A Proteomic Style Approach To Characterize a Grass Mix Product Reveals Potential Immunotherapeutic Benefit

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Background: Grass allergy immunotherapies often consist of a mix of different grass extracts, each containing several proteins of different physiochemical properties; however, the subtle contributions of each protein are difficult to elucidate. This study aimed to identify and characterize the group 1 and 5 allergens in a 13 grass extract and to standardize the extraction method.

Methods: The grass pollens were extracted in isolation and pooled and also in combination and analyzed using a variety of techniques including enzyme-linked immunosorbent assay, liquid chromatography-mass spectrometry, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Results: Gold-staining and IgE immunoblotting revealed a high degree of homology of protein bands between the 13 species and the presence of a densely stained doublet at 25–35 kD along with protein bands at approximately 12.5, 17, and 50 kD. The doublet from each grass species demonstrated a high level of group 1 and 5 interspecies homology. However, there were a number of bands unique to specific grasses consistent with evolutionary change and indicative that a grass mix immunotherapeutic could be considered broad spectrum.

Conclusions: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and IgE immunoblotting showed all 13 grasses share a high degree of homology, particularly in terms of group 1 and 5 allergens. IgE and IgG enzyme-linked immunosorbent assay potencies were shown to be independent of extraction method.

Key Words: allergens, characterization, extraction, homology and standardization, mass spectrometry

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Grass pollen is one of the most common and prevalent causes of allergic symptoms and has been found to be the sensitizing agent in at least 40% of allergic individuals worldwide.[1] Allergen exposure triggers the production of allergen-specific IgE antibodies and the recruitment of eosin-ophils through the action of T-cell cytokines, predominantly IL-4 and IL-5. Cross-linked allergen-specific IgE on the surface of mast cells and basophils triggers the release of inflammatory mediators such as histamine and leukotri-enes,[2,3] which cause early symptoms such as rhinitis, among others.[4,5]

Allergen-specific immunotherapy (SIT), where the individual is repeatedly exposed to increasing amounts of the allergen, resulting in a desensitization to the allergen, is the only causative allergy treatment to date.[6] Although the precise underlying immunologic mechanism is yet to be fully elucidated, an increase in the production of IgG antibodies, particularly IgG4, has been demonstrated in parallel with increasing antigen dose.[7] A report by Vrtala and coworkers[8] also showed that immunization with allergens induced blocking IgGl antibodies that bound to the same IgE epitopes, thus inhibiting IgE-mediated histamine release and the associated inflammatory response. Other immunologic effects described as a result of SIT include a shift in the T_H2 response typical in allergy to T_H1[9] and a reduction in the number of circulating basophils.[10]

The grass family (Poaceae) consists of more than 10,000 species and during the last few decades many species have been investigated intensively for allergenicity. The majority of proteins recognized as the major causes of grass allergy have been identified in the Pooideae subfamily found in temperate zones.[11] Other allergens have been identified in the Chloridoideae and Panicoideae subfamilies[12] of subtropical regions. To date, 13 groups of grass pollen allergens have been identified from different grass species,[11] of which groups 1 and 5 are considered major allergens. Both group 1 and group 5 allergens are expressed in several grass species and IgEs against these proteins represent up to 80% or more of the specific IgE in patients allergic to grass.[13] These allergens have been conserved in the Pooideae species[11] and hence share a high degree of homology in their amino acid sequences, sometimes up to 90% and 75% for group 1 and group 5, respectively.[13] However, molecular differences resulting in isoforms of these 2 allergens in different species have been observed.[14,15] This observation, taken together with the involvement of minor allergens, the nonuniform geographical distribution of grasses, and the polyexposure of each individual to multiple pollens, strongly suggests that there will be heterogeneity in the sensitization profile of each patient. Thus, immunotherapy with mixed extracts may ensure that different patients benefit equally.

However, controversy exists on the benefits of an immunotherapeutic mix containing more than 5 grass species; in addition, the reproducibility of extracting different pollen species in combination has been debated.[16] Hence, this study

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investigated a sublingual and subcutaneous immunotherapeutic consisting of 13 different grass species from the Pooideae subfamily (Table 1). The effects of extracting the grass pollens in isolation and in combination were studied by analyzing their respective total protein content, IgE reactivity, and chromatographic profiles. The suspected group 1 and group 5 allergens were isolated and a proteomics style approach was used to match peptide sequence to online databases.

