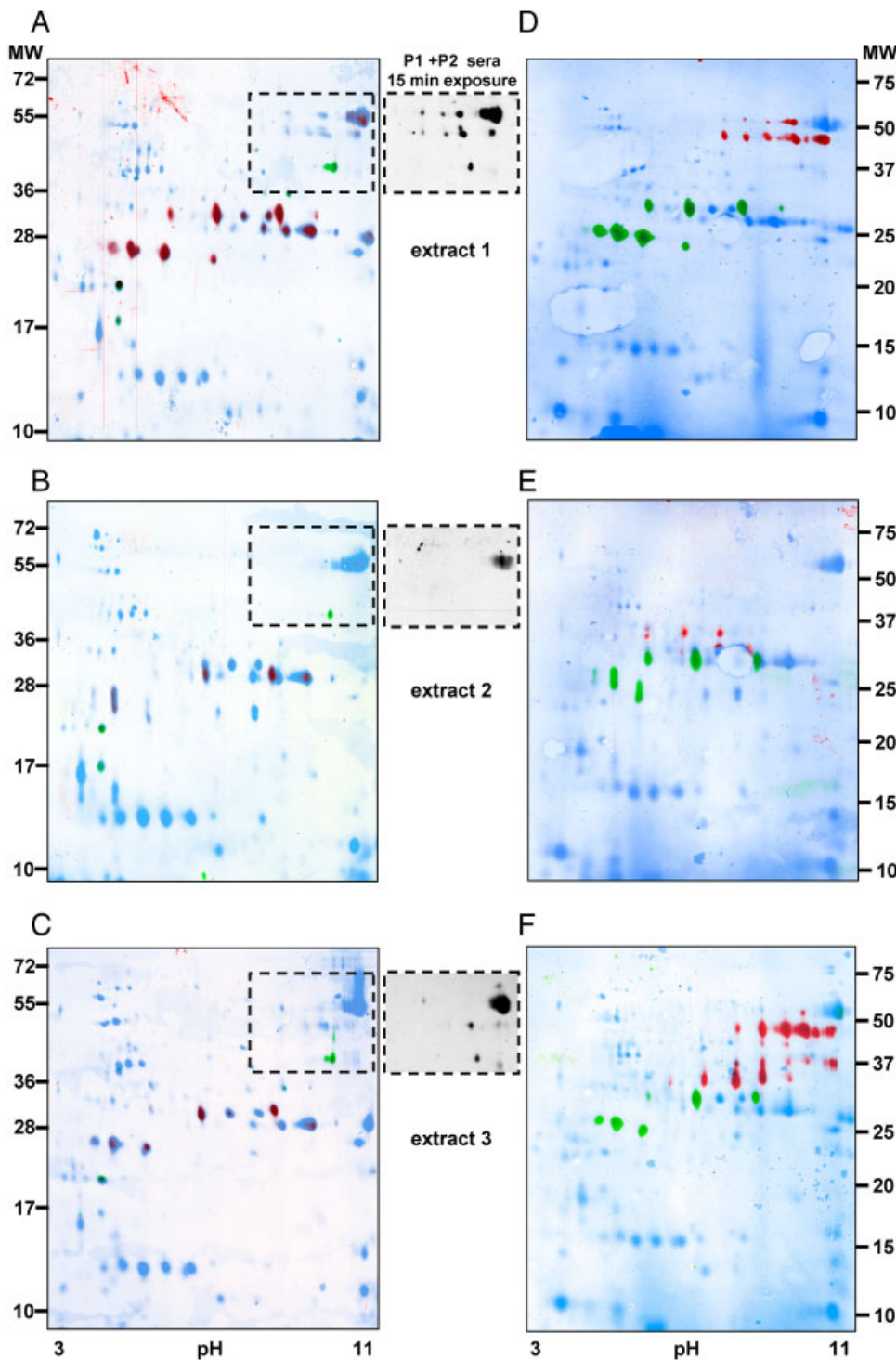


Figure 5. 2-D Western blot analysis of extracts with patients' sera (A–C), Phl p 5- and 13-specific antibodies (D–F). Pollen extracts (60 µg) from different suppliers were separated on 18 cm pH 3–11NL IPG strips and 12.5% polyacrylamide gels, transferred to NC membranes and total proteins (blue) were visualized by Sypro Ruby blot staining. Blots (A–C) were incubated with control sera (green) and reprobed with pooled patients' sera (P1+P2, red). Prolonged exposure time reveals additional differences of spots identified as Phl p 13. In another experiment, blots (D–F) were incubated subsequently with Phl p 5-(green) and Phl p 13-(red) directed mAbs followed by ECL detection. Equal exposure times were applied in each set of experiments. Pictures were overlaid as described in Section 2.

Possible means to circumvent different allergen compositions are the addition of defined recombinant allergens to a basic extract or finally, the replacement of natural extracts with mixtures of recombinant allergens [27, 28]. Component-resolved diagnostics by use of arrays with single allergens is already available and is performed to some extent

[29]. As a logical consequence, patients could be treated individually with those components to which they display IgE-reactivity and clinical symptoms. Nonetheless, also for these future strategies, it is prerequisite to identify those isoforms that can trigger the desired immunodeviation being immunogenic but not allergenic.

Our study clearly demonstrates that neither the International Union of Immunological Societies allergen standards nor mAbs alone suffice to define and to standardize complex allergen extracts. Nonetheless, standardization is a recommendation and a concern of the regulatory authorities in order to improve allergy diagnostic and specific immunotherapy (EMA/CHMP/BWP/304831/2007). For this, attempts such as the CREATE approach [30] are performed to achieve improved standards by comparing different allergen preparations and assays. In this regard, our multiplex approach using 2-D DIGE, immunoblotting and MS might be suitable to unravel the structural variability in complex pollen extracts.

