

ORIGINAL PAPER

Heterogeneity of commercial timothy grass pollen extracts

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Summary

Background The diagnosis and specific immunotherapy of allergy is currently performed with allergen extracts prepared from natural allergen sources.**Objective** To analyse commercial timothy grass pollen allergen extracts used for *in vivo* diagnosis regarding their qualitative and quantitative allergen composition and *in vivo* biological activity.**Methods** Antibodies specific for eight timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 7, Phl p 12, Phl p 13) were used to detect these allergens in timothy grass pollen extracts from four manufacturers by immunoblotting. ELISA assays were developed and used to quantify the three major allergens (Phl p 1, Phl p 2, Phl p 5) in the extracts. The magnitude of skin responses to the four extracts was studied by skin prick testing in 10 grass pollen-allergic patients.**Results** The allergen extracts showed broad variations in protein compositions and amounts (24.1–197.7 µg/mL extract). Several allergens could not be detected in certain extracts or appeared degraded. A considerable variability regarding the contents of major allergens was found (Phl p 1: 32–384 ng/mL; Phl p 2: 1128–6530 ng/mL, Phl p 5: 40–793 ng/mL).

Heterogeneous skin test results were obtained with the extracts in grass pollen-allergic patients.

Conclusions Timothy grass pollen extracts from different manufacturers exhibit a considerable heterogeneity regarding the presence of individual allergens and hence yield varying *in vivo* test results. Problems related to the use of natural grass pollen allergen extracts may be circumvented by using defined recombinant grass pollen allergens.**Keywords** allergen measurement, grass pollen allergy, recombinant allergen, skin prick test

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Introduction

Substantial progress has been made in the field of allergen characterization during the last 20 years through the application of immunochemical and molecular biological techniques [1, 2]. Today, the most common and important allergens have been characterized regarding their structure and immunological properties. Recombinant allergens closely resembling the properties of the natural allergens have been produced and can now be used for the diagnosis and therapy of allergy (www.allergome.org) [3–5]. Nevertheless, natural allergen extracts are currently in use for routine diagnostic and therapeutic purposes.

In most cases, allergen extracts are prepared from natural allergen sources using aqueous extraction [6]. It

has been recognized earlier that it is important to control allergen extracts regarding their allergenic activity and potency. For this purpose, methods of 'allergen standardization' have been introduced. One of the first *in vitro* standardization procedures has been the quantification of protein nitrogen units (PNU), a method already introduced in 1933 [7]. However, this technique does not provide direct information about the allergenic activity of a given extract. Therefore, several other methods for standardization of allergen extracts have been developed. They include biological standardization by skin testing in allergic volunteers [8, 9], characterization of extracts by biochemical methods such as CIE, SDS-PAGE, IEF [10, 11], semi-quantitative determinations of allergens by CRIE and immunoblotting [10, 12] using pooled sera of allergic

subjects or determination of individual allergens by monoclonal antibodies [13, 14]. Other standardization methods are radio-allergoadsorbent test (RAST) inhibition [10, 11, 15] and basophil activation tests [10, 16, 17].

In principle, these standardization methods allow the determination of both the allergenic potency of a given extract and the concentrations of certain allergens within the extract. One major problem is that allergen manufacturers use different in-house methods for standardization and display their results using a variety of company-specific units. Consequently, the products of different companies cannot be compared [18]. Several studies have also indicated problems regarding the quality of allergen extracts. For example, it has been described that skin prick testing (SPT) extracts contain endotoxins and bacterial materials and can yield false-negative as well as false-positive results [19–23].

In this study, we were interested in analysing and comparing commercial allergen extracts used for skin test diagnosis of allergy regarding the presence, quality, concentration and ratio of individual allergens. We have selected timothy grass pollen extracts because grass pollen represents one of the most important allergen sources world-wide [24, 25]. Furthermore, the allergens of timothy grass have been well characterized and produced as recombinant allergens [25].

Timothy grass pollen extracts used for SPT from four leading allergen manufacturers were studied regarding allergen contents using antibody probes specific for eight different grass pollen allergens. Quantitative ELISAs for the major grass pollen allergens (groups 1, 2 and 5) were developed to determine the concentrations of the major grass pollen allergens. To examine the clinical relevance of the *in vitro* data for the allergologist's practice, we further compared the extracts *in vivo* by SPT in grass pollen-allergic patients.

Materials and methods

Recombinant and natural allergens, allergen extracts

Purified recombinant Phl p 1, 2, 5, 6, 7 and 12 that had been expressed in *Escherichia coli* were obtained from BIOMAY (Vienna, Austria). Recombinant Phl p 13 was expressed in *E. coli* and purified to homogeneity as described [26]. Natural Phl p 4 was purified from *Phleum pratense* pollen extracts as published previously [27].

Pollen from timothy grass (*P. pratense*) was purchased from Allergen Phadia (Välinge, Sweden) and an aqueous pollen extract was prepared [28].

Commercial SPT solutions for the diagnosis of grass pollen allergy (1, Allergopharma 177, 50 000 BE/mL, Ch. B. 30005760; 2, ALK prick SQ 225, 10 HEP, Ch. B. 0000060793; 3, ALYOSTAL ST-IR 0661, 100 IR/mL, Ch. B. 10829; 4: Hal 07–11, 10 000 AU/mL, Ch. B. B1218038)

were purchased from four major European manufacturers. Extracts were stored at 4 °C as recommended by the manufacturer. For *in vitro* experiments, glycerol and phenol were removed from the extracts by dialysis against Aqua dest. using Dispo Dialyzers (cut-off 100 Da, Spectra/Por® CE, Spectrum, TX, USA) under identical conditions. The total protein contents of the pollen extracts were determined using the method of Bradford [29].

