

REGULAR ARTICLE

Identification of grass pollen allergens by two-dimensional gel electrophoresis and serological screening

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Approximately 50% of allergic patients are sensitized against grass pollen allergens. The characterization of specific immunoglobulin E (IgE) reactivity to allergen components in pollen-allergic patients is fundamental for clinical diagnosis and for immunotherapy. Complex allergen extracts are commonly used in diagnostic tests as well as in immunotherapy preparations, but their composition in single allergenic molecules is only partially known. Diagnostic tests which utilize recombinant or immuno-purified allergens have been made available in clinical practice. They allow to obtain specific profiles of IgE reactivity, but the panel of available molecules is far from complete. Here, we used a proteomic approach in order to detect grass allergens from a natural protein extract. A five-grass pollen extract used for diagnosis and immunotherapy was resolved by two dimensional gel electrophoresis (2-DE), and assayed with 9 sera from pollen-allergic patients whose sensitization profile was dissected by using IgE reactivity to recombinant allergens. 2-DE immunoreactivity patterns were matched with IgE reactivity to identify protein spots as candidate allergens. Identity was confirmed by mass spectrometry analysis. We identified 6 out of 8 expected clinically relevant allergens in the natural grass extract. Moreover, we identified different molecular isoforms of single allergens, thus obtaining a more detailed profile of IgE reactivity. Some discrepancies in protein isoform profile and sera immunoreactivity between recombinant and native allergen 5 from *Phleum pratense* were observed and a new putative allergen was described. The proteomic approach applied to the analysis of a natural allergen allows the comprehensive evaluation of the sensitization profile of allergic patients and the identification of new allergens.

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Abbreviations: IgE, immunoglobulin E; kU_A/L, kilo arbitrary Unit/L; Phl p5, allergen 5 from *Phleum pratense*; RAST, Radio Allergo Sorbent Test

1 Introduction

Grass pollen is one of the most important airborne allergen sources worldwide, approximately 50% of allergic patients are sensitized against their allergens. Characterization of specific immunoglobulin E (IgE) reactivity to single allergen components in pollen-allergic patients has implications in clinical diagnosis, in the

design of patient-tailored immunotherapy and in the elucidation of the mechanisms of sensitization to multiple allergens.

About 20 species from five subfamilies of grass are considered to be causes of allergy, and the allergenic relationships closely follow their phylogenetic relationships [1]. The most allergenic grasses are *Dactylis*, *Festuca*, *Poa*, *Lolium*, *Phleum*, and an extensive cross-reactivity among them is expected [2, 3]. For example *Lolium*, *Festuca*, *Poa* and *Dactylis* have a virtually complete allergen identity, while in *Phleum pratense* some antigens are present that are not shared by other grass species. On this basis, in order to test sensitivity to grass in general it is enough to test patients for their sensitivity to allergens of *Phleum pratense* [4].

Allergens are named according to the following rules [5]: (i) first 3 letters indicate the genus; (ii) a letter, in lower case, stands for the species, and (iii) an Arabic number indicates the group of allergen (for example allergen 5 from *Phleum pratense* is indicated as Phl p 5). The same number is assigned to structurally homologous allergens belonging to different species.

Allergen extracts containing a mixture of allergenic and non-allergenic molecules are prevalently used in common diagnostic tests as well as in immunotherapy preparations, but they are difficult to standardize because their composition is only partially known.

Allergen-specific immunotherapy is an efficacious treatment of allergic disease [6, 7]. The immunological mechanisms behind its effect are not fully understood, and may imply the change of the phenotypic T lymphocyte response to allergens (the so-called “Th2-Th1 shift”) and/or the activation of regulatory T cells [8]. Among the potential limitation of this approach *de novo* induction of new specific IgE reactivity can occur to allergenic components present in the extract used for immunotherapy [9, 10, 11, 12]. Furthermore, the efficacy of the treatment is heavily conditioned by the appropriateness of the allergenic composition of the extract used for immunotherapy in a given patient.

Therefore, in order to improve the management of immunotherapy, a mixture of recombinant allergens, tailor-made for each patient and based on *in vitro* measurements of IgE reactivity to individual specific allergens, has been suggested as an alternative to the crude pollen extracts that are used today in diagnosis and immunotherapy [13]. This might allow a more efficient characterization of the individual sensitization profile and the choice of a more suitable treatment, which could prevent the development of new IgE reactivities.