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5 References

- [1] Andersson, K., Lidholm, J., Characteristics and immunobiology of grass pollen allergens. *Int. Arch. Allergy Immunol.* 2003, **130**, 87–107.
- [2] Pfaar, O., Anders, C., Klimek, L., Clinical outcome measures of specific immunotherapy. *Curr. Opin. Allergy Clin. Immunol.* 2009, **9**, 208–213.
- [3] Moingeon, P., Hrabina, M., Bergmann, K. C., Jaeger, S. *et al.*, Specific immunotherapy for common grass pollen allergies: pertinence of a five grass pollen vaccine. *Int. Arch. Allergy Immunol.* 2008, **146**, 338–342.
- [4] Suphioglu, C., What are the important allergens in grass pollen that are linked to human allergic disease? *Clin. Exp. Allergy* 2000, **30**, 1335–1341.
- [5] Allakhverdi, Z., Bouguermouh, S., Rubio, M., Delespesse, G., Adjuvant activity of pollen grains. *Allergy* 2005, **60**, 1157–1164.
- [6] Gilles, S., Mariani, V., Bryce, M., Mueller, M. J. *et al.*, Pollen allergens do not come alone: pollen associated lipid mediators (PALMS) shift the human immune systems towards a T(H)2-dominated response. *Allergy Asthma Clin. Immunol.* 2009, **5**, 3–.
- [7] Brecker, L., Wicklein, D., Moll, H., Fuchs, E. C. *et al.*, Structural and immunological properties of arabinogalactan polysaccharides from pollen of timothy grass (*Phleum pratense* L.). *Carbohydr. Res.* 2005, **340**, 657–663.
- [8] Larenas-Linnemann, D., Cox, L. S., European allergen extract units and potency: review of available information. *Ann. Allergy Asthma Immunol.* 2008, **100**, 137–145.
- [9] Sander, I., Fleischer, C., Meurer, U., Bruning, T. *et al.*, Allergen content of grass pollen preparations for skin prick testing and sublingual immunotherapy. *Allergy* 2009, **64**, 1486–1492.
- [10] Petersen, A., Suck, R., Lindner, B., Georgieva, D. *et al.*, Phl p 3: structural and immunological characterization of a major allergen of timothy grass pollen. *Clin. Exp. Allergy* 2006, **36**, 840–849.
- [11] Schmidt, H., Gelhaus, C., Latendorf, T., Nebendahl, M. *et al.*, 2-D DIGE analysis of the proteome of extracts from peanut variants reveals striking differences in major allergen contents. *Proteomics* 2009, **9**, 3507–3521.
- [12] Schmidt, H., Gelhaus, C., Lucius, R., Nebendahl, M. *et al.*, Enrichment and analysis of secretory lysosomes from lymphocyte populations. *BMC Immunol.* 2009, **10**, 41.
- [13] Schmidt, H., Krause, S., Gelhaus, C., Petersen, A. *et al.*, Detection and structural characterization of natural Ara h 7, the third peanut allergen of the 2S albumin family. *J. Proteome Res.* 2010, **9**, 3701–3709.
- [14] Rogerieux, F., Godfrin, D., Senechal, H., Motta, A. C. *et al.*, Modifications of *Phleum pratense* grass pollen allergens following artificial exposure to gaseous air pollutants (O(3), NO(2), SO(2)). *Int. Arch. Allergy Immunol.* 2007, **143**, 127–134.
- [15] Fischer, S., Grote, M., Fahlbusch, B., Muller, W. D. *et al.*, Characterization of Phl p 4, a major timothy grass (*Phleum pratense*) pollen allergen. *J. Allergy Clin. Immunol.* 1996, **98**, 189–198.
- [16] Fahlbusch, B., Muller, W. D., Rudeschko, O., Jager, L. *et al.*, Detection and quantification of group 4 allergens in grass pollen extracts using monoclonal antibodies. *Clin. Exp. Allergy* 1998, **28**, 799–807.
- [17] Petersen, A., Becker, W. M., Schlaak, M., Characterization of isoforms of the major allergen Phl p V by two-dimensional immunoblotting and microsequencing. *Int. Arch. Allergy Immunol.* 1992, **98**, 105–109.
- [18] Becker, W. M., Bufe, A., Petersen, A., Schlaak, M., Molecular characterization of timothy grass pollen group V allergens. *Int. Arch. Allergy Immunol.* 1995, **107**, 242–244.
- [19] Lowenstein, H., Sparholt, S. H., Klysner, S. S., Ipsen, H. *et al.*, The significance of isoallergenic variations in present and future specific immunotherapy. *Int. Arch. Allergy Immunol.* 1995, **107**, 285–289.
- [20] Suck, R., Hagen, S., Cromwell, O., Fiebig, H., The high molecular mass allergen fraction of timothy grass pollen (*Phleum pratense*) between 50–60 kDa is comprised of two major allergens: Phl p 4 and Phl p 13. *Clin. Exp. Allergy* 2000, **30**, 1395–1402.
- [21] Petersen, A., Suck, R., Hagen, S., Cromwell, O. *et al.*, Group 13 grass allergens: structural variability between different grass species and analysis of proteolytic stability. *J. Allergy Clin. Immunol.* 2001, **107**, 856–862.
- [22] Gavrovic, M. D., Trtic, T., Vujcic, Z., Petrovic, S. *et al.*, Comparison of allergenic potentials of timothy (*Phleum pratense*) pollens from different pollen seasons collected in the Belgrade area. *Allergy* 1997, **52**, 210–214.
- [23] Corti, V., Cattaneo, A., Bachi, A., Rossi, R. E. *et al.*, Identification of grass pollen allergens by two-dimensional gel

- electrophoresis and serological screening. *Proteomics* 2005, 5, 729–736.
- [24] Fenaille, F., Nony, E., Chabre, H., Lautrette, A. *et al.*, Mass spectrometric investigation of molecular variability of grass pollen group 1 allergens. *J. Proteome. Res.* 2009, 8, 4014–4027.
- [25] Focke, M., Marth, K., Flicker, S., Valenta, R., Heterogeneity of commercial timothy grass pollen extracts. *Clin. Exp. Allergy* 2008, 38, 1400–1408.
- [26] Mothes, N., Heinzkill, M., Drachenberg, K. J., Sperr, W. R. *et al.*, Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. *Clin. Exp. Allergy* 2003, 33, 1198–1208.
- [27] Lundberg, M., Chen, Z., Rihs, H. P., Wrangsjo, K., Recombinant spiked allergen extract. *Allergy* 2001, 56, 794–795.
- [28] Heiss, S., Mahler, V., Steiner, R., Spitzauer, S. *et al.*, Component-resolved diagnosis (CRD) of type I allergy with recombinant grass and tree pollen allergens by skin testing. *J. Invest. Dermatol.* 1999, 113, 830–837.
- [29] Hiller, R., Laffer, S., Harwanegg, C., Huber, M. *et al.*, Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. *FASEB J.* 2002, 16, 414–416.
- [30] van Ree, R., Chapman, M. D., Ferreira, F., Vieths, S. *et al.*, The CREATE project: development of certified reference materials for allergenic products and validation of methods for their quantification. *Allergy* 2008, 63:310–326.