Skin prick testing of allergic patients

Grass pollen-allergic patients ($n = 10$) suffering from allergic rhinoconjunctivitis and/or asthma to grass pollen were characterized by case history and serology. Skin prick testing was performed on the patient's forearms with disposable lancets from ALK (Horsholm, Denmark) using histamine and sodium chloride (ALK) as positive and negative controls. For each SPT, a fresh lancet was used and the distance between the SPT sites was kept to 4 cm [30].

Skin prick testing was carried out in a single-blinded way, where the product identities were unknown to the investigator. Reactions were recorded 20 min after testing by transferring the ballpoint pen-surrounded weal area with a scotch tape to paper. The mean weal areas were calculated with the image-processing program ImageJ [31].

Production and purification of allergen-specific antibodies

Phl p 1-peptide-specific monoclonal antibodies were obtained by immunizing mice with the KLH-coupled peptides derived from the sequence of Phl p 1 [32] (Charles River, Kißlegg, Germany).

After determination of the antibody isotype (IgM) by ELISA using isotype-specific antibodies (BD PharMingen, San Diego, CA, USA), the monoclonal anti-Phl p 1-peptide antibodies were examined for specificity for Phl p 1 and homologous group 1 allergens from different grass pollens by ELISA. Monoclonal IgM antibodies specific for group 1 allergens were purified from hybridoma supernatants using Hi Trap purification columns from Amersham (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocol, and protein contents were determined by measuring the optical density at 280 nm.

A peptide derived from a surface-exposed area from Phl p 5 was synthesized (sequence: CAEEVKVIPAGELQVIE KVDAAFKVAATAANAAPANDK), coupled to KLH and used to immunize rabbits with CFA as an adjuvant (Charles River) as described in references [32, 33].

IgG fractions from allergen-specific rabbit antisera were purified using an Immuno Pure IgG Purification Kit from Pierce (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Grass pollen allergen-specific rabbit antisera were raised by immunizing rabbits with purified allergens

(Phl p 1 [32], Phl p 4 [27], Phl p 5 [34], Phl p 6 [35], Phl p 7 [36], Phl p 13 [26], Profilin: Phl p 12 [37]) using Freund's complete adjuvant (CFA) and giving two booster injections with Freund's incomplete adjuvant (Charles River).

Biotinylation

Purified polyclonal rabbit IgG antibodies specific for group 5 allergens were labelled with Biotin using *N*-succinimidyl-*N*-(6-(biotinylamino)caproyl)-6-aminocaproate (Fluka Biochemica, Buchs, Switzerland), according to the manufacturer's instructions in a concentration of 250 µg biotin for 1 mg purified antibodies. rPhl p 2 was labelled as described in reference [38].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting

The protein composition of timothy grass pollen extracts was evaluated analysing equal volumes (24 µL/lane) by SDS-PAGE (15% separating gel [39]) and subsequent silver staining (Silver Stain Kit, Bio Rad Laboratories Inc., CA, USA) or immunoblotting (Schleicher & Schüll, Dassel, Germany) [40].

For identification of allergens in the extracts, immunoblots were developed with rabbit antisera specific for seven different grass pollen allergens (Phl p 1 [32], Phl p 4 [27], Phl p 5 [34], Phl p 6 [35], Phl p 7 [36], Phl p 13 [26], Profilin: Phl p 12 [37]) using the corresponding pre-immune sera for control purposes.

Bound rabbit IgG antibodies were detected with a ¹²⁵I-labelled donkey anti-rabbit antiserum (Amersham Biosciences) [41].

Nitrocellulose-blotted Phl p 2 was detected with a human Phl p 2-specific monoclonal antibody [38] using human IgG1 (Sigma, Vienna, Austria, I5154) for control purposes. The specificity of the grass pollen allergen-specific antibodies was demonstrated by testing each antiserum with dot-blotted aliquots (1 µg/dot) of the purified allergens.

In addition, serum from a grass pollen-allergic individual containing IgE antibodies against the major timothy grass pollen allergens, Phl p 1, Phl p 2 and Phl p 5 was used to probe nitrocellulose-blotted grass pollen extracts. Bound IgE was detected with ¹²⁵I-labelled anti-human IgE (Phadia, Uppsala, Sweden) [41].

Development of enzyme-linked-immunosorbent serologic assays for the detection and quantification of major grass pollen allergens

The sandwich ELISA for the detection and quantification of group 1 grass pollen allergens is based on an antibody pair. As a catching antibody, a mouse monoclonal IgM antibody against the rPhl p 1-derived peptide 4 (sequence: GYKDVKPPFSGMTGCGNTPIFKSGRGC [32]) was used to

coat the wells of Maxi Sorp (NUNC, Roskilde, Denmark) ELISA plates at 4 °C overnight [100 µL, c = 5 µg/mL diluted in 0.1 M carbonate buffer, pH 9.6 (CB)].

The wells were washed two times with phosphate-buffered saline (PBS) containing 0.05% v/v Tween (PBST) and afterwards blocked with 2% w/v bovine serum albumin (BSA) in PBST for 3 h (150 µL/well).