Diagnostic tests which utilize recombinant or immunopurified allergens have been made available in clinical practice. For example, a specific serum IgE dosage based on the ELISA technique and using recombinant (rPhl p 1, 2, 5, 6, 7, 11, 12) and native purified (Phl p 4) allergens of *Phleum pratense* is available to the routine laboratory (Unicap®, Pharmacia Diagnostics AB, Uppsala, Sweden). This test allows detailed profiles of IgE reactivity to be obtained, but the panel of presented molecules is far from being complete. Moreover, these recombinant allergens are produced in *Escher-*

ichia coli, so differences in post-translational modifications (*i.e.* glycosylation) occur. Carbohydrate-reactive IgE antibodies have been attributed to grass pollen sensitization and found to cross-react with glycan structures from other allergen sources, particularly vegetables [1].

To partially overcome these limits and have an alternative technique with complementary information to the conventional ELISA test, we used a proteomic approach for the detection of single grass allergens from a natural protein extract. Grass pollen proteins extract were resolved by 2-DE, and assayed with sera from pollen-allergic patients who were characterized in terms of reactivity to single allergenic molecules. The combination of immunological specificity with 2-DE protein separation and MS analysis allows the identification of additional unknown protein spots as well as allergen isoforms and fragments. We were able to identify known allergens in complex natural grass extracts used for diagnostic and immunotherapy purposes, and to obtain a detailed profile of IgE reactivity in a group of patients allergic to grass, including in single cases specificities not recognized by the ELISA technique.

2 Materials and methods

2.1 Patient sera

Allergic sera were from patients sensitized to grass pollen and affected by hay fever with or without bronchial asthma. Control sera were either from healthy, non-allergic patients or from patients allergic to house dust mite. Sera were separated from whole blood, collected by venipuncture.

Allergen specific serum IgE were measured with a standard Radio Allergo Sorbent Test (RAST) assay of general clinical use (Unicap® specific IgE, Pharmacia Diagnostics AB, Uppsala, Sweden) and expressed as kilo arbitrary unit / L (kU_A/L), according to the manufacturer's instructions.

2.2 Reagents

If not otherwise specified, all the chemicals were from Sigma, St. Louis, MO, USA.

2.3 2-DE

A grass pollen protein extract was obtained from Lofarma SpA, Milan, Italy. This was an “in-house” reference preparation of a mixture of pollens from five different plants (*Phleum pratense*, *Lolium italicum*, *Dactylis glomerata*, *Festuca elatior*, *Poa pratensis*) and it is used as starting material for skin-prick test reagents and for immunotherapy preparations.

Proteins were extracted by acetone precipitation and resuspended in a buffer containing (8 M urea, 4% w/v CHAPS, 65 mM DTT, 0.2% v/v IPG-buffer (pH 3–10 NL) (Amersham Biosciences, Uppsala, Sweden) and 0.05% w/v bromophenol blue.

Samples corresponding to 210 µg of total protein as evaluated by Protein Quantitation Assay (Bio-Rad Laboratories, Hercules, CA, USA) were applied to 7 cm IPG strips (pH 3–10NL) (Amersham Biosciences) by in-gel rehydration at 18°C. Focusing was performed with an IPGphor system (Amersham Biosciences) at 50 µA maximum per IPG strip with a gradient voltage (5000 V maximum) for a total of 25 kVh. Strips were equilibrated for 15 min in 50 mM pH 8.8 Tris-HCl buffer containing 6 M urea, 30% v/v glycerol, 2% v/v SDS and 2% w/v DTT, then for 15 min in the same buffer replacing DTT by 2.5% w/v iodoacetamide. The strips were then transferred onto either 10%–20% gradient or 12% T 3·3% C polyacrylamide SDS-PAGE gels for the second dimension separation. In selected experiments, recombinant *Phleum pratense* p 5 allergen (Biomay, Wien, Germany) was analyzed by 2-DE and Western blot (see below). Preparative 2-DE gels for protein spot excision (see below) were generated by loading 150 µg of total protein.

2.4 Western blot analysis

Proteins resolved by 2-DE were electrotransferred onto nitrocellulose membranes and incubated over night with patient sera (1:4 or 1:10 working dilution depending on the level of *Phleum*-specific IgE). Immunoreactivity was revealed by incubation with goat anti-human IgE conjugated with horse raddish peroxidase (Sigma), followed by ECL (Amersham Biosciences) reaction and film exposure. Images were acquired using a Molecular Dynamics Personal SI Laser Densitometer (Amersham Biosciences) and 2-DE protein patterns analyzed using ImageMaster 2D Platinum V 5.0 software (Amersham Biosciences). M_r were estimated by comparison with M_r reference markers (Precision, Bio-Rad) and pI values assigned to detected spots by calibration as described in the Amersham Biosciences guide lines.