PROTEOMICS

Supporting Information for Proteomics

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Characterization of *Phleum pratense* pollen extracts by 2-D DIGE and allergen immunoreactivity

Characterization of *Phleum pratense* pollen extracts by 2D-DIGE and allergen immunoreactivity

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Supplemental Tables

Table S1. Timothy grass (*Phleum pratense*) pollen grains used in this study.

the pollen batches were made pseudoanonymous			
Manufacturer	Allergon	ARTU Biologicals	Greer Laboratories
Origin	Sweden	USA, MN	USA, MN
Batch	011308102 (SP-17/2)	24PP28-7C	P235947-2
Collection date	September 2008	July 2001	July 2007
Shelf life	September 2013	not stated	July 2017
Collection mode	cut flower heads	vacuum, sieve	vacuum, sieve
Vacuum drying	<38°C, >24 h	35 - 41°C, 12 – 24 h	3.95 % humidity (<4.5%)
Storage	2-8°C	not specified	<0°C
Delipidation	no	no	no
Contaminants	0.1% pollen 0.0% mold spores	1% pollen 1% mold spores	0.37% pollen and/or mold spores

Table S2. Identified proteins by mass spectrometry.

The table lists the spot number (a) corresponding to figure S2 with the number of iterant identifications (b), the respective accession number of the proteins in NCBI (c) and Uni-Prot (d), the theoretical molecular weights (MW) (e) and isoelectric points (*pI*) (f), the number of matched peptides (g) and the sequence coverage (h). The total MASCOT score (i) indicates the probability of the identification (>95%, if MASCOT ≥ 70). The last three columns display the volume ratios of single protein spots in the assigned pollen extracts. Decreased and increased spot abundance ratios are marked in red and blue, respectively.