MATERIALS AND METHODS

Grass Pollen Extracts

Grass pollens were purchased from Allergon (Ängelholm, Sweden) and Pharmallerga (Lisov, Czech). The combined extracts were prepared by roller mixing 2.31 g of pollen (from each of the 13 grass species) in 600 mL of extraction buffer (Pool A). This was followed by centrifugation at 3000*g* for 10 minutes and the supernatant was clarified by passage through a 0.2-μm syringe filter (Millipore, Watford, UK). Five percent single pollen extracts were prepared by roller mixing 2.31 g of pollen in 46.15 mL of phosphate extraction buffer (1 μM Na_2HPO_4, 271.89 nM KH_2PO_4, 8.56 μM NaCl, 0.5% phenol, 2 M HC1, 2 M NaOH) at 2–8°C for 18 hours and clarifying as described for pool A. One milliliter of each single pollen extract was then combined to give a pool of 13 grass extracts (pool B) and the remainder of the single pollen extracts retained for testing.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Aliquots of the pollen extracts (single pollen extracts, pool A and pool B) were denatured by heating at 100°C for 2 minutes in sample buffer containing sodium dodecyl sulfate. The proteins were then resolved on a 10%–20% Tris-HC1 Criterion gel (Bio-Rad, Hemel Hempstead, UK) and the gels electrophoresed according to the manufacturer's protocol. The separated proteins were transferred onto polyvinyldifluoride membranes using a semidry apparatus (Bio-Rad). The membranes were either stained with colloidal gold stain (BioRad) for total protein profile or used for Western blotting.

TABLE 1. Pollens in the 13 Grass Extract

Grass	Species	Abbreviation
Colonial Bent	*Agrostis capillaris*	*Agr ca*
Brome	*Bromus inermis*	*Bro i*
Orchard	*Dactylis glomerata*	*Dac g*
Crested Dogstail	*Cynosurus cristatus*	*Cyn cr*
False Oat	*Arrhenatherum elatius*	*Arr e*
Fescue Meadow	*Festuca pratensis*	*Fesp*
Foxtail Meadow	*Alopecurus pratensis*	*Alo p*
Meadow	*Poa pratensis*	*Poa p*
Rye	*Lolium perenne*	*Lol p*
Timothy	*Phleum pratense*	*Phl p*
Sweet Vernal	*Anthoxanthum odoratum*	*Ant o*
Yorkshire Fog	*Holcus lanatus*	*Hol I*
Cultivated Rye	*Secale cereale*	*Sec c*

IgE and IgG Western Blotting

The membranes were blocked with 10% milk diluent (KPL, Middlesex, UK) prepared in Dulbecco phosphate buffered saline (DPBS). The membranes were then washed with DPBS–0.3% Tween 20 and incubated overnight at 4°C with sera. IgE sera ($n > 2$) from individuals allergic to grass was screened by PlasmaLab (Everett, WA) and evaluated in-house before pooling was diluted 1:5 (vol/vol in 5% milk diluent) or an international standard IgE sera sourced from the Center for Biologies Evaluation and Research (CBER; $n = 5$) was used. Alternatively, IgG sera raised in rabbits immunized with purified grass extracts diluted 1:1000 (vol/vol) were used. The membranes were washed again and incubated with biotinylated goat anti-human IgE or goat anti-rabbit IgG (diluted 1:1000) for 1 hour at room temperature. After being washed, the membranes were incubated with streptavidin peroxidase (Sigma, Poole, UK) diluted 1:1000 for another hour at room temperature. After the final wash, the color was developed with the addition of 1 component 3,3′,5,5′-tetramethylbenzidine membrane peroxidase substrate (KPL). The color reaction was stopped by washing the membranes with distilled water.

Isoelectric Focusing (IEF)

For IEF, ampholine PAG plate gels (pH 3.5–9.5), pI standard (pH 4.7–10.6; Pharmacia Biotech AB), and broad range pI standard (pH 3–10; GE Healthcare, Buckinghamshire, UK) were used. The gel was mounted onto a Multiphor II electrophoresis unit cooling system (Pharmacia Biotech AB) set at 7°C and 20 μL of each pollen extract and 5 μL of pI standards were focused for 500 V, 25 mA, and 10 W for 30 minutes, followed by 2000 V, 20 mA, and 25 W and finally 2000 V, 20 mA, and 30 W. Immediately after IEF, the gel was fixed, washed, and visualized with Coomassie Brilliant Blue G250. The gel was destained and scanned.

IgE and IgG ELISA Potency Determination

Potency ELISAs were performed to measure the IgE and IgG reactivity of the 2 types of grass extracts (ie, pools A and B). IgE reactivity was determined by competing solid-phase grass extract with soluble samples for IgE antibodies. Briefly, microtitre plates (Corning) were incubated overnight at 2–8°C with a freeze-dried 12 grass extract in DPBS containing magnesium chloride and calcium chloride. The plates were washed with DPBS-Tween 20 and blocked with 1% bovine serum albumin solution in coating buffer. After washing, samples were then loaded on, followed by human anti-grass IgE sera, and incubated for 2 hours with continuous shaking at 20°C. The plates were washed once again and incubated with goat anti-human IgE horseradish peroxidase. The color was then developed by adding 3,3′,5,5′-tetramethylbenzidine peroxidase substrate. The reaction was stopped with 1 M orthophosphoric acid and the plates read at 450 nm.