2.5 Protein identification by MALDI-TOF MS analysis

Protein spots were visualized by staining preparative gels with MS compatible silver stain [14]. Spots of interest were excised from gels (either by manual or automated excision (ProXCISION; PerkinElmer, Monza, Italy); reduced, alkylated and digested overnight with bovine trypsin (Roche Milan, Italy), as previously described [15]. One µL aliquots of the supernatant were used for MS analysis using the dried droplet technique and CHCA as matrix. MS spectra were obtained on a MALDI-TOF Voyager-DE STR (Applied Biosystem) mass spectrometer. Alternatively, gel fragments were further extracted and resulting peptide mixtures subjected to a single desalting/concentration step before MS analysis over µZip-TipC₁₈ (Millipore Corporation, Bedford, MA, USA).

Spectra were internally calibrated using trypsin autolysis products and processed via Data Explorer software. Proteins were unambiguously identified by searching a comprehensive non-redundant protein database using both ProFound

and Mascot programs [16, 17]. One missed cleavage per peptide was allowed, and an initial mass tolerance of 50 ppm was used in all searches.

2.6 Protein identification by LC-ES/-MS/MS

Ten µL of trypsin digested sample were injected in an Ultimate+Switchos+Famos System (LC Packings, Amsterdam, The Netherlands) equipped with a 20 µL injection loop. Peptide separations occurred on a RP nano column (75 µm i.d. × 15 cm), packed with C₁₈ PepMap100 material (Lcpackings, Amsterdam, The Netherlands). A gradient of eluents A (H₂O with 2% v/v ACN, 0.1% v/v formic acid) and B (ACN with 2% v/v H₂O with 0.1% v/v formic acid) was used to achieve separation, following: 8% B (at t_0 0.2 µL/min flow rate) to 48% B (at 60 min, 0.2 µL/min flow rate). Eluting peptides were ionized through a Pico Tip FS360-20-10 (New Objective, Cambridge, MA, USA) supported by a nano ESI on-line source and further introduced on an Ion Trap Esquire 3000 plus (Bruker, Bremen, Germany). Nitrogen was used as drying gas (10–12 L/min at 120°C). Analyses were performed in positive ion mode, the HV Potential was set up around 1.5–1.7 kV. Other mass spectrometer parameters were as follows: Trap Drive, 84; Capillary Exit, +225 V; Skimmer, –40.0 V; and Multiplier Voltage, 1950 V. Full scan mass spectra ranging from m/z 140 to 2000 Da were collected and in each duty cycle, the two most intense (at least 1×10^5) peaks in the range from 350 to 1200 were selected for MS/MS. The collision energy was ramped from 30 to 200% of the selected fragmentation amplitude (1.0V).

3 Results

3.1 Sera reactivity characterization

Allergen specificity of patients sera were measured with a standard assay that uses mostly recombinant (p 1, 2, 5–7, 11, 12) and one purified (p 4) *Phleum pratense* allergens. Results were expressed as kU_A/L and values >0.35 kU_A/L were considered positive (Table 1, positive values are underlined). Sera 2 and 6 showed strong reactivity against most of the tested allergens. Sera 5 and 7 had a strong reactivity against Phl p 5 and Phl p 7 respectively, but all the other reactivities were weak. Phl p 1 allergen was recognized by all sera and Phl p 4 by all sera except serum 9. Sera 1, 2, 5–7 shared the reactivity against Phl p 5 and 6; Phl p 2, 11 and 12 were recognized by about 50% of the sera; while reactivity against Phl p 7 was restricted to two sera (# 6 and 7).

3.2 Serological reactivity on 2-DE maps

The grass pollen protein extract was resolved by 2-DE, and separately assayed with sera from nine pollen-allergic patients (Fig. 1). Since a consistent proportion of grass pollen allergic patients is also sensitized to house dust mite, sera