All subsequent steps were performed with PBST/0.5% w/v BSA as dilution buffer, using 100 µL/well. After blocking, standards starting from 1000 to 3.9 ng rPhl p 1 per mL or grass pollen extracts were diluted serially in 1 : 2 steps and added to the wells for 2 h at 37 °C. After five washings, the wells were incubated with purified rabbit anti-Phl p 1 IgG-antibodies (1 : 1000) at 4 °C overnight and with anti-rabbit Ig, horseradish peroxidase-linked antibodies from donkey (Amersham Life Science, NA 934, 1 : 2000 diluted) for 0.5 h at 37 °C and 0.5 h at 4 °C. Between each incubation step, five washings were performed. ABTS (Sigma, A-1888) was used as a substrate for the detection of bound donkey antibodies. A modified ELISA protocol using rabbit anti-Phl p 1 peptide antibodies as catching antibodies instead of the monoclonal peptide-specific antibody has also been developed and yielded identical results (data not shown).

The group 2-allergen-specific ELISA is a competition-ELISA based on purified rPhl p 2 and a human Phl p 2-specific monoclonal antibody [38].

The group 5 allergen-specific ELISA is based on rabbit IgG antibodies raised against the Phl p 5 derived-peptide (sequence: CAEEVKVIPAGELQVIEKVDAAFKVAATAAN AAPANDK) as catching antibodies (0.5 µg/100 µL CB) and biotinylated rabbit anti-rPhl p 5a IgG as a detecting reagent. Washing and blocking steps were as described for the group 1 ELISA. Aliquots of 100 µL consisting of serial 1 : 2 dilutions of rPhl p 5 (starting from 500 to 3.9 ng/mL) or of grass pollen extracts were added to the plates for 2 h, 37 °C. After washing, 100 µL biotinylated purified rabbit IgG antibodies against recombinant Phl p 5a (dilution 1 : 5000) were added overnight at 4 °C. After washing, detection was performed as described for the Phl p 2 ELISA using streptavidin peroxidase [38].

Statistical methods

Inter- and intra-assay variability was assessed by descriptive methods. The geometric mean coefficient of variation across concentrations was determined. Number of replicates was chosen such that the difference between the upper 95% confidence interval of the pooled coefficient of variation and the estimate (projected to be within the range of 10–20%) would be <5%.

No curve fitting was conducted for the standard curve. Concentrations were obtained by linear interpolation between contiguous points of the standard curve.

Results

Qualitative and quantitative heterogeneity of commercial timothy grass pollen extracts

The total protein contents in the four commercial extracts showed a high variability (Table 1). The protein contents were the lowest in the extracts from company 3 (24.1 µg/mL) and the highest in the extract from company 4 (197.7 µg/mL). The qualitative protein composition of the extracts was compared by SDS-PAGE and subsequent silver-staining (Fig. 1). Each of the four commercial extracts contained protein bands of comparable intensity at 10 kDa corresponding to the molecular weights of groups 2 and 6 allergens. However, the pattern of protein bands between 15 and 70 kDa was highly heterogeneous. Bands between 25 and 30 kDa, corresponding to the molecular weights of groups 1 and 5 allergens, were well visible only in extract 4 and the intensities of bands between 15 and 20 kDa differed considerably among the extracts. An unusual prominent band of 66–68 kDa, presumably representing serum albumin, was stained only in extract 4 but in none of the other three commercial extracts. The in-house extract contained densely packed protein bands between 5 and 80 kDa.

Table 1. Contents of group 1, 2 and 5 allergens in grass pollen extracts from four companies

	ng/mL extract			Total protein (µg/mL)	Percentage weight/ total protein		
	Phl p 1	Phl p 2	Phl p 5		Phl p 1	Phl p 2	Phl p 5
Extract 1	114.2	5618.3	40	77.9	0.15	7.21	0.05
Extract 2	97.9	2802.3	356	28.0	0.35	10.01	1.27
Extract 3	32.5	1128.3	391	24.1	0.13	4.68	1.62
Extract 4	384.4	6530.9	793	197.7	0.19	3.30	0.40

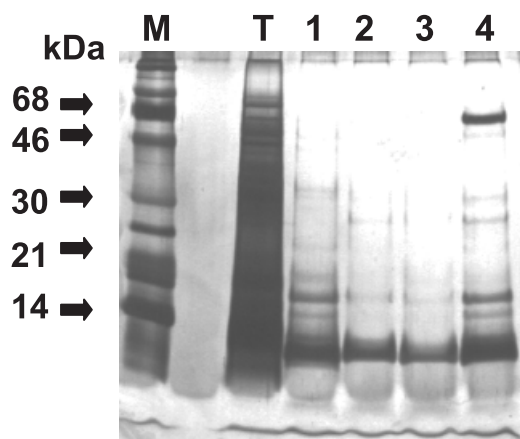


Fig. 1. Silver-stained SDS-PAGE containing in-house (lane T) and commercial timothy grass pollen extracts from four different companies (lanes 1–4). Lane M contains a molecular weight marker (kDa).

Commercial timothy grass pollen extracts differ from each other regarding the presence of certain allergen molecules

In a first experiment, the specificity of the allergen-specific antibodies was documented by a dot-blot assay using purified recombinant or natural timothy grass pollen allergens (rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5a, rPhl p 6, rPhl p 7, rPhl p 12, rPhl p 13) (Fig. 2a). The antibodies reacted specifically with the allergen against which they had been raised (Fig. 2a).