IgG reactivity was determined by measuring the *Lol p 1* content—the major allergen of rye grass pollen found to be distributed between other grass species—[17] using an in-house competition ELISA in conjunction with time-resolved

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fluorescence. Briefly, microtitre plates (Thermo Fisher Scientific, UK) were coated with 50 μg/mL staphylococcal protein-A in DPBS. After the plate was washed with DPBS–0.1% Tween 20, the wells were incubated with rabbit anti-Lol p 1 serum at 37°C for 1 hour. This was followed by the addition of a mixture of grass extract and Europium-labeled purified Lol p 1 and incubated at 37°C for 1.5 hours. The plate was washed again and the reaction developed with enhancement solution. The plate was then read using a time-resolved fluorescence spectrometer (PerkinElmer, Waltham, MA).

Trypsin In-Gel Digestion and Peptide Extraction for Proteomic Style Assessment

The heavily stained protein doublet at 25–37 kD was excised as 2 singlets from Coomassie blue-stained SDS-PAGE protein profiles of each of the 13 grasses and in-gel digestion carried out with trypsin. Gel pieces were washed twice with 50 μL of acetonitrile (ACN) and reduced with 50; uL of 10 mM dithiothreitol/25 mM NH_4HCO_3 at 56°C for 1 hour. The dithiothreitol was removed and cysteines were alkylated in the dark with 50 μL of 55 mM iodoacetimide/ 25 mM NH_4HCO at room temperature for 45 minutes before the addition of trypsin. The iodoacetimide was removed, and the gel pieces were washed with ACN and dried for 30 minutes in a Speedivac. Proteins were digested with 5 μL of 25 ng/μL trypsin along with 45 μL of 25 mM NH_4HCO_3 on ice for 45 minutes followed by overnight incubation at 37°C or a minimum of 4 hours at 48°C. The reaction was terminated with trifiuoroacetic acid to 1% vol/vol and the supernatant retained.

Fifty microliters of 20 mM NH_4HCO_3 was added to the gel pieces before sonication for 5 minutes and then allowed to stand for 20 minutes at room temperature before centrifuging; this step was repeated twice and the retained supernatants were pooled together. The sample was then acidified with 50 μL of 5% formic acid/50% ACN and concentrated down to approximately 25 μL using a Speedivac. The extracted pep-tides were then fractionated and separated on a PepMap100 C18 reverse-phase column (Dionex, Sunnyvale, CA) using an Ultimate U3000 nano-LC system (Dionex) equipped with a 20-μL injection loop. Peptide separation was performed using a linear gradient from 100% solvent A (97.9% water, 2% ACN, 0.1% formic acid) to 56% solvent B (90% ACN, 9.9% water, and 0.1% formic acid) at a flow rate of 350 nL/min.

Tandem Mass Spectrometry and Interpretation of MS/MS Data Sets

The eluted peptides were directly analyzed by tandem mass spectrometry using an LTQ Orbitrap FT-MS (Thermo Scientific) fitted with a nanospray ion source and using stainless steel nano-bore emitters (both Proxeon Biosystems, Odense, Denmark). Tandem mass spectra were collected in a data-dependent fashion by collecting one full MS scan *(m/z* range: 375-1800) followed by MS/MS spectra of the 5 most abundant precursor ions (in ion trap), both in the Orbitrap detector. The resulting MS/MS spectra were then used to search against an annotated UniProtKB/Swiss-Prot database (release version 57) using the SEQUEST protein identification algorithm as implemented within Bio Works v3.3 (Thermo Scientific). Stringent filtering criteria used for positive protein identifications were Xcorr values $>$ 1.9 for +1 spectra, 2.2 for +2 spectra, and 3.75 for +3 spectra and a delta correlation cutoff of 0.1.

Reverse-Phase and Size-Exclusion Chromatography

The proteins within the pooled extracts were separated and analyzed with both reverse-phase and size-exclusion chromatography. The former was on a Jupiter C_4 300 Ä column (Phenomenex, Cheshire, UK) with an injection volume of 10 μL using a 0.1 M phosphate buffer mobile phase (sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate anhydrous; pH 6.8 $\pm$ 0.5) and a flow rate of 1 mL/min. The size exclusion was carried out on a BioSep 4000 column (Phenomenex) using the same mobile phase described above. The following aqueous standards were dissolved in the mobile phase to give a 2 mg/ml solution: dextran blue, thyroglobulin, apoferritin, B-amylase, albumin, and carbonic anhydrase (Sigma).

RESULTS

SDS-PAGE and Western Blot

The pooled grass extracts exhibited identical total protein profiles containing the same number of bands of similar staining intensity while also highly comparable with the 13 single grass extracts, each of which separated into at least 12 bands (Fig. 1A). Both the CBER and in-house grass standards were also comparable and separated into at least 17 bands in the 10–100 kD range. Western blotting of the proteins with an in-house batch of IgE sera (Fig. 1B) and FDA/ CBER-supplied IgE sera (Fig. 1C) demonstrated the allergenic profiles to be highly comparable and also with the total protein. A prominently stained doublet in the 25–37 kDa range was present in all samples along with bands of $\sim$12.5, 17, and 50 kDa.