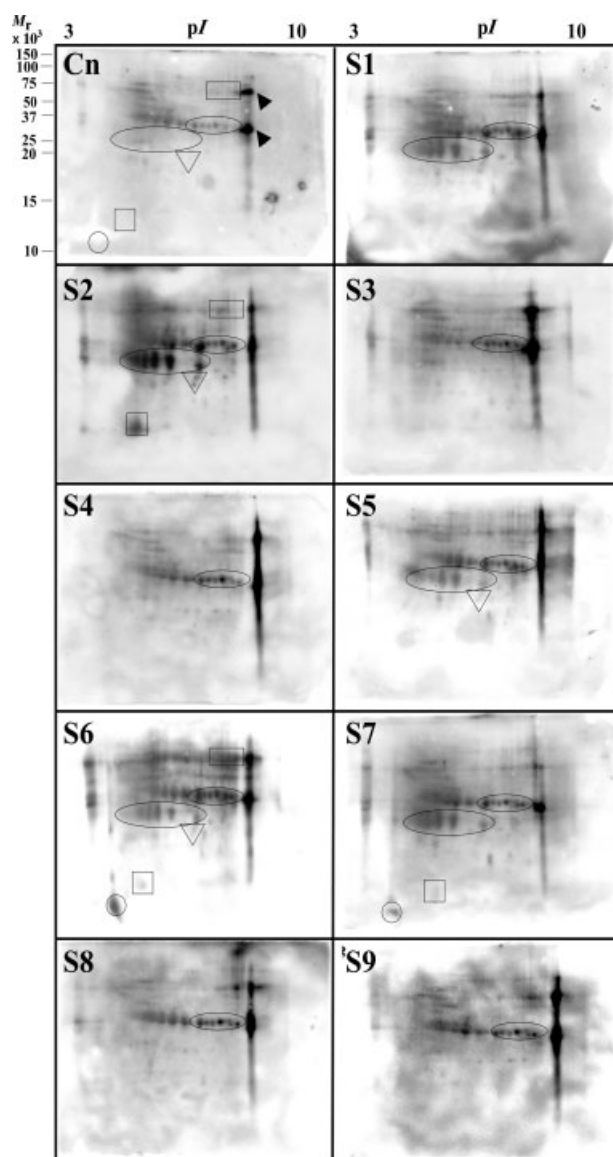
Table 1. Patient attributes and sera RAST reactivity against *Phleum pratense* pollen allergens

Serum	Sex/age	Diagnosis	Phl p 1 ^{a)}	Phl p 2 ^{a)}	Phl p 4 ^{a)}	Phl p 5 ^{a)}	Phl p 6 ^{a)}	Phl p 7 ^{a)}	Phl p 11 ^{a)}	Phl p 12 ^{a)}
1	M/31	RC BA	<u>6.31</u>	<u>2.25</u>	<u>6.52</u>	<u>10.6</u>	<u>0.56</u>	0.1	0.1	0.1
2	M/12	–	<u>97.9</u>	<u>36.3</u>	<u>110</u>	<u>110</u>	<u>59.8</u>	0.1	<u>5.54</u>	<u>15.6</u>
3	M/14	–	<u>5.4</u>	<u>2.85</u>	<u>0.9</u>	0.1	0.1	0.1	0.1	0.1
4	M/39	RC	<u>6.13</u>	0.1	<u>2.01</u>	0.1	0.1	0.1	0.1	0.1
5	M/24	RC BA	<u>10.5</u>	0.1	<u>7.05</u>	<u>24.9</u>	<u>6.02</u>	0.1	<u>6.78</u>	<u>1.67</u>
6	M/33	–	<u>110</u>	<u>75.9</u>	<u>97.6</u>	<u>110</u>	<u>59.8</u>	<u>110</u>	<u>34.2</u>	<u>21</u>
7	F/58	–	<u>7.09</u>	0.1	<u>3.47</u>	<u>6.7</u>	<u>2.82</u>	<u>25.8</u>	<u>3.17</u>	<u>0.63</u>
8	F/15	–	<u>9.8</u>	0.1	<u>0.94</u>	0.1	0.1	0.1	0.1	0.1
9	F/35	RC	<u>3.68</u>	0.1	0.1	0.1	0.1	0.1	0.1	<u>0.6</u>

^{a)} value expressed as kU_A/L

RC, rhino-conjunctivitis

BA, bronchial asthma

**Figure 1.** Serological reactivity on 2-DE maps. Grass pollen Mix5 proteins were resolved by 2-DE (first dimensions were performed on a pH 3–10NL range; second dimensions were performed on 10–20% T 3–3% C polyacrylamide gradient gels), transferred onto nitrocellulose and assayed with ten characterized sera: one from a house dust mite-allergic patient (Cn) and nine from pollen-allergic patients (S1–S9). Specific immunostaining shown by sera from allergic-patients are highlighted by different shapes. Possible reactivity: small ellipse = Phl p 1; big ellipse = Phl p 5; rectangle = Phl p 4; square = Phl p 6; circle = Phl p 7; triangle = unknown. Non-specific cross reactivity is pointed by arrowheads in panel Cn.