Each of the antibodies recognized the corresponding natural allergen in the nitrocellulose-blotted in-house timothy grass pollen extract (Fig. 2b). The highly cross-reactive timothy grass pollen profilin, Phl p 12, could not be detected in any of the commercial extracts. Phl p 4, Phl p 7 and Phl p 13 were absent from certain extracts (Phl p 4: extract 2; Phl p 7: extracts 1, 3; Phl p 13: extract 3). The amounts of major allergens Phl p 1, Phl p 5 and Phl p 6 showed considerable variability. The major timothy grass pollen allergen, Phl p 1, was not even detectable by immunoblotting in extract 3. Certain extracts contained low-molecular-weight allergen-derived moieties (e.g., Phl p 5 in extract 1) presumably representing degradation products. Only Phl p 2 could be detected in each of the four extracts although at varying intensities.

Commercial timothy grass pollen extracts contain varying concentrations of major allergens

Figure 3 shows the standard dose-response curves with mean values and SDs for the ELISAs used for the quantification of rPhl p 1, rPhl p 2 and rPhl p 5. The Phl p 2-specific competition-ELISA has been described earlier [38]. Low inter- and intra-assay variations could be detected for the rPhl p 1 ELISA (15% inter-, 8% intra-assay variation) and for the rPhl p 5 ELISA (13% inter-, 7% intra-assay variation). No binding was detected when unrelated allergens such as purified rBet v 1, birch pollen- or mugwort pollen-extract were used instead of the target allergens (data not shown). Despite sequence homology between Phl p 5 and Phl p 6 and reported cross-reactivity, we did not obtain binding when rPhl p 6 was used in the Phl p 5 ELISA (data not shown). These results demonstrate the specificity and robustness of the ELISA assays that were used for the quantification of groups 1, 2 and 5 allergens in the commercial extracts (Table 1).

A summary of the contents of groups 1, 2 and 5 allergens determined with the ELISAs in the four commercial grass pollen extracts is shown in Table 1. We determined the protein contents of the different grass pollen extracts in parallel. Based on the total protein amounts, the contents of groups 1, 2 and 5 allergens were calculated in ng/100 µg grass pollen extract or as percentage weight per total protein.

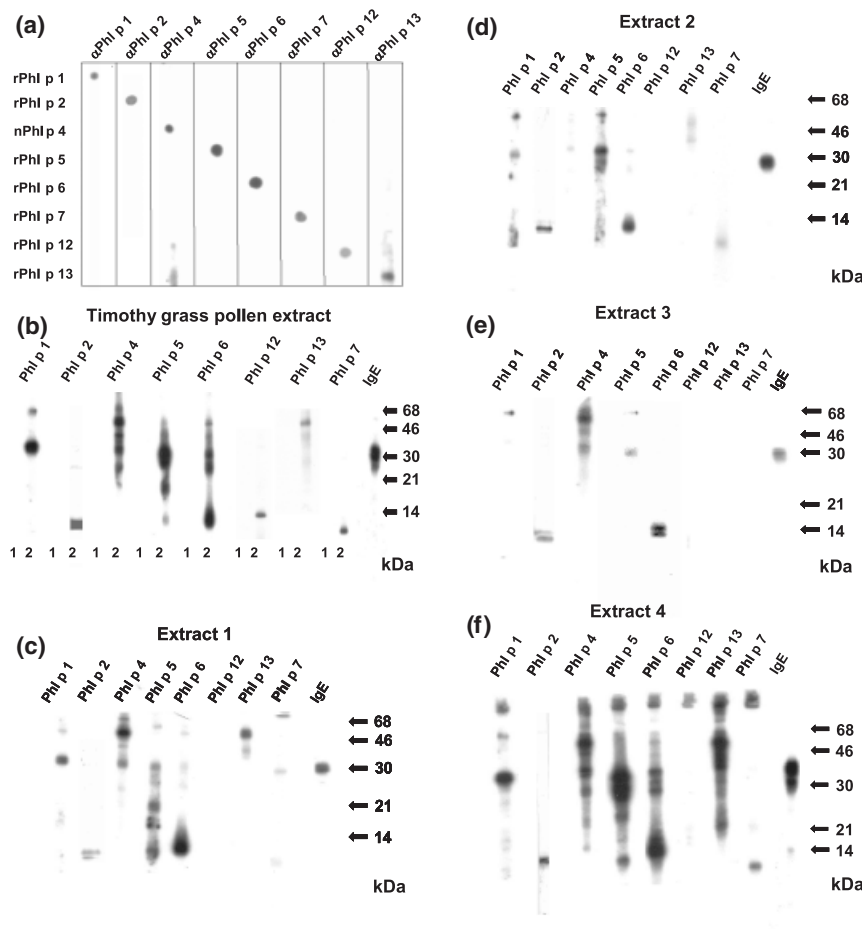


Fig. 2. (a) Specificity of grass pollen allergen-specific antibodies. Dot blots containing purified grass pollen allergens (y-axis) were probed with antibodies (top). (b–f) Nitrocellulose-blotted grass pollen extracts [(b) in-house extract, (c–f) extracts from companies 1–4] were probed with allergen-specific antibodies or with IgE from a grass pollen-allergic patient (lanes IgE). In the in-house timothy grass pollen extract in (b), the reactivity of the pre-immune (lanes 1) or immune sera (lanes 2) is shown for the rabbit antisera specific for Phl p 1, Phl p 4, Phl p 5, Phl p 6, Phl p 7, Phl p 12, and Phl p 13. Phl p 2 was detected with a human Phl p 2-specific monoclonal antibody (lane 2) using human IgG1 (lane 1) for control purposes. Molecular weights in kDa are indicated on the right margin.