Figure 1B, C additionally highlights the presence of the considered major allergens Phi p 1 and Phi p 5[13] in each of the 13 grass species and both pooled extracts. However, significant minor allergens are evident with molecular weights ranging from 15 to 50 kDa.

Isoelectric Focusing

The grass pollen extracts (sample identities found in Table 2) were also analyzed on a precast IEF gel (Fig. 2) and each exhibited a highly comparable profile especially in the 3.5-5.85 pi range. Similar to the SDS-PAGE, both pools A and B separated into multiple bands of identical isoelectric points.

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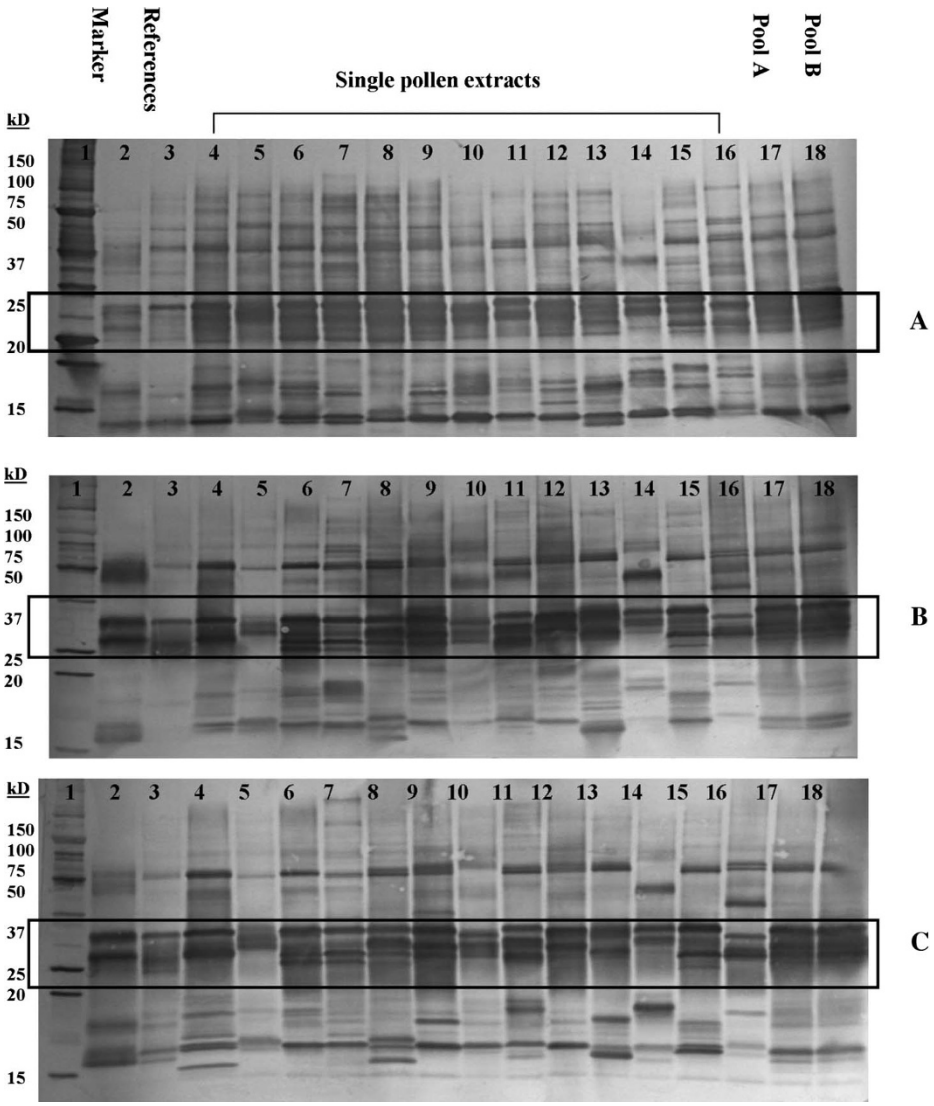

FIGURE 1. Total protein and IgE reactivity profiles of the grass pollen extracts. A, Proteins gold-stained or Western-blotted with (B) ATL IgE sera or (C) CBER IgE sera. Lane 1, molecular weight marker; lane 2, CBER grass standard; lane 3, in-house grass standard; lanes 4-16, single pollen extracts in the order listed in Table 1; lane 17, pollens extracted singly and pooled; lane 18, pollens extracted together.

IgE and IgG ELISA Potency Determination

The 2 pooled grass extracts when assayed on IgE and IgG reactivity ELISAs returned highly comparable potencies (Table 3; QAU/mL [quality assurance unit - arbitrary in-house potency unit]).