from patients monosensitized to house dust mite were included in our assays as controls (Fig. 1). A common cross reactivity against the most abundant proteins not fully resolved at alkaline pI was visible both in the non-allergic controls (data not shown) and in the house dust mite allergic patients (Fig. 1 Cn, arrowheads). The two major protein spots of the vertical streak have been identified by MS analysis as Phl p 4 and Phl p 1 (upper and lower spot, respectively). On the contrary, sera from different allergic patients showed specific immunostaining (Fig. 1). Immunoreactivity patterns matched with both specific IgE test results and allergens theoretical pI and M_r (see Table 2) allowed us to hypothesize specific protein spots as candidate allergens. In particular, spots included in the ellipses might correspond to Phl p 1 and Phl p 5 (Fig. 1, small and big ellipses, respectively); spots highlighted by squares and circles might be Phl p 6 and Phl p 7, respectively (Fig. 1); while spots included in the rectangle might be Phl p 4. Another two spots of immunoreactivity present in 2-DE probed with sera 2, 5 and 6 were not assigned (Fig. 1, triangles).

In order to confirm this hypothetical attribution, and to identify proteins, the spots of interest (Fig. 2, #1–15) were excised from preparative 2-DE gels and analyzed by MALDI-TOF MS. Due to the low amount of protein recovered from gel, spots #8 and 11 were not identified by MALDI-TOF MS and therefore were analyzed by LC-ESI MS/MS.

Table 2. A: Proteins identified by MALDI-TOF MS analysis

Spot	Protein name	Accession number	pI/M _r		Matched peptide	Sequence coverage	Mascot score
			exp.	theor.			
1	group 5 isoallergen 1 [<i>Dactylis glomerata</i>]	gi 14423120	5.96/24 720	5.61/26 300	15	72%	164
2	pollen allergen Phl p 5b precursor	gi 2851457	4.93/27 186	8.75/28 001	14	58%	107
	pollen allergen Lol p VA precursor	gi 4416516	4.93/27 186	5.62/29 767	8	31%	77
3	group 5 isoallergen 1 [<i>Dactylis glomerata</i>]	gi 14423120	4.93/25 267	5.61/26 300	11	53%	104
	pollen allergen Phl p 5b precursor	gi 2851457	4.93/25 267	8.75/28 001	8	42%	101
	pollen allergen Lol p VA precursor	gi 4416516	4.93/25 267	5.62/29 767	6	25%	82
4	pollen allergen Phl p 5b precursor	gi 2851457	4.59/27 620	8.75/28 001	14	63%	138
	pollen allergen Lol p VA precursor	gi 4416516	4.59/27 620	5.62/29 767	11	54%	105
5	pollen allergen Phl p 5b precursor	gi 2851457	4.59/25 053	8.75/28 001	9	46%	95
	pollen allergen Lol p VA precursor	gi 4416516	4.59/25 053	5.62/29 767	7	28%	84
6	calcium-binding pollen allergen Phl p 7	gi 14423846	3.72/10 336	4.19/8676	4	41%	69
7	Phl p 5a allergen precursor [<i>Phleum pratense</i>]	gi 29500897	6.10/32 059	7.94/28 286	11	35%	85
	pollen allergen Lol p 1 precursor (Lol p I)	gi 126385	6.10/32 059	5.52/28 437	6	29%	71
8	n.i.		6.01/15 913				
9	pollen allergen Phl p 6 precursor	gi 1171011	4.91/10 014	5.31/13 920	9	70%	121
10	pollen allergen Lol p 2-A	gi 126386	4.90/9396	5.25/10 880	6	46%	77
12	grass pollen allergen Phl p 4	gi44844852	6.74/62 981	9.19/55 624	16	28%	111
13	pollen allergen Phl p 1	gi3901094	6.74/29 070	7.53/28 203	13	49%	141
	major group I allergen Hol 1 [<i>Holcus lanatus</i>]	gi3860384	6.74/29 070	6.46/28 347	8	33%	72
14	major group I allergen Hol 1 [<i>Holcus lanatus</i>]	gi3860384	6.39/30 282	6.46/28 347	14	52%	140
	pollen allergen Phl p 1	gi3901094	6.39/30 282	7.53/28 203	13	50%	124
15	major group I allergen Hol 1 [<i>Holcus lanatus</i>]	gi3860384	6.25/30 127	6.46/28 347	14	55%	164
	pollen allergen Phl p 1 precursor	gi1171008	6.25/30 127	6.14/28 457	13	47%	137