We found that the contents of Phl p 1 allergens in the four commercial grass pollen preparations ranged between 32.5 and 384.4 ng/mL (134–349 ng/100 µg, equivalent to 0.13–0.35% weight/total amount).

The Phl p 2 allergen concentrations ranged between 1128.3 and 6530.9 ng/mL (3303–10 008 ng/100 µg or 3.3–10.1% weight/total amount), and the concentrations of Phl p 5 varied between 40 and 793 ng/mL (51.3–1622 ng/100 µg or 0.05–1.62% weight/total amount).

Commercial timothy grass pollen extracts exhibit different allergenic activity in vivo

Whether the varying allergen concentrations in the commercial allergen extracts affect biological test results in clinical practice was studied by SPT experiments in 10 grass pollen-allergic patients (Table 2).

If the different commercial grass pollen extracts were identical in terms of the contents of the individual allergens, one would expect that each of the four extracts induces an identical weal reaction in a given patient at the same time. However, the weal reactions induced with the four extracts showed variability in the same patients. For example, extract 1 induced a weal area of 13 mm² in patient 4 whereas extract 2 induced a weal area of 70 mm² in the same patient. In patient 2, extract 1 induced the largest weal area (96 mm²) whereas extract 2 induced only a 20 mm² weal area.

If only the allergen concentration between the different extracts was subject to variability and the relation of the individual allergen concentrations was constant, one would expect that the biological activity just differs for a certain constant factor. However, this was not the case. Extract 1 was approximately sevenfold more potent in patient 2 than in patient 4 whereas extract 2 was

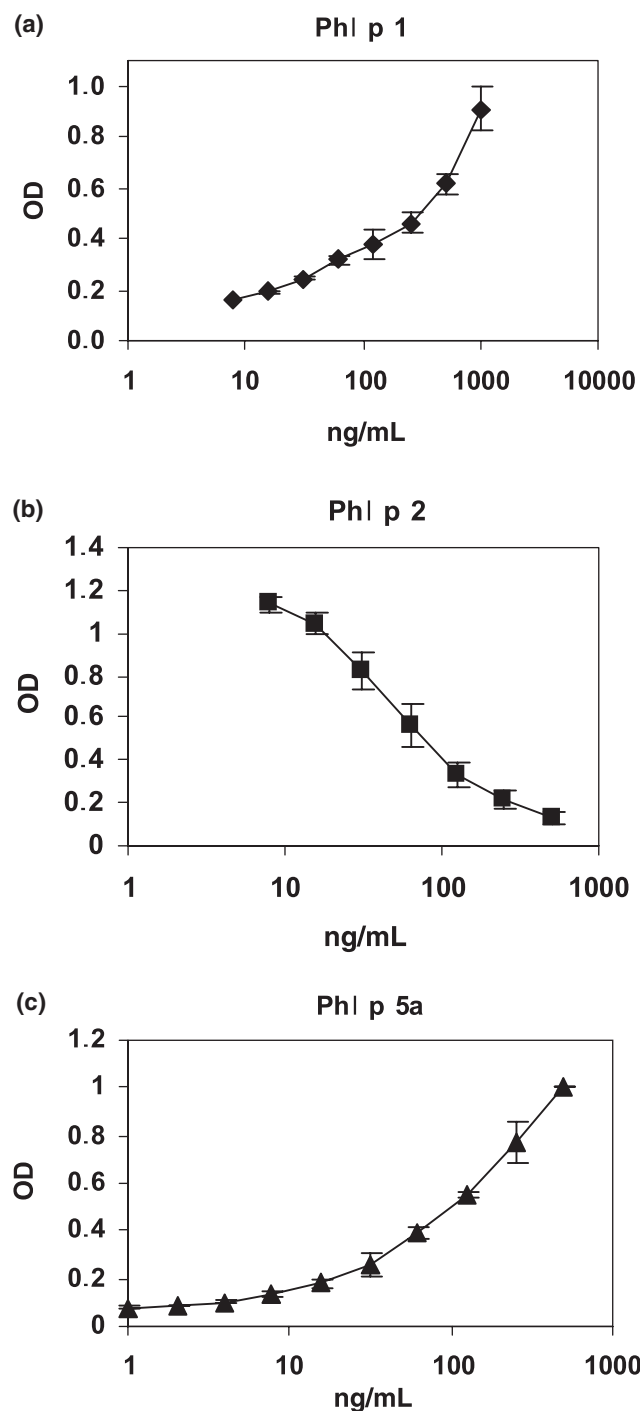


Fig. 3. Dose-response curves with inter-assay SDs for quantification of rPhl p 1 (a), rPhl p 2 (b) and rPhl p 5a (c). The x-axes show the allergen concentrations in a logarithmic scale and the y-axes show the corresponding optical density (OD) values.

3.5-fold more potent in patient 4 than in patient 2. Similar results were found for the other patients and can only be explained by the high variability regarding the concentrations of individual allergens and by the presence of different ratios of the allergens in the commercial extracts.

Table 2. Summary of skin prick test results mean weal areas in mm²

Extract	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
1	14	96	80	13	52	156	25	26	78	184
2	26	20	92	70	30	86	22	14	68	110
3	32	40	62	36	46	143	17	13	56	90
4	27	89	175	40	15	191	26	39	75	130
Histamine	31	33	69	39	17	17	16	15	30	21

Discussion

Our analysis of four commercial extracts produced by European allergen manufacturers for SPT has revealed several substantial weaknesses in the tested products that may have important implications for diagnosis, monitoring of therapy and even treatment of grass pollen allergy.