Chromatograms

The size-exclusion and reverse-phase chromatograms of the pooled extracts overlay very closely with identical peaks and troughs (Fig. 3A, B).

Mass Spectrometry

The densely stained protein bands between 25 and 37 kDa molecular weight (MW) were trypsin digested and subjected to liquid chromatography-mass spectrometry (LC-MS). The resulting peptides were identified by comparing the MS/MS spectra generated against that of known sequences in the SEQUEST database. The tryptic digests yielded multiple peptides from each grass species with the exception of Cultivated Rye, which yielded only one peptide. A total of 157 peptides were analyzed by LC-MS and compared against SEQUEST, returning a total of 156 matches (data not shown). At least one peptide from each of the 13 species demonstrated homology to *Hol l 1,* with the exception of the Cultivated Rye peptide, which showed homology only to Rye grass. The

TABLE 2. Sample Identity of the IEF Gel

Lane No.	Sample Identity	Lane No.	Sample Identity
1	Marker	9	Meadow
2	Cultivated rye	10	Rye Grass
3	Brome	11	Bent
4	Orchard	12	Sweet Vernal
5	Dogstail	13	Yorkshire Fog
6	Oat	14	Timothy
7	Fescue	15	PoolB
8	Foxtail	16	Pool A

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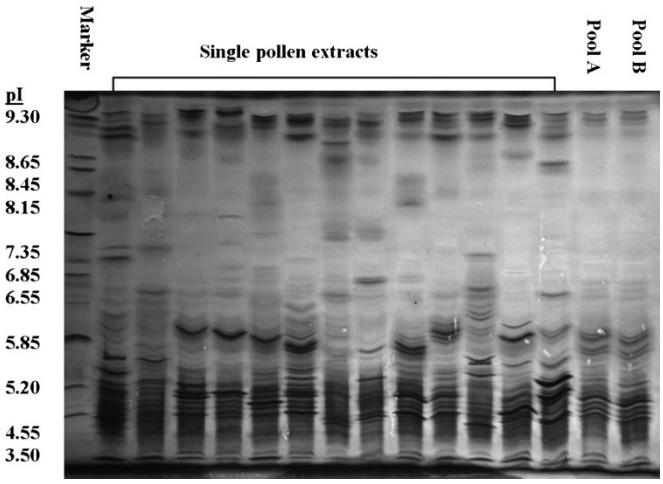

FIGURE 2. Coomassie Blue-stained IEF analysis of grass pollen extracts (sample identities as in Table 2).

peptide sequences that yielded a match against different species are summarized in Table 4.

DISCUSSION

In allergen-specific immunotherapy, patients allergic to grass pollen are often treated with immunotherapeutics consisting of extracts prepared from a mixture of grasses. The composition of allergens in extracts can vary depending on the allergen source, manufacturing process, and storage conditions. Variability can be controlled to a degree by using reproducible extraction and processing procedures. Therefore, in this study, we investigated the impact of extracting 13 different grass pollens together. In addition, we assessed interspecies variability for both major group 1 and group 5 allergens (Table 1) present in the 13 grass immunotherapeutic extract. Molecules extracted from each of these major allergens were characterized using a combination of different analytical methods.

Grass pollens from 13 different pollens when extracted together exhibited indistinguishable gold-stained and IgE reactivity profiles to the pollens extracted singly and combined when separated on SDS-PAGE, both resolving into at least 24 protein bands spanning the 10–100 kD range. The IEF Coomassie Brilliant Blue G250-stained profiles also yielded similar results. The size-exclusion and reverse-phase chromatographic profiles of the pooled extracts (Fig. 3 A, B) show that they contain proteins of the same size and hydrophobicity in the same proportion. Furthermore, ELISA showed the 2 pools to be of highly comparable IgE and IgG reactivities, indicating that extracting the pollens in combination does not negatively affect epitope recognition by antibodies. These observations demonstrate the equivalence of extracting grass pollens in combination or in isolation and pooling.

TABLE 3. IgE and IgG Potency of the Pooled Grass Extracts (QAU/mL)

Sample	IgE Potency	IgG Potency
Pool A	109.1	1346
	111.2	1431
	109.9	1566
Pool B	99.08	1014
	107.1	1471
	116.5	2074

SDS-PAGE and immunoblotting with IgE sera demonstrated the presence of highly similar molecular weight proteins among the grasses, indicating that the grasses share significant homology. Interspecies IgE cross-reactivity was demonstrated in Fig. 1C, which shows that all 13 grass species reacted with the 5-grass CBER-supplied sera. These findings support other studies that report the presence of a high degree of shared epitopes among these grasses using monoclonal antibodies.[18,19]