B: Proteins identified by LC-ESI MS/MS analysis

Spot	Protein name	Accession number	pI/M _r		Sequenced peptide	Mascot score
			exp.	theor.		
11	Phl p 5A protein – common timothy (fragment)	gi 422005	6.02/14 589	8.51/26 546	YDAYVATLSEALR	85

n.i. = not identified

exp. = experimental

theor. = theoretical

Table 2 shows the results of protein spot identifications. Protein spots #1–5 all belong to the group 5 isoallergen, including protein isoforms from three different grasses, namely *Dactylis glomerata* (Dac g 5.1), *Lolium perenne* (Lol p VA), and *Phleum pratense* (Phl p 5b). The fact that more than one protein was identified in the same spot was expected since isoallergens induce identical immunoreactivity, show high protein homology and were all present in the native extract. We identified proteins belonging to *Lolium perenne* instead of *Lolium italicum*, but this was not surprising because only one protein belonging to the latter is present in the database. Another two spots were identified as Phl p 5 allergen: spot #7 contains variant *a* of Phl p 5, while spot #11 was probably a fragment of degradation of Phl p 5a as inferred by experimental M_r being much lower than the theoretical M_r (Table 2). In spot #7 the presence of the group 1 allergen (Lol

p 1) was also identified. Group 1 allergens Phl p 1 and Hol 1 were found in spots #13–15. Unexpectedly, Hol 1 is an allergen from a grass (*Holcus lanatus*) supposedly absent in the native extract we used. Spot #6, 9, 10, and 12 corresponded to calcium-binding pollen allergen 7 (Phl p 7), to Phl p 6, to allergen of a group 2 (Lol p 2A), and to Phl p 4, respectively. Spot #8 was also not identified by LC-ESI MS/MS analysis.

In the case of protein spots belonging to the isoallergen group 1 a weak reactivity was observed in control sera from patients allergic to house dust mite (Fig. 1, Cn, small ellipse) and from non-allergic individuals (data not shown). In order to set up an analysis criteria able to identify specific reactivity against group 1 isoallergens, we evaluated by image analysis the intensity of the immunoreactivity measured as OD of the spot volume. The volume of the spots identified as isoallergen group 1 were quantified in three different control sera