spot No. ^{a)}	replicates ^{b)}	Protein Name	Species	Acc. No. ^{c)}	UniProt No. ^{d)}	MW ^{e)}	<i>pI</i> ^{f)}	matched peptides ^{g)}	sequence coverage ^{h)}	Mascot score ⁱ⁾	extract 1/ extract 2	extract1/ extract 3	extract 3/ extract 2
1	1	putative aconitate hydratase, cytoplasmic	Oryza sativa	gi 222624156	Q6YZX6	99391	5.8	12	11	103	1.89	-2.12	3.11
2	1	putative aconitate hydratase, cytoplasmic	Oryza sativa	gi 222624156	Q6YZX6	99391	5.8	16	16	162	1.24	-2.40	2.62
3	2	alpha 1,4-glucan phosphorylase H isozyme	Oryza sativa	gi 12025466	Q9FPE6	91833	7.4	7	10	81	-3.58	1.01	-4.79
4	3	alpha 1,4-glucan phosphorylase H isozyme	Oryza sativa	gi 12025466	Q9FPE6	91833	7.4	8	12	96	-3.87	-1.31	-3.67
5	1	alpha 1,4-glucan phosphorylase H isozyme	Triticum aestivum	gi 14916632	Q9FPE6	93952	7.3	20	22	132	-3.60	-1.93	-2.53
6	1	alpha 1,4-glucan phosphorylase H isozyme	Triticum aestivum	gi 14916632	Q9FPE6	93952	7.3	21	22	182	-3.04	2.05	-2.01
7	1	alpha 1,4-glucan phosphorylase H isozyme	Triticum aestivum	gi 14916632	Q9FPE6	93952	7.4	19	20	161	-2.14	-2.14	-1.16
8	1	pollen allergen Phl p 11/12	Phleum pratense	gi 3914432	O24650	14255	4.9	2	17	194	1.03	-1.75	-2.12
9	1	pollen allergen Phl p 11/12	Phleum pratense	gi 3914432	O24650	14255	4.9	5	52	229	1.00	-1.28	-2.35
10	1	calreticulin	Hordeum vulgare	gi 439588	Q40041	47558	4.5	4	11	196	2.93	1.28	-1.02
11	2	phosphoglucumutase	Triticum aestivum	gi 18076790	Q8VX48	62978	5.7	23	35	265	3.04	-1.33	3.53
12	1	Os01g0125800 protein	Oryza sativa	gi 115434270	Q9AWU6	67097	6.5	12	14	163	11.14	1.37	7.22
13	2	Os01g0125800 protein	Oryza sativa	gi 115434270	Q9AWU7	67097	6.5	13	14	230	12.45	1.06	7.13
14	2	Os01g0125800 protein	Oryza sativa	gi 115434270	Q9AWU8	67097	6.5	14	18	218	12.02	-1.88	17.72
15	2	NADP-dependent malic enzyme	Hordeum vulgare	gi 209405432	B6DEN9	63309	5.6	23	39	372	-5.57	-1.60	3.75
16	1	NADP-dependent malic enzyme	Hordeum vulgare	gi 209405432	B6DEN9	63309	5.6	20	33	245	-2.65	1.38	-3.38
17	1	predicted protein (phosphoglycerate mutase)	Populus trichocarpa	gi 224140653	B9IGY9	61371	5.4	3	6	89	1.37	1.54	-1.08
18	1	phosphoglycerate mutase	Zea mays	gi 551288	B8A306	60763	5.3	4	10	250	1.72	-1.14	1.73
19	1	Bp2A protein	Triticum turgidum	gi 133872360	A8QPL0	25773	5.9	5	26	174	1.64	-1.01	1.23
20	1	UTP--glucose-1-phosphate uridylyltransferase	Hordeum vulgare	gi 6136111	Q43772	51783	5.2	21	41	459	1.48	3.06	-1.20
21	1	enolase	Oryza sativa	gi 218184182	B8BFV2	48356	5.4	9	22	286	1.18	-1.19	-1.00
22	1	enolase	Zea mays	gi 195619804	B6T3P9	48386	5.6	5	17	334	1.18	-1.37	1.61
23	1	putative uncharacterized protein	Zea mays	gi 223947323	C0P429	51441	5.7	19	38	295	2.12	-1.21	2.56
24	2	rab GDP dissociation inhibitor alpha	Zea mays	gi 226502766	B8A2P2	50634	5.4	10	29	133	2.55	-2.25	5.68
25	2	rab GDP dissociation inhibitor alpha	Zea mays	gi 195637100	B8A2P2	50634	5.4	9	23	176	1.63	-3.62	4.41
26	1	hypothetical protein OsI_01694	Oryza sativa	gi 125525704	Q9M5K3	48512	6.3	2	6	81	2.54	-3.51	2.99
27	1	pollen allergen Phl p 4	Phleum pratense	gi 54144332	Q5ZQK5	56979	8.8	4	10	117	5.47	4.78	-1.19
28	1	pollen allergen Phl p 4 precursor	Phleum pratense	gi 82492267	Q5ZQK5	58590	9.2	2	3	71	5.57	4.59	-1.19
29	1	pollen allergen Phl p 4	Phleum pratense	gi 189014266	B2ZWE8	56147	9.1	12	23	342	-1.38	1.05	-3.92
30	1	pollen allergen Phl p 4	Phleum pratense	gi 189014268	B2ZWE9	55889	9.2	10	20	277	-1.59	1.03	-2.70
31	1	pollen allergen Phl p 4	Phleum pratense	gi 54144334	Q5ZQK4	56741	9.2	9	17	308	1.21	-1.10	1.57
32	1	pollen allergen Phl p 4	Phleum pratense	gi 54144334	Q5ZQK4	56741	9.2	11	22	399	1.14	-1.28	-1.08
33	1	polygalacturonase (Phl p 13)	Phleum pratense	gi 4826572	Q9XG86	42579	7.8	7	12	133	8.80	2.92	3.65
34	1	polygalacturonase (Phl p 13)	Phleum pratense	gi 4826572	Q9XG87	42579	7.8	8	24	121	4.33	2.47	2.29
35	2	polygalacturonase (Phl p 13)	Phleum pratense	gi 4826572	Q9XG88	42579	7.8	8	22	141	22.73	1.85	5.39
36	1	polygalacturonase (Phl p 13)	Phleum pratense	gi 4826572	Q9XG89	42579	7.8	7	22	77	36.41	1.20	15.89
37	1	polygalacturonase (Phl p 13)	Phleum pratense	gi 4826572	Q9XG91	42579	7.8	12	32	193	21.32	1.04	12.12
38	1	polygalacturonase (Phl p 13)	Phleum pratense	gi 4826572	Q9XG93	42579	7.8	7	24	125	4.68	-3.15	5.14
39	2	polygalacturonase (Phl p 13)	Phleum pratense	gi 4826572	Q9XG90	42579	7.8	9	28	152	22.41	-1.18	17.76
40	2	polygalacturonase (Phl p 13)	Phleum pratense	gi 4826572	Q9XG92	42579	7.8	8	24	110	18.23	-1.88	14.34
41	1	polygalacturonase (Phl p 13)	Phleum pratense	gi 4826572	Q9XG93	42579	7.8	5	14	133	5.57	-1.46	5.23
42	1	polygalacturonase-like protein	Oryza sativa	gi 125581161	P35339	45764	9.3	1	5	122	7.33	1.66	6.26
43	2	exopolygalacturonase	Oryza sativa	gi 218190250	P35339	40600	7.9	2	8	97	8.63	2.18	10.93
44	2	predicted protein (polygalacturonase activity)	Populus trichocarpa	gi 222849203	B9HPN0	44771	8.9	4	9	118	15.42	1.86	13.42
45	1	predicted protein (polygalacturonase activity)	Populus trichocarpa	gi 222849203	B9HPN0	44771	8.9	2	6	100	18.75	3.30	9.15
46	1	eukaryotic initiation factor 4A	Pennisetum glaucum	gi 63139086	Q4U474	47277	5.4	20	41	312	5.80	-1.16	4.84
47	1	citrate synthase 4	Zea mays	gi 226503683	B6TAE5	50808	6.3	11	22	170	1.67	-1.15	2.64
48	4	citrate synthase	Daucus carota	gi 8928010	O80433	52908	7.0	13	23	175	2.80	-1.56	4.39
49	1	monodehydroascorbate reductase	Oryza sativa	gi 115480733	Q652L6	46758	5.5	4	12	137	1.39	-1.18	1.21
50	1	RNA helicase	Arabidopsis thaliana	gi 3776021	Q9CAI7	50232	5.2	3	9	172	-1.14	-2.09	-1.27
51	1	monodehydroascorbate reductase	Oryza sativa	gi 4666287	Q652L6	46716	5.5	9	19	191	2.95	-1.38	3.02
52	1	putative uncharacterized protein	Zea mays	gi 219363507	B4FLJ3	46411	6.6	8	23	279	4.32	-1.45	4.87
53	1	adenosine kinase	Zea mays	gi 4582787	Q9XGC6	36465	5.2	7	29	144	4.80	1.95	3.39
54	1	phosphoglycerate kinase, cytosolic	Triticum aestivum	gi 129916	P12783	42153	5.6	11	31	526	1.09	1.08	-1.02
55	1	phosphoglycerate kinase, cytosolic	Triticum aestivum	gi 129916	P12783	42153	5.6	6	19	355	1.60	-1.05	1.58
56	1	phosphoglycerate kinase, cytosolic	Triticum aestivum	gi 129916	P12783	42153	5.6	10	27	416	1.41	1.07	1.20
57	1	succinyl-CoA ligase beta subunit-like protein	Solanum tuberosum	gi 76573373	Q3HRW0	33722	4.9	3	6	234	1.88	-1.64	2.63
58	2	peroxidase 65	Oryza sativa	gi 125583637	A3ABT9	46268	6.3	5	11	87	-1.03	-1.49	-1.27
59	6	putative fructose-bisphosphate aldolase	Phleum pratense	gi 5419990	Q9XGH5	25028	7.8	10	44	380	19.16	1.21	19.81
60	2	putative fructose-bisphosphate aldolase	Phleum pratense	gi 5419990	Q9XGH5	25028	7.8	6	22	98	8.83	-1.71	8.91
61	1	glyceraldehyde 3-phosphate dehydrogenase	Daucus carota	gi 51703306	Q6RUQ2	36768	7.1	7	20	150	3.07	-1.53	4.12
62	6	putative fructose-bisphosphate aldolase	Phleum pratense	gi 5419990	Q9XGH5	25028	7.8	12	49	441	17.93	-1.72	27.42
63	6	glyceraldehyde 3-phosphate dehydrogenase	Daucus carota	gi 51703306	Q6RUQ2	36768	7.1	11	30	222	3.11	-4.81	12.23
64	1	hypothetical protein (polygalacturonase activity)	Zea mays	gi 226502274	B4FY93	44631	9.0	3	10	87	7.89	-1.55	9.17
65	3	exopolygalacturonase	Zea mays	gi 218190250	P35339	40600	7.9	2	8	124	12.20	-1.56	8.04
66	1	elongation factor 2 (OsI_03688)	Oryza sativa	gi 125527658	O23755	92956	6.2	8	10	148	6.29	-1.01	6.05
67	5	putative fructose-bisphosphate aldolase	Phleum pratense	gi 5419990	Q9XGH5	25028	7.8	9	37	288	-5.95	1.89	-15.46
68	1	putative fructose-bisphosphate aldolase	Phleum pratense	gi 5419990	Q9XGH5	25028	7.8	6	23	304	-1.22	-2.83	1.24