Our results also reveal the presence of a densely stained protein doublet at 25–37 kDa in all 13 grasses and densely stained proteins at approximately 12.5, 17, and 50 kDa. With use of a proteomics style approach, tryptic digests and LC-MS analysis of the 25–35 kDa doublet as singlets showed the higher and lower molecular weight bands in this doublet to be group 1 and group 5 homologs, respectively (Table 4). With the exception of the Cultivated Rye extract, the higher molecular weight bands of this doublet from all 13 species demonstrated homology to *Hol l 1, Phl p 1,* and *Pha a 1* (Canary Grass; Phalariscanariensis) and to a lesser degree *Lol p 1,* whereas the lower molecular weight bands demonstrated homology to the *Pha a 5, Phl p 5,* and *Lol p 5.* This strongly suggests the excised singlets to be group 1 and group 5 allergens. The discovery of such a high level of interspecies homogeneity is not surprising as members of the Pooideae grass family are considered to be homogenous.[13] The presence of the group 1 and group 5 doublet in all 13 grass extracts signifies the importance of standardization of common allergens to ensure that the allergens responsible for eliciting an allergic response are always present in the immunotherapeutic extract.

However, our results also reveal heterogeneity in the total protein and allergenic profiles of the grasses both in terms of molecular weight and staining intensity. For example, in addition to the prominently stained doublet within the 25–37 kDa range, some of the grasses also contain a similarly stained third and sometimes fourth band at approximately 25 and 27 kDa. A similar observation is evident in the lower molecular weight region where all extracts have a densely gold-stained 10 kDa protein band; however, in some extracts this band is present as a doublet. Furthermore, in the IgE immunoblots a triplet of approximately 10 kDa bands is present in some extracts (Fig. 1). This variation in protein content suggests that each grass species contributes unique properties to an immunotherapeutic mix. As yet unidentified, these bands may be due to the presence of multiple isoforms of the same allergen because of alternative splicing or post-transcriptional modification. In 2009, Chabre et al[20] demonstrated the presence of several primary sequence variants, glycosylated forms, and hence several isoforms of

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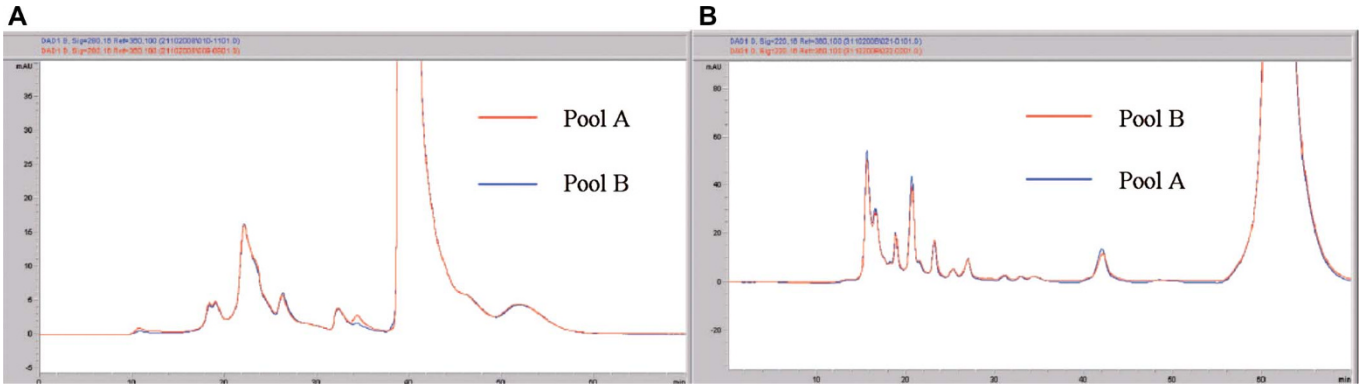

FIGURE 3. A, Size-exclusion chromatogram and (B) reverse-phase chromatogram.

group 1 and group 5 allergens within each grass species. Other studies have also reported quantitative and qualitative differences between the allergen content of Pooideae species.[21–23]

Different sensitization profiles may mean minor allergens are more relevant immunotherapeutic targets in certain patients; hence, standardization based solely on major allergens may be limited in providing data for a wider allergen-sensitive population. A recent study reporting on the efficacy of SIT found that, of 746 patients, 73% sensitized to the major allergen benefited from treatment; however, only 16% of those sensitized to the minor allergens reported the same benefit.[24] Although relatively lower, the figure reveals the benefit of the inclusion of minor allergens. Furthermore, the immunoblots presented here demonstrate that the minor allergens are different across different grass species and this is