First, we found that the four commercial timothy grass pollen extracts showed an almost 10-fold variation regarding the total protein contents. Second, we demonstrated a considerable variability regarding the contents of major allergens. Finally, we obtained evidence that defined allergens were degraded and several important allergens were lacking in certain extracts.

For example, we could demonstrate that the Phl p 1 content was generally low in all four extracts and differed up to a factor of 12, pointing to the fact that the most frequently recognized timothy grass pollen allergen, Phl p 1, seems to be under-represented in the extracts. The content of Phl p 5, another frequently recognized allergen, showed the greatest variability up to a factor of 20 and was degraded in some extracts. Phl p 2 could be detected in each of the four extracts although the concentrations of Phl p 2 varied up to a factor of 6. Only the timothy grass pollen extract from company 4 contained all examined allergens, except the highly cross-reactive timothy grass pollen profilin, Phl p 12, which could not be detected in any of the commercial extracts.

There are several explanations for the observed variations. The differences regarding allergen contents may be caused by factors related to the raw materials (i.e., pollen) used by the manufacturers [6]. For example, it has been demonstrated that allergen contents in pollen may vary depending on different plant cultivars [42], climate conditions [43] and stages of pollen maturation [44]. Furthermore, there is evidence that the expression of certain allergens is under the influence of growth and environmental factors [45, 46]. It is also known that the major grass pollen allergens rapidly migrate to the pollen exine under humid conditions [28], so that storage conditions may affect allergen concentrations in pollen grains.

Finally, and perhaps most importantly, different methods of extraction and treatment of extracts will have a major influence on the presence of allergens and their possible degradation [8, 47].

Although a positive skin test result was obtained with each of the four grass pollen extracts in each of the 10 tested grass pollen-allergic patients, we noted an obvious variability of test results. We do not think that these observed differences can be explained by variabilities due to the SPT performance. Skin prick test gives reproducible results at different application sites and shows no relevant variability during the day time [48, 49]. To prevent carryover, we always used sterile disposable lancets from the same company [50], changed lancets between SPT sites [51] and the distance between the SPT sites was large enough, so that proximity effects can be excluded [30].

Using the allergen extracts evaluated by us, it is therefore impossible to draw conclusions as to whether a patient is highly sensitive to grass pollen or not. Moreover, test results obtained with different extracts cannot be compared and thus cannot be used for the monitoring of a change in sensitivity under treatment (e.g., allergen-specific immunotherapy, pharmacological treatment). One should also consider the possibility that patients with a low degree of exclusive sensitization to Phl p 1 or Phl p 5 or to one of the minor components may be negative when tested with certain of the extracts.

Another important problem related to natural allergen extracts is the occurrence of allergen isoforms with varying biological activity in natural allergen preparations [52, 53]. It has been reported that natural allergens consist of isoforms with high, low or even no allergenic activity and varying T cell-stimulating activity [53, 54]. We are well aware of the fact that such isoforms or degradation products with reduced allergenic activity cannot be accurately differentiated from highly allergenic materials using animal-derived antibody probes as used in our study. Nevertheless, antibody-based measurements reliably detect differences regarding the concentrations of the corresponding epitopes in different allergen extracts. In our study, we measured allergen molecules in diagnostic skin test extracts but one must assume that the raw material, i.e., allergen extracts, used for the preparation of skin test solutions will also be used for the preparation of therapeutic vaccines. The differences in the presence of allergens may have even more important implications for such therapeutic extracts because allergens that are lacking or that are present in small amounts will fail to induce protective immune responses against these components [55]. For example, it has been noted that immunotherapy treatment with natural grass pollen extracts indeed induces a highly heterogeneous IgG response against individual grass pollen allergens, which may be one explanation for the partly unpredictable effect of immunotherapy [55].

It is impossible to influence the natural composition of allergen extracts. Methods of standardization can only reveal the compositions of natural allergen extracts but

cannot influence them. One possibility to overcome the lack of certain components in extracts would be the addition of defined recombinant allergen as has been done for the improvement of diagnostic latex extracts by addition of recombinant Hev b 5 [56]. Alternatively, we would suggest to replace natural grass pollen allergen extracts either with mixtures of recombinant allergens [57] or to use recombinant hybrid allergens [58] that incorporate the relevant IgE epitopes of a complex allergen source within one or a few molecules. A hybrid molecule containing the IgE epitopes of timothy grass pollen has already been used for the successful skin test diagnosis of grass pollen allergy recently [59] and might be applied to overcome the problems seen with the natural grass pollen extracts.