spot No. ^{a)}	replicates ^{b)}	Protein Name	Species	Acc. No. ^{c)}	UniProt No. ^{d)}	MW ^{e)}	pI ^{f)}	matched peptides ^{g)}	sequence coverage ^{h)}	Mascot score ⁱ⁾	extract 1/ extract 2	extract1/ extract 3	extract 3/ extract 2
69	1	polygalacturonase (Phl p 13)	Phleum pratense	gi 4826572	Q9XG93	42579	7.8	6	19	156	-2.51	-4.28	1.77
70	4	Phl p5a allergen precursor	Phleum pratense	gi 29500897	Q84UI2	28269	7.9	16	63	457	2.56	1.30	2.15
71	2	pollen allergen Phl p 1	Phleum pratense	gi 28373838	P43213	26611	6.2	11	36	350	-1.44	1.22	-2.62
72	1	pollen allergen Phl p 1	Phleum pratense	gi 28373838	P43213	26611	6.2	7	24	355	2.24	1.19	1.99
73	3	pollen allergen Phl p 1	Phleum pratense	gi 28373838	P43213	26611	6.2	10	36	238	-2.61	1.38	-3.58
74	1	pollen allergen Phl p 1	Phleum pratense	gi 28373838	P43213	26611	6.2	8	24	348	-1.57	1.00	-1.79
75	1	pollen allergen Phl p 1	Phleum pratense	gi 28373838	P43213	26611	6.2	11	44	299	-1.01	1.30	-1.52
76	2	pollen allergen Phl p 1	Phleum pratense	gi 3901094	P43213	28584	7.5	6	19	263	-1.90	1.94	-3.18
77	1	pollen allergen Phl p 1	Phleum pratense	gi 3901094	Q40967	28584	7.5	10	35	400	-1.51	1.34	-2.46
78	1	pollen allergen Phl p 1	Phleum pratense	gi 3901094	Q40967	28584	7.5	5	17	276	-1.61	1.34	-2.74
79	2	pollen allergen Phl p 1	Phleum pratense	gi 3901094	P43213	28584	7.5	10	32	300	-2.77	-1.07	-2.39
80	2	pollen allergen Phl p 1	Phleum pratense	gi 3901094	P43213	28584	7.5	15	55	385	-5.37	-1.18	-4.38
81	2	pollen allergen Phl p 1	Phleum pratense	gi 3901094	P43213	28584	7.5	8	22	340	3.96	1.30	-1.84
82	1	pollen allergen Phl p 1	Phleum pratense	gi 3901094	Q40967	28584	7.5	9	28	382	-1.10	-1.03	-1.27
83	2	pollen allergen Phl p 1	Phleum pratense	gi 3901094	P43213	28584	7.5	11	22	306	-1.35	-1.02	-3.13
84	1	pollen allergen Phl p 1	Phleum pratense	gi 3901094	Q40967	28584	7.5	6	19	202	1.08	1.63	-1.59
85	1	pollen allergen Phl p 1	Phleum pratense	gi 3901094	Q40967	28584	7.5	4	17	149	1.30	1.93	-1.51
86	1	ribosomal protein S3	Daphniphyllum	gi 130076566	BOL4P1	54409	10.37	6	10	83	14.02	1.03	11.03
87	4	pollen allergen Phl p 5a	Phleum pratense	gi 29500897	Q84UI2	28269	7.9	15	58	721	-7.52	-1.18	-6.24
88	6	pollen allergen Phl p 5a	Phleum pratense	gi 29500897	Q84UI2	28269	7.9	18	70	809	6.05	1.21	5.96
89	1	pollen allergen Phl p 5a	Phleum pratense	gi 29500897	Q84UI2	28269	7.9	5	21	284	8.66	6.60	1.12
90	1	pollen allergen Phl p 5a	Phleum pratense	gi 29500897	Q84UI2	28269	7.9	4	20	88	1.15	-1.63	1.50
91	1	Os01g0913600 protein	Oryza sativa	gi 115441785	Q8S081	24874	4.5	2	14	90	10.89	2.74	3.44
92	2	Os01g0913600 protein	Oryza sativa	gi 115441785	Q8S081	24874	4.5	5	28	180	2.67	3.26	-2.02
93	1	14-3-3 E protein	Hordeum vulgare	gi 83271058	A1X810	29533	4.8	8	34	243	1.56	1.07	1.76
94	1	14-3-3 protein	Triticum aestivum	gi 40781605	Q08G36	29388	4.8	8	36	278	1.58	-1.19	1.73
95	1	pollen allergen Phl p 5.0204	Phleum pratense	gi 3309043	Q9SBE0	26252	5.6	2	8	80	-1.36	-1.17	1.81
96	3	pollen allergen Phl p 5b	Phleum pratense	gi 3309043	Q40963	26252	5.6	16	73	424	-1.36	-2.45	5.77
97	2	pollen allergen Phl p 5b	Phleum pratense	gi 3309043	Q40963	26252	5.6	13	65	871	1.38	-1.90	4.45
98	3	pollen allergen Phl p 5b	Phleum pratense	gi 3309043	Q40963	26252	5.6	15	87	390	10.24	1.60	5.50
99	2	pollen allergen Phl p 5b	Phleum pratense	gi 3309043	Q81343	28869	6.0	2	10	172	-3.26	1.23	-4.49
100	1	pollen allergen Phl p 5b	Phleum pratense	gi 3309043	Q40963	26252	5.6	11	60	508	-6.57	-1.64	-3.49
101	5	pollen allergen Phl p 5b	Phleum pratense	gi 3309043	Q40963	26252	5.6	9	50	360	-9.07	-1.37	-7.96
102	1	pollen allergen Phl p 5b	Phleum pratense	gi 3309043	Q40963	26252	5.6	7	40	92	7.83	1.68	3.55
103	2	pollen allergen Phl p 5b	Phleum pratense	gi 3309043	Q40963	26252	5.6	17	87	648	5.41	1.51	4.86
104	5	pollen allergen Phl p 5b	Phleum pratense	gi 3309043	Q40963	26252	5.6	17	87	639	54.07	3.34	9.43
105	1	pollen allergen Phl p 5b	Phleum pratense	gi 3309043	Q40963	26252	5.6	19	90	928	35.85	2.98	7.43
106	2	pollen allergen Phl p 5b	Phleum pratense	gi 3309043	Q40963	26252	5.6	3	16	258	1.75	-1.55	2.02