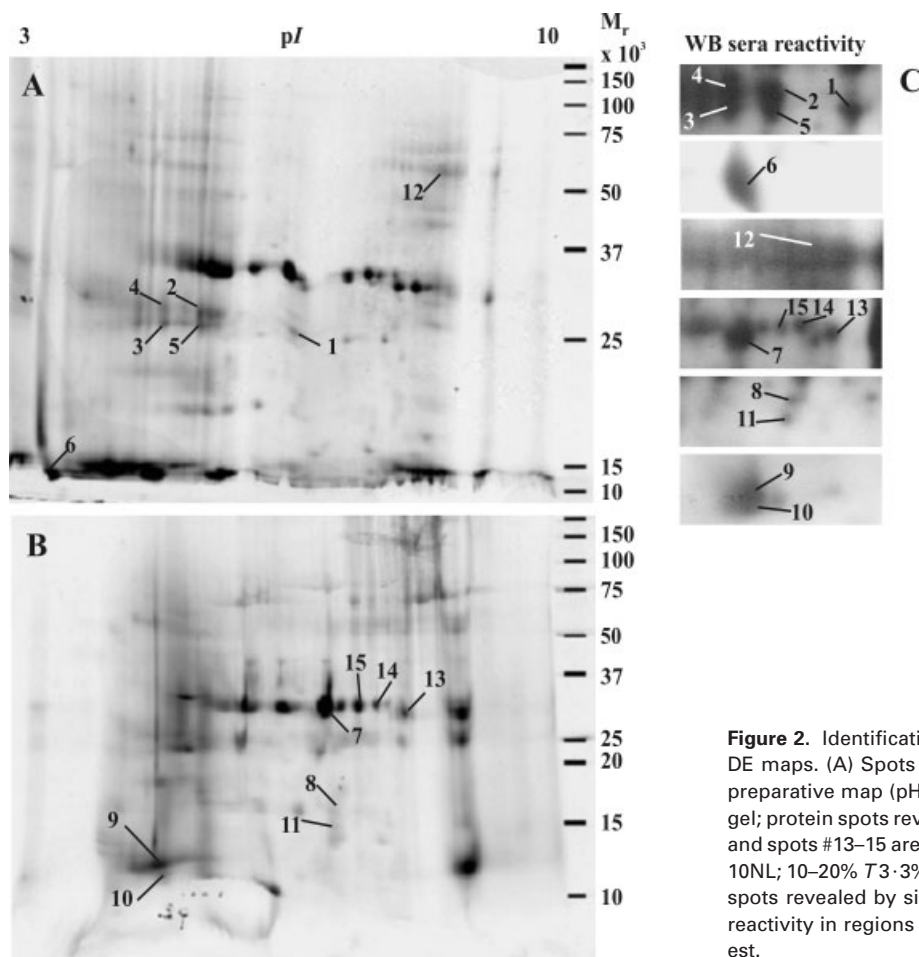


Figure 2. Identification of spots of interest in preparative 2-DE maps. (A) Spots #1–6 and spot #12 are indicated on the preparative map (pH 3–10NL; 12% T3·3% C polyacrylamide gel; protein spots revealed by silver staining), (B) Spots #7–11 and spots #13–15 are indicated on the preparative map (pH 3–10NL; 10–20% T3·3% C polyacrylamide gradient gel; protein spots revealed by silver staining), (C) Western blots of sera reactivity in regions corresponding to protein spots of interest.

from patients allergic to house dust mite and compared to the volume of the same spots identified by the nine sera characterized for Phl p 1 specificity. The immunoreactivity of sera known to have anti-Phl p 1 reactivity was more than twice that of non-specific sera (Fig. 3). This difference resulted to be statistically significant as evaluated by Student's *t*-test ($p < 0.03$).

Finally, in order to investigate possible differences between recombinant and natural allergens, we compared reactivity of serum 2 (including Phl p 5 specificity) *versus* Phl p 5 present in the native complete extract and *versus* recombinant rPhl p 5, respectively. We observed by Ponceau red staining that in the recombinant material some protein products were not recognized by serum 2 (Fig. 4, ellipse). By MS analysis these smaller and acidic protein products were found to be Phl p 5 degradation products that do not carry IgE immunogenic epitopes (data not shown). Interestingly, serum 2 recognized in recombinant Phl p 5 an extra spot missing in the natural extract (Fig. 4, arrowhead). On the contrary, the spot indicated by an empty arrow in Fig. 4 and identified as Phl p 5a (see spot #7 in Fig. 2), was missing in the rPhl p 5 protein. The spot with vertical streak at alkaline

pI recognized by serum 2 in the natural extract corresponded to the non-specific sera reactivity against the most abundant protein present in the extract (see also Fig. 1 panel Cn lower arrowhead).

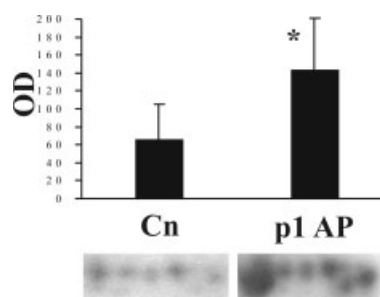


Figure 3. Evaluation of immunoreactivity against protein spots belonging to Isoallergen Group 1. Bar histograms representation of the mean of the spot volumes evaluated as OD with SD bars ($n = 3$ Cn and $n = 9$ p1AP). Data were analyzed by Student's *t*-test. * = $p < 0.03$. p1AP = group 1-specific allergic patient sera. In the lower part of the figure examples of corresponding immunoreactivities are shown.

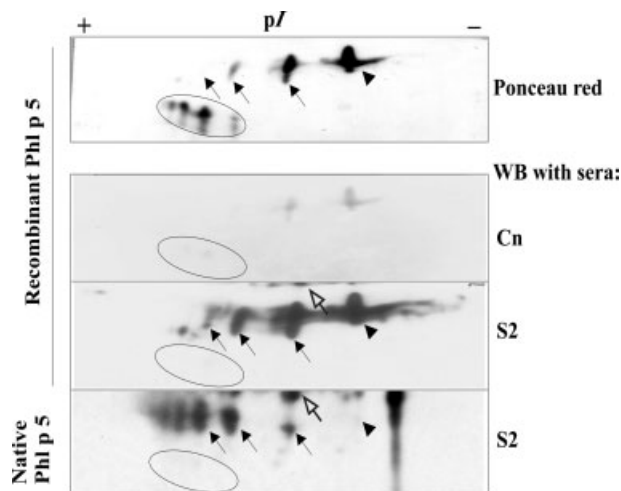


Figure 4. Comparison of serum 2 reactivity against native and recombinant Phl p 5 allergen. Native and recombinant material were resolved by 2-DE (first dimensions were performed on a pH 3–10NL range; second dimensions were performed on 12% T 3·3% C polyacrylamide gels), transferred onto nitrocellulose and assayed with different sera. Upper panel shows Ponceau red staining of rPhl p 5. Lower panels show Western blot reactivity obtained with control serum (Cn) and serum 2 (S2, characterized for Phl p 5 reactivity) on recombinant and natural material. Ellipse indicates Phl p 5 degradation products not recognized by sera; arrows indicate reactivity shared among recombinant and native Phl p 5; empty arrows indicate Phl p 5a, not present in recombinant material; arrowheads indicate serum 2 reactive material present in recombinant but not in natural Phl p 5; The spot with a vertical streak at alkaline pI in the native Phl p 5 sample is non-specific sera reactivity.