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References

- Valenta R, Kraft D. From allergen structure to new forms of allergen-specific immunotherapy. *Curr Opin Immunol* 2002; 14:718–27.
- Ferreira F, Hawranek T, Gruber P, Wopfner N, Mari A. Allergic cross-reactivity: from gene to the clinic. *Allergy* 2004; 59:243–67.
- Mari A. Importance of databases in experimental and clinical allergology. *Int Arch Allergy Immunol* 2005; 138:88–96.
- Schmid-Grendelmeier P, Cramer R. Recombinant allergens for skin testing. *Int Arch Allergy Immunol* 2001; 125:96–111.
- Linhart B, Valenta R. Molecular design of allergy vaccines. *Curr Opin Immunol* 2005; 17:646–55.
- Esch RE. Allergen source materials and quality control of allergenic extracts. *Methods* 1997; 13:2–13.
- Stull A, Cooke RA, Tennant JA. The allergen content of pollen extracts. Its determination and its deterioration. *J Allergy* 1933; 4:455–67.
- Malling HJ. Skin prick testing in biological standardization of allergenic products. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M* 1997; 91:157–63.
- Aas K, Backman A, Belin L, Weeke B. Standardization of allergen extracts with appropriate methods. The combined use of skin prick testing and radio-allergosorbent tests. *Allergy* 1978; 33:130–7.
- Gjesing B, Jager L, Marsh DG, Löwenstein H. The international collaborative study establishing the first international standard for timothy (*Phleum pratense*) grass pollen allergenic extract. *J Allergy Clin Immunol* 1985; 75:258–67.
- Ford AW, Rawle FC, Lind P, Spiekma FT, Löwenstein H, Platts-Mills TA. Standardization of *Dermatophagoides pteronyssinus*:

- assessment of potency and allergen content in ten coded extracts. *Int Arch Allergy Appl Immunol* 1985; **76**:58–67.
- 12 Tovey ER, Baldo BA. Standardization of allergens. Qualitative definition of house dust mite extracts following electroblotting and detection of components with antibody and lectin probes. *Int Arch Allergy Appl Immunol* 1984; **75**:322–9.
 - 13 van Ree R. Value of monoclonal antibody-based assays: advantages and drawbacks. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M* 1994; **87**:127–35.
 - 14 Chapman M. Monoclonal antibody-based immunoassays: quantitative methods for allergen standardization. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M* 1994; **87**:120–4.
 - 15 Yman L, Ponterius G, Brandt R. RAST-based allergen assay methods. *Dev Biol Standard* 1975; **29**:151–65.
 - 16 Lichtenstein LM, Osler AG. Studies on the mechanisms of hypersensitivity phenomena. IX. Histamine release from human leukocytes by ragweed pollen antigen. *J Exp Med* 1964; **120**:507–30.
 - 17 Kaul S, Luttkopf D, Kastner B et al. Mediator release assays based on human or murine immunoglobulin E in allergen standardization. *Clin Exp Allergy* 2007; **37**:141–50.
 - 18 Eichler I, Götz M, Jarisch R, Eichler G, Moss R. Reproducibility of skin prick testing with allergen extracts from different manufacturers. *Allergy* 1988; **43**:458–63.
 - 19 Trivedi B, Valerio C, Slater JE. Endotoxin content of standardized allergen vaccines. *J Allergy Clin Immunol* 2003; **111**:777–83.
 - 20 Valerio CR, Murray P, Arlian LG, Slater JE. Bacterial 16S ribosomal DNA in house dust mite cultures. *J Allergy Clin Immunol* 2005; **116**:1296–300.
 - 21 Akkerdaas JH, Wensing M, Knulst AC et al. How accurate and safe is the diagnosis of hazelnut allergy by means of commercial skin prick test reagents? *Int Arch Allergy Immunol* 2003; **132**:132–40.
 - 22 Schwartz HJ. Problems with skin testing in patients with allergic bronchopulmonary aspergillosis. *Ann Allergy Asthma Immunol* 1995; **75**:533–5.
 - 23 Van der Veen MJ, Mulder M, Wittman AM et al. False positive skin prick test response to commercially available dog dander extracts caused by contamination with house dust mite (*Dermaphagoides pteronyssinus*) allergens. *J Allergy Clin Immunol* 1996; **98**:1028–34.
 - 24 Suphioglu C. What are the important allergens in grass pollen that are linked to human allergic disease? *Clin Exp Allergy* 2000; **30**:1335–41.
 - 25 Anderson K, Lidholm J. Characteristics and immunobiology of grass pollen allergens. *Int Arch Allergy Immunol* 2003; **130**:87–107.
 - 26 Swoboda I, Grote M, Verdino P et al. Molecular characterization of polygalacturonases as grass pollen-specific marker allergens: expulsion from pollen via submicronic respirable particles. *J Immunol* 2004; **172**:6490–500.
 - 27 Stumvoll S, Lidholm J, Thunberg R et al. Purification, structural and immunological characterization of a timothy grass (*Phleum pratense*) pollen allergen, Phl p 4, with cross-reactive potential. *Biol Chem* 2002; **383**:1383–96.
 - 28 Vrtala S, Grote M, Duchene M et al. Properties of tree and grass pollen allergens: reinvestigation of the linkage between solubility and allergenicity. *Int Arch Allergy Immunol* 1993; **102**:160–9.
 - 29 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**:248–54.
 - 30 Nelson HS, Knoetzer J, Bucher B. Effect of distance between sites and region of the body on results of skin prick tests. *J Allergy Clin Immunol* 1996; **97**:596–601.
 - 31 Abramoff MD, Magelhaes PJ, Ram SJ. Image processing with ImageJ. *Biophoton Int* 2004; **11**:36–42.
 - 32 Focke M, Mahler V, Ball T et al. Nonanaphylactic synthetic peptides derived from B cell epitopes of the major grass pollen allergen, Phl p 1, for allergy vaccination. *FASEB J* 2001; **15**:2042.
 - 33 Focke M, Linhart B, Hartl A et al. Non-anaphylactic surface-exposed peptides of the major birch pollen allergen, Bet v 1, for preventive vaccination. *Clin Exp Allergy* 2004; **34**:1525–33.
 - 34 Vrtala S, Susani M, Sperr WR et al. Immunologic characterization of purified recombinant timothy grass pollen (*Phleum pratense*) allergens (Phl p1, Phl p2, Phl p 5). *J Allergy Clin Immunol* 1996; **97**:781–7.
 - 35 Vrtala S, Fischer S, Grote M et al. Molecular, immunological, and structural characterization of Phl p 6, a major allergen and P-particle-associated protein from timothy grass (*Phleum pratense*) pollen. *J Immunol* 1999; **163**:5489–96.
 - 36 Westritschnig K, Focke M, Verdino P et al. Generation of an allergy vaccine by disruption of the three-dimensional structure of the cross-reactive calcium-binding allergen, Phl p 7. *J Immunol* 2004; **172**:5684–92.
 - 37 Vrtala S, Wiedemann P, Mittermann I et al. High-level expression in *Escherichia coli* and purification of recombinant plant profilins: comparison of IgE-binding capacity and allergenic activity. *Biochem Biophys Res Commun* 1996; **226**:42–50.
 - 38 Marth K, Focke M, Flicker S, Valenta R. Human monoclonal antibody-based quantifications of group 2 grass pollen allergens. *J Allergy Clin Immunol* 2004; **113**:470–4.
 - 39 Lämml U. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970; **227**:680–5.
 - 40 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**:4350–4.
 - 41 Valenta R, Duchene M, Ebner C et al. Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992; **175**:271–3.
 - 42 Castro AJ, de Dios Alche J, Cuevas J, Romero PJ, Alche V, Rodriguez-Garcia MI. Pollen from different olive tree cultivars contains varying amounts of the major allergen Ole e 1. *Int Arch Allergy Immunol* 2003; **131**:164–73.
 - 43 Hjeltners M, Schumacher MJ, Van Hage-Hamsten M. Heterogeneity of pollen proteins within individual *Betula pendula* trees. *Int Arch Allergy Immunol* 1995; **108**:368–76.
 - 44 Mittermann I, Swoboda I, Pierson E et al. Molecular cloning and characterization of profilin from tobacco (*Nicotina tabacum*): increased profilin expression during pollen maturation. *Plant Mol Biol* 1995; **27**:137–46.
 - 45 Midoro-Horiuti T, Brooks EG, Goldblum RM. Pathogenesis-related proteins of plants as allergens. *Ann Allergy Asthma Immunol* 2001; **87**:261–71.
 - 46 Hayek B, Reichenauer T, Kraft D, Valenta R. Elevated levels of ozone increase allergen contents in pollen of rye (*Secale cereale* L.). *Allergy* 1999; **54**:OP10–85.