107	1	pollen allergen Phl p 5b	Phleum pratense	gi 2851457	Q40963	28042	8.8	12	66	255	-10.07	-1.66	-7.41
108	4	pollen allergen Phl p 5b	Phleum pratense	gi 3309043	Q40963	26252	5.6	15	87	512	16.50	3.77	3.71
109	1	biostress-resistance-related protein	Triticum aestivum	gi 29409364	Q84VJ1	34277	8.9	4	15	98	-10.87	-2.34	-23.98
110	6	small Ras-related GTP-binding protein	Triticum aestivum	gi 16903082	Q944C6	25460	6.7	17	51	331	-1.36	1.53	1.01
111	4	pollen allergen Phl p 1	Phleum pratense	gi 3901094	P43213	28584	7.5	5	14	228	-17.61	-1.26	-16.22
112	6	pollen allergen Phl p 1	Phleum pratense	gi 3901094	P43213	28584	7.5	9	27	275	-36.67	-4.74	-9.91
113	3	pollen allergen Phl p 1	Phleum pratense	gi 3901094	P43213	28584	7.5	5	14	265	-70.85	-3.24	-29.13
114	1	pollen allergen Phl p 1	Phleum pratense	gi 3901094	P43213	28584	7.5	2	9	92	-28.38	-3.88	-7.59
115	2	pollen allergen Phl p 1	Phleum pratense	gi 3901094	P43213	28584	7.5	10	41	101	-16.21	-2.88	-19.91
116	2	pollen allergen Phl p 1	Phleum pratense	gi 3901094	P43213	28584	7.5	5	14	123	-10.32	-2.68	-6.27
117	1	ascorbate peroxidase	Hordeum vulgare	gi 15808779	Q945R5	27964	5.1	6	28	276	-1.21	-1.40	2.15
118	1	triosephosphate isomerase, cytosolic	Hordeum vulgare	gi 2507469	P34937	26948	5.4	3	15	120	-1.19	1.18	-1.55
119	1	triosephosphate isomerase, cytosolic	Hordeum vulgare	gi 2507469	P34937	26948	5.4	6	21	312	1.10	-1.03	-1.42
120	1	carbonic anhydrase, chloroplastic	Hordeum vulgare	gi 729003	P40880	35736	8.9	6	23	133	3.08	1.04	2.61
121	2	carbonic anhydrase, chloroplastic	Hordeum vulgare	gi 729003	P40880	35736	8.9	5	14	131	3.43	-2.24	5.56
122	1	peptidylprolyl isomerase	Triticum aestivum	gi 133741925	A7XDG5	26084	9.4	5	31	81	-4.60	-1.03	-4.08
123	2	peptidyl-prolyl cis-trans isomerase	Digitalis lanata	gi 1563719	Q96416	18458	8.5	9	45	159	1.89	1.59	2.77
124	2	pollen allergen Phl p 11	Phleum pratense	gi 47606039	Q8H6L7	16111	5.0	15	79	618	-1.75	1.42	-4.38
125	1	pollen allergen Phl p 11	Phleum pratense	gi 47606039	Q8H6L7	16111	5.0	7	41	369	-1.58	-1.11	-2.27
126	2	pollen allergen Phl p 11	Phleum pratense	gi 47606039	Q8H6L7	16111	5.0	12	86	348	-1.28	1.27	-2.11
127	1	pollen allergen Phl p 11	Phleum pratense	gi 47606039	Q8H6L7	16111	5.0	9	53	438	-1.72	1.27	-2.32
128	4	actin-depolymerizing factor 1	Oryza sativa	gi 125540584	Q6EUH7	15577	5.6	4	29	143	7.02	-1.66	13.29
129	4	actin-depolymerizing factor 1	Oryza sativa	gi 125540584	Q6EUH7	15577	5.6	8	43	162	4.25	-2.00	4.96
130	1	actin-depolymerizing factor 9	Oryza sativa	gi 218199609	B8B622	16250	5.5	3	21	299	3.82	-1.16	2.19
131	3	pollen allergen Phl p 12	Phleum pratense	gi 3914432	O24650	14255	4.9	6	53	346	-1.29	1.26	-2.83
132	1	pollen allergen Phl p 2	Phleum pratense	gi 157830392	P43214	10809	4.6	3	54	103	-2.70	1.23	-2.95
133	1	pollen allergen Lol p 2-A	Lolium perenne	gi 126386	P14947	10873	5.3	5	38	139	-2.08	1.23	-5.42
134	5	pollen allergen Phl p 6	Phleum pratense	gi 1171011	P43215	13912	5.3	8	74	278	-3.81	1.70	-5.14
135	2	pollen allergen Phl p 6	Phleum pratense	gi 1171011	P43215	13912	5.3	6	62	203	-3.88	2.44	-9.00
136	2	pollen allergen Phl p 6	Phleum pratense	gi 1171011	P43215	13912	5.3	4	50	161	-1.69	1.07	-2.19
137	3	pollen allergen Phl p 6	Phleum pratense	gi 1171011	P43215	13912	5.3	12	100	520	-1.69	1.08	-3.50
138	3	pollen allergen Phl p 6	Phleum pratense	gi 1171011	P43215	13912	5.3	7	62	418	-2.64	1.41	-19.63
139	3	pollen allergen Phl p 6 allergen	Phleum pratense	gi 3004465	O6S868	14592	5.7	7	43	409	1.16	-1.04	-1.30
140	2	thioredoxin H	Triticum aestivum	gi 27461140	Q8GVD3	12856	5.3	4	33	136	-1.58	-1.08	-1.54
141	3	pollen allergen Phl p 3	Phleum pratense	gi 39841264	Q69B42	10952	8.9	5	51	228	-1.38	1.51	-1.52
142	1	pollen allergen Phl p 3	Phleum pratense	gi 39841264	Q69B42	10952	8.9	4	54	166	-1.12	1.15	-1.15
143	4	pollen allergen Phl p 3	Phleum pratense	gi 39841264	Q69B42	10952	8.9	6	67	232	-3.83	-1.12	-2.91
144	2	pollen allergen Phl p 3	Phleum pratense	gi 39841264	Q69B42	10952	8.9	2	26	158	-1.03	-1.14	-1.05