4 Discussion

The use of recombinant allergens allows the fine dissection of the sensitization pattern (*i.e.*, the IgE reactivity) of patients allergic to antigens contained in the natural extracts which are commonly used in clinical practice. This has brought a relevant improvement to the comprehension of the pathogenesis of allergies, and will foster the use of patient-tailored immunotherapy. However, several aspects have to be considered when comparing the information that can be retrieved from the analysis of IgE reactivity towards recombinant molecules, *versus* that derived with natural extracts.

In particular, recombinant allergens can display features depending on the expression system used [18]. Moreover, in recombinant preparations allergens isoforms are missing [19, 20, 21], which may have biological and immunological relevance [19, 20].

Here, we used an approach which integrated the characterization with recombinant allergens of the IgE reactivity profile of grass allergic patients together with the 2-DE capability in resolving allergen isoforms from native extracts [19, 20, 21, 22]. A similar approach was previously used for the

identification of sesame seed and hazelnut food allergens [23, 24], and in the characterization of *Phleum pratense* allergens belonging to group 5 and 13 [21, 25].

We were able to identify six (p 1, p 2, p 4, p 5, p 6, p 7) out of eight pollen antigens on the basis of sera with known IgE serological reactivity. Furthermore, MS analysis showed that several immunoreactive protein spots of the native extract contained allergens belonging to the same group but from different grass (*i.e.* spot #3 contains Dac g 5.1, Phl p 5b and Lol p VA); while in one case we found in a single spot an allergen belonging to a different group of a different plant. At the moment we are not able to define whether the epitope recognized by IgE was present in a specific component of the protein spot or shared by different proteins in the spot.

Strikingly, 2-DE analysis revealed several isoforms of allergens belonging to group 1 and group 5 in the native extract. The issue of isoform specific IgE reactivity is presently under investigation.

Our results show differences in biochemical and immunological features of recombinant *versus* natural Phl p 5. We failed to identify the other two allergens (p 11 and p 12) for which reactivity was expected on the basis of the specific IgE assay. It has been reported that p 11 allergen might carry structural epitopes based on disulfide bonds and tertiary structure [26, 27]. According to this observation, the failure in anti-p 11 detection may be referred to the fact that 2-DE was performed under reducing and denaturing conditions, where structural epitopes are lost.

We show here that in principle 2-DE serological screening could be a good tool for detecting simultaneously in a single assay IgE immunoreactivity to all allergens and allergen isoforms included in raw extracts of common clinical use. However, the sensitivity of this approach is not sufficient to identify allergens with weak immunoreactivity at ELISA test. This might be due to different mechanisms. For instance, the low IgE-prevalence may imply that less relevant and/or rare allergens might have not been identified by the quite limited panel of sera we used. Moreover, the nature of single allergens may imply they are not detected by the assay we used (*e.g.*, structural epitope cannot be identified by Western blotting).

In our opinion, our approach could help to develop standardized techniques allowing the comparison of allergenic components of different commercial extracts for diagnosis and immunotherapy. Nowadays these preparations are routinely compared by means of non-harmonized internal standard or, at most, in terms of one single major allergen content (*e.g.*, p 5 allergen).

As previously reported the 2-DE serological screening approach allows the identification of new allergens [23, 25]. Here we described a putative new allergen that was recognized by 3 of the tested sera, but we were unable to identify the corresponding protein. This might depend on either the low amount of protein recovered from gel or on the limited number of grass proteins present in the database.

5 Concluding remarks

In conclusion, we show in this pilot study that information derived from the diagnosis of allergies with recombinant allergens can be integrated with immunoreactivity analysis of 2-DE gels of native allergen extracts. The comparison of the IgE reactivity profile obtained with these different diagnostic reagents allows the exploitation of the advantages of each methodology and a comprehensive picture of the advantages and of the limits of each one of them to be obtained. Further studies are needed to explore the potential applications of this innovative approach.

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