TABLE 4. Peptide Homology Generated From LC-MS

Grass	MWof Digested Segment (kDa)	Peptide	Peptide Homology			
Bent	33.9	K.STWYGKPTGAGPK.D	Hol l 1	Lol p 1	Pha a 1	Phl p1
		K.YAVFEAALTK.A	Lol p 5a	Phl p 5b		
Yorkshire Fog	33.5	K.YPDGTKPTFHVEK.G	Hol l1	Pha a 1		
Brome	31.7	K.YAVFEAGLTK.A	Pha a5.1	Pha a5.3	Pha a5.4	
		K.GSNPNYLALLVK.Y	Hol l1	Pha al	Phl p1	
Dogstail	29.4	K.STWYGKPTGAGPK.D	Hol l1	Lol pi	Pha al	Phl p1
		K.YDAYVATLSEALR.I	Phl p5a			
Cultivated Rye	26.6	K.FTVFESAFNK.A	Lol p5a			
False Oat	32.7	K.GKDKWIELK.E	Hol l1	Lol p1	Pha al	Phl p1
	28.6	K.GSNPNYLALLVK.Y	Hol l1	Pha al	Phl pl	
		K.FTVFEGAFNK.A	Pha a 5.1	Pha a 5.3	Pha a 5.4	
Fescue	32.6	K.GSNPNYLALLVK.Y	Hol l1	Pha al	Phl pl	
		K.TFVETFGTATNK.A	Lol p5b			
	28.3	K.YV N/D GDGDVVAVDIK.E	Lol p1	Pha al	Phl pl	
		K.IAATAANAAPTNDK.F	Lol p5a			
Foxtail	32	K.GSNPNYLALLVK.Y	Hol l1	Pha al	Phl p5b	
	29	K.IPAGELQIIDKIDAAFK.V	Phl p5b			
Meadow	33.9	K.GSNPNYLALLVK.Y	Hol l1	Pha al	Phl p5b	
	29.5	K.YAVFEAALTK.A	Lol p5a	Phl p5b		
Orchard	33	K.GSNPNYLALLVK.Y	Hol l1	Pha al	Phl pl	
		K.LAYEAAQGATPEAK.Y	Lol p 5b			
Rye	33	K.GSNPNYLALLVK.Y	Hol l1	Pha al	Phl pl	
		K.LAYEAAQGATPEAK.Y	Lol p 5b			
	28.8	K.YAVFEAALTK.A	Lol p5a	Phl p5b		
Sweet Vernal	33.8	K.GSNPNYLALLVK.Y	Hol l1	Pha al	Phl p1	
		K.YV N/D GDGDVVAVDIK.E	Lol p1	Pha a 1	or Phl p 1 (if N)	
	30	R.VIAGALEVHAVK.P	Lol p5a	Phl p5b		
Timothy	32	K.GSNPNYLALLVK.Y	Hol l 1	Pha a 1	Phlp 1	
	27.7	K.YV N/D GDGDVVAVDIK.E	Lol p1	Pha al	or Phl pl (if N)	
		K.YAVFEAALTK.A	Lol p5a	Phi p5b		

N/D means Asn (N) to Asp (D) deamidation possible.

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supported in findings by Hrabina et al who show that minor allergens show sequence identity, but little cross-reactivity with group 1 allergens.[13] A therapeutic product that contains a broad spectrum of efficacy that the inclusion of minor allergens may confer may be considered beneficial to the pool of patients sensitized to those minor allergens such as Phl p 7 and Phl p 12 while providing equal benefit to patients sensitized to the major allergens Phl p 1 and Phl p 5 as shown in Fig. 1B, C.

Another important factor to consider in patient sensitization profiles is highlighted by a recent study that reported an outbreak of adverse reactions to olive immunotherapy because of the high concentrations of a minor olive allergen *Ole e* 9, in certain batches of olive pollen extracts.[25] Patients living in olive-growing areas were found to be highly sensitized to *Ole e* 9 compared with those outside of these regions where *Ole e* 1, a major allergen, is the relevant allergen. Occurrences such as this further emphasizes the need for allergen standardization as a variation in protein content may lead to adverse events or a reduction in the reactivity/immulnotherapeutic effect. This also indicates an immunotherapeultic based on a 13-grass mixture may allow desensitization to a broader range of epitopes than one with less as it better reflects natural exposure conditions.