Table S3. Immunological and biochemical properties of *Phleum pratense* pollen allergen groups characterized to date (modified according to refs 1-3).

name	IgE prevalence	MW (kDa) exp.	pI exp.	identified in this publication	function	PTMs, characteristics
Phl p 1	90%	31-35 26-28 ^s	6.2-8.5	yes	similar to expansins	glycoprotein, disulfide bonds
Phl p 2	50%	11	4.7	yes	-	-
Phl p 3	50%	11	9.0	yes	-	-
Phl p 4	80%	50-67	9.0-9.5	yes	-	glycoprotein, disulfide bonds
Phl p 5a	90%	27-33	4.2-9.0	yes	-	-
Phl p 5b	90%			yes	RNAse	1 cysteine residue, dimerization
Phl p 6	60-70%	13	4.3-5.5	yes	-	in 2D: two protein spots pI 5.2 and 5.5
Phl p 7	10%	9	n.d.	no	Ca ²⁺ -binding protein	1 cysteine residue
Phl p 11	40%	20	n.d.	yes	Trypsin inhibitor	glycoprotein, disulfide bonds
Phl p 12	15-30%	14	4.4-4.9	yes	profilin	2 cysteine residues
Phl p 13	60%	55-60	6.5-7.4	yes	polygalacturonase	glycoprotein, disulfide bonds

^s experimentally obtained using mass spectrometry

References

- (1) Andersson K, Lidholm J. Characteristics and immunobiology of grass pollen allergens. *Int Arch Allergy Immunol* 2003 Feb;130(2):87-107.
- (2) Behrendt H, Becker WM. Localization, release and bioavailability of pollen allergens: the influence of environmental factors. *Curr Opin Immunol* 2001 Dec;13(6):709-15.
- (3) Petersen A, Dresselhaus T, Grobe K, Becker WM. Proteome analysis of maize pollen for allergy-relevant components. *Proteomics* 2006 Dec;6(23):6317-25.