- 47 Nelson HS, Ikle D, Buchmeier A. Studies of allergen extract stability: the effects of dilution and mixing. *J Allergy Clin Immunol* 1996; **98**:382–8.
- 48 Demoly P, Bousquet J, Manderscheid JC, Dreborg S, Dhivert H, Michel FB. Precision of skin prick and puncture tests with nine methods. *J Allergy Clin Immunol* 1991; **88**:758–62.
- 49 Vichyanond P, Nelson HS. Circadian variation of skin reactivity and allergy skin tests. *J Allergy Clin Immunol* 1989; **83**:1101–6.
- 50 Illi S, Garcia-Marcos L, Hernando V, Guillen JJ, Liese A, von Mutius E. Reproducibility of skin prick test results in epidemiologic studies: a comparison of two devices. *Allergy* 1998; **53**:353–8.
- 51 Piette V, Bourret E, Bousquet J, Demoly P. Prick tests to aeroallergens: is it possible simply to wipe the device between tests? *Allergy* 2002; **57**:940–2.
- 52 Chang ZN, Liu CC, Tam MF, Peng HJ, Tsai JJ, Han SH. Characterization of the isoforms of the group I allergen of *Cynodon dactylon*. *J Allergy Clin Immunol* 1995; **95**:1206–14.
- 53 Gelhar K, Petersen A, Schramm G, Becker WM, Schlaak M, Bufe A. Investigation of different recombinant isoforms of grass group-V allergens (timothy grass pollen) isolated by low-stringency cDNA hybridization – antibody binding capacity and allergenic activity. *Eur J Biochem* 1997; **247**:217–23.
- 54 Würtzen PA, Bufe A, Wissenbach M *et al.* Identification of isoform-specific T-cell epitopes in the major timothy grass pollen allergen, Phl p 5. *Clin Exp Allergy* 1999; **29**:1614–25.
- 55 Mothes N, Heinzkill M, Drachenberg KJ *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**:1198–208.
- 56 Lundberg M, Chen Z, Rihs HP, Wrangsjo K. Recombinant spiked allergen extract. *Allergy* 2001; **56**:794–5.
- 57 Heiss S, Mahler V, Steiner R *et al.* Component resolved diagnosis (CRD) of type I allergy with recombinant grass and tree pollen allergens by skin prick testing. *J Invest Dermatol* 1999; **113**:830–7.
- 58 Linhart B, Hartl A, Jahn-Schmid B *et al.* A hybrid molecule resembling the epitope spectrum of grass pollen for allergy vaccination. *J Allergy Clin Immunol* 2005; **115**:1010–6.
- 59 Metz-Favre C, Linhart B, Focke-Tejkl M *et al.* Skin test diagnosis of grass pollen allergy with a recombinant hybrid molecule. *J Allergy Clin Immunol* 2007; **120**:315–21.