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REFERENCES

1. Freidhoff LR, Ehrlich-Kautzky E, Grant JH, Meyers DA, Marsh DG. A study of the human immune response to *Lolium perenne* (rye) pollen and its components, Lol, p. I and Lol, p. II (rye I rye II). Prevalence of reactivity to the allergens and correlations among skin test, IgE antibody, and IgG antibody data. *J Allergy Clin Immunol*. 1986;78:1190–1201.
2. Segal DM, Taurog JD, Metzger H. Dimeric immunoglobulin E serves as a unit signal for mast cell degranulation. *Proc Natl Acad Sci USA*. 1977;74:2993–2997.
3. He SH, Xie H, Zhang XJ, Wang XJ. Inhibition of histamine release from human mast cells by natural chymase inhibitors. *Ada Pharmacol Sin*. 2004;25:822–826.
4. Naclerio RM, Meier HL, Kagey-Sobotka A, Adkinson NF Jr, Meyers DA, Norman PS, Lichtenstein LM. Mediatorrelease after nasal airway challenge with allergen. *Am Rev Respir Dis*. 1983;128:597–602.
5. Creticos PS, Peters SP, Adkinson NF Jr, Naclerio RM, Hayes EC, Norman PS, Lichtenstein LM. Demonstrationof peptide-leukotriene release following nasal antigen challenge in vivo. *N Engl J Med*. 1984;310:1626–1630.
6. Varney VA, Gaga M, Frew AJ, Aber VR, Kay AB, Durham SR. Usefulness of immunotherapy in patients with severe summer hay fever uncontrolled by antiallergic drugs. *BMJ*. 1991;302:265–269.
7. Bullock RJ, Barnett D, Howden ME. Immunologic and clinical responses to parenteral immunotherapy in peanut anaphylaxis–a study using IgE and IgG4 immunoblot monitoring. *Allergol Immunopathol (Madr)*. 2005;33:250–256.
8. Vrtala S, Ball T, Spitzauer S, Pandjaitan B, Suphioglu C, et al. Immunization with purified natural and recombinant allergens induces mouse IgGl antibodies that recognize similar epitopes as human IgE and inhibit the human IgE-allergen interaction and allergen-induced basophil degranulation. *J Immunol*. 1998;160:6137–6144.
9. Ebner C, Siemann U, Bohle B, Willheim M, Wiedermann U, et al. Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Phl p 1, a major grass pollen allergen. *Clin Exp Allergy*. 1997;27:1007–1015.
10. Mikkelsen S, Bibby BM, Dolberg MK, Dahl R, Hoffmann HJ. Basophil sensitivity through CD63 or CD203c is a functional measure for specific immunotherapy. *Clin Mol Allergy*. 2010;8:2.
11. Andersson K, Lidholm J. Characteristics and immunobiology of grass pollen allergens. *Int Arch Allergy Immunol*. 2003;130:87–107.
12. Suphioglu C, Singh MB, Knox RB. Peptide mapping analysis of group I allergens of grass pollens. *Int Arch Allergy Immunol*. 1993;102:144–151.
13. Hrabina M, Peltrew G, Van Reez R, Moingeon P. Grass pollen allergens. *Clin Exp Allergy Rev*. 2008;8:7–11.
14. Esch RE, Klapper DG. Cross-reactive and unique grass group I antigenic determinants defined by monoclonal antibodies. *J Allergy Clin Immunol*. 1987;79:489–495.
15. Muller WD, Karamfilov T, Kahlert H, Stuwe HT, Fahlbusch B, et al. Mapping of T-cell epitopes of Phl p 5: evidence for crossreacting and non-crossreacting T-cell epitopes within Phl p 5 isoallergens. *Clin Exp Allergy*. 1998;28:1538–1548.
16. Committee for Medicinal Products for Human Use (CHMP) Guideline on Allergen Products: Production and Quality Issues. EMEA/CHMP/BWP/304831/2007.
17. Standring R, Spackman V, Porter SJ. Distribution of a major allergen of rye grass (Lolium perenne) pollen between other grass species. *Int Arch Allergy Appl Immunol*. 1987;83:96–103.
18. Kahn CR, Marsh DG. Monoclonal antibodies to the major Lolium perenne (rye grass) pollen allergen Lol p I (Rye I). *Mol Immunol*. 1986;23:1281–1288.
19. Smith PM, Avjioglu A, Ward LR, Simpson RJ, Knox RB, Singh MB. Isolation and characterization of group-I isoallergens from Bermuda grass pollen. *Int Arch Allergy Immunol*. 1994;104:57–64.
20. Chabre H, Gouyon B, Huet A, Boran Bodo V, Nony E, et al. Molecular variability of group 1 and 5 grass pollen allergens between Pooideae species: implications for immunotherapy. *Clin Exp Allergy*. 2010;40:505–519.
21. Moingeon P, Hrabina M, Bergmann K, Jaeger S, Frati F, Bordas V, Peltre G. Specific immunotherapy for common grass pollen allergies: pertinence of a five grass pollen vaccine. *Int Arch Allergy Immunol*. 2008;146:338–342.
22. Marth K, Focke M, Flicker S, Valenta R. Human monoclonal antibody-based quantification of group 2 grass pollen allergens. *J Allergy Clin Immunol*. 2004;113:470–474.
23. Fahlbusch B, Muller WD, Rudeschko O, Jager L, Cromwell O, Fiebig H. Detection and quantification of group 4 allergensin grass pollen extracts using monoclonal antibodies. *Clin Exp Allergy*. 1998;28:799–807.
24. Schmid-Grendelmeier P. Rekombinante Allergene, routine diagnostik Oder Wissenschaft? *Hautarzt*. 2010;61:946–953.
25. Duffort O, Palomares O, Lombardero M, Villalba M, Barber D, Rodriguez R, Polo F. Variability of Ole e 9 allergen in olive pollen extracts: relevance of minor allergens in immunotherapy treatments. *Int Arch Allergy Immunol*. 2006;140:131–138.

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