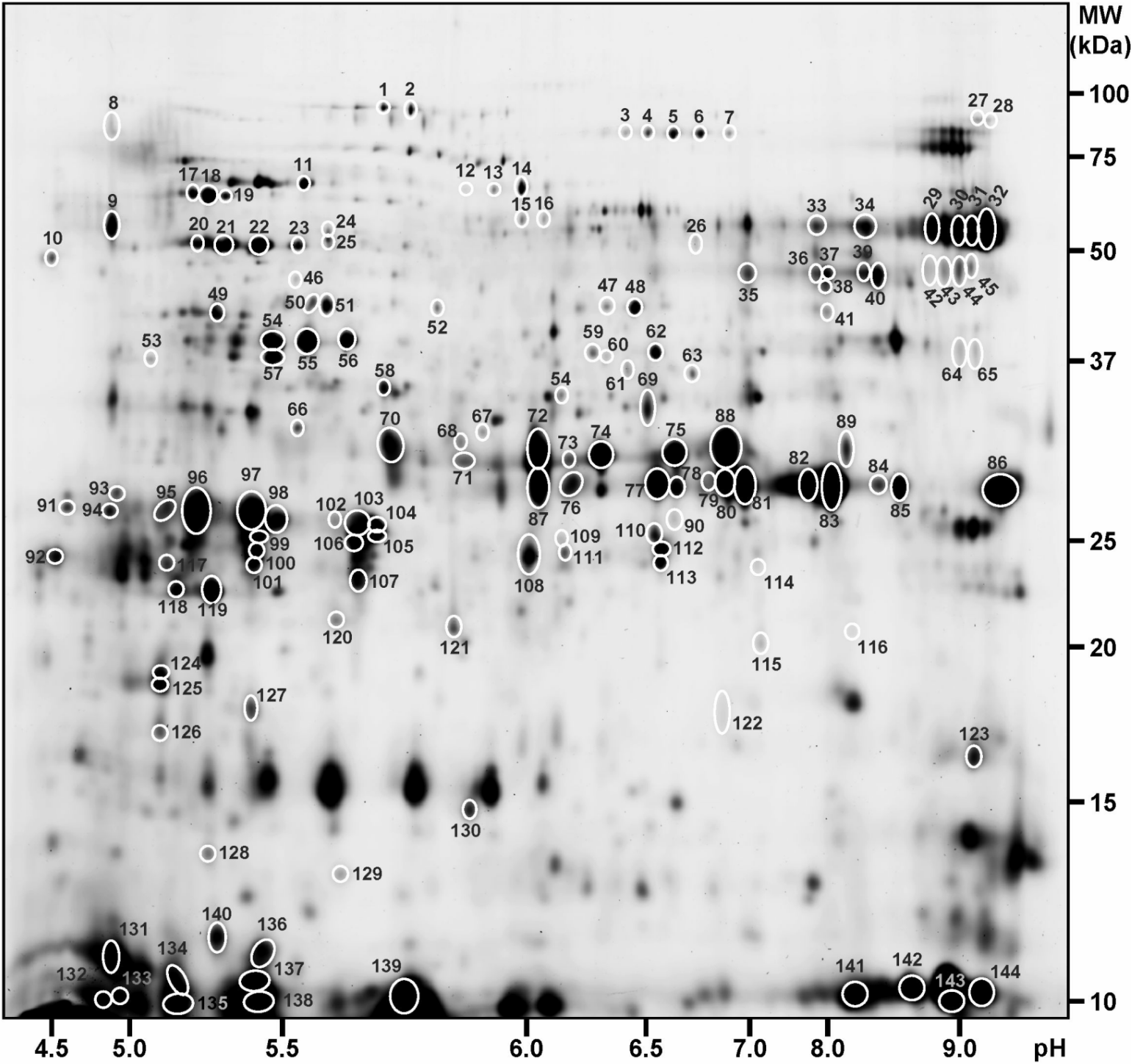


Fig. S1: Annotated 2D map of a *Phleum* pollen mixture. For the overall 2D map, 66.7 µg of each pollen extract were mixed and a total of 200 µg protein separated on a pH 3-11NL IPG strip in the first and a 12.5% SDS-PAGE in the second dimension. Proteins were stained with Flamingo Pink and selected spots were assigned for picking using the Image Master 6.0 software. In total, 144 spots were identified by mass spectrometry (MS+MS/MS) and annotated according to table S2.

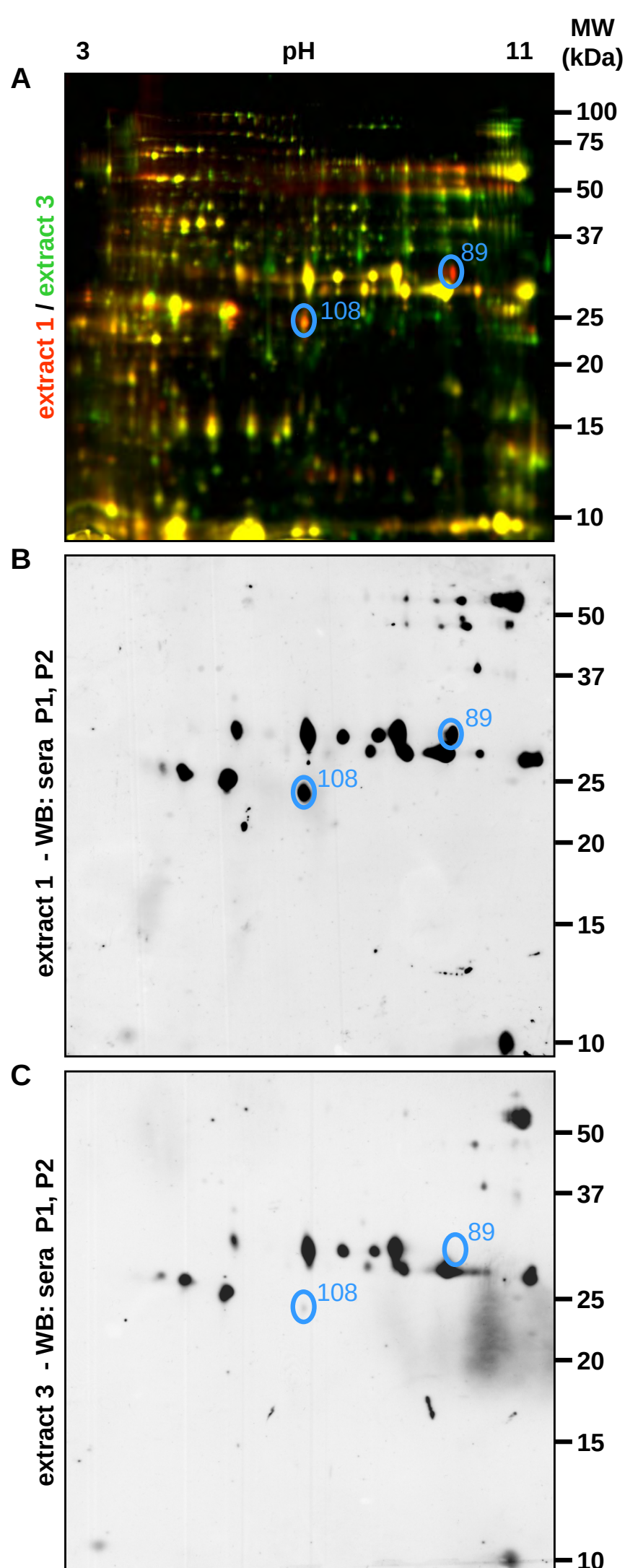


Fig. S2: Differences in IgE-reactive isoforms of Phl p 5. Pollen extracts 1 and 3 were compared in a 2D-DIGE experiment (A) or by 2D-Western blotting using pooled sera of pollen allergic patients under comparable conditions (P1, P2) (B,C). The annotated spot #89 was identified by mass spectrometry as Phl p 5a and spot #108 as Phl p 5b.