



Letter

Phleum pratense manganese superoxide dismutase identified by proteomic: a new candidate grass allergen

The identification of allergen-specific sensitization is a clue to establish the correct diagnosis and treatment of allergic diseases.

Currently available forms of diagnosis still rely prevalently on allergen extracts prepared from various allergen-containing biological sources (pollen, fruits, animal hair, etc). These extracts contain different allergenic and nonallergenic components and are often awkward to standardize. Diagnosis based on single allergens obtained by recombinant cloning technology or as purified components has progressively increased in recent years. There are advantages and limits to the 2 approaches, whose discussion is beyond the purpose of the present work. However, a possible limit of an allergen-component-based diagnosis is related to the actual availability of each component, which is relevant to identify the entire population of patients sensitized to a given allergen source. This phenomenon is difficult to quantify, because for most common allergen sources, at least 1 allergen component has been shown to perform as a highly efficient marker of sensitization for the entire allergen source.^{1,2} Nevertheless, there might be uncommon patients who have a diagnosis based on a positive reaction to an allergen extract and a coherent clinical history but a negative score when tested with the set of allergen components available to the clinical laboratory. These patients are experiments of nature demonstrating the clinical relevance of a sensitizing component missing from the diagnostic toolbox based on a component-resolved diagnosis and showing shortcuts to the discovery of new allergens.

The authors previously used a proteomic approach to detect grass allergens from a natural protein extract. They identified 6 of 8 expected clinically relevant allergens in the natural grass extract, including different molecular isoforms of single allergens.³

In the present work, the authors investigated the IgE reactivity of 1 patient (number 101) with seasonal allergic rhinoconjunctivitis and a positive skin prick test reaction to grass extract who had a negative score for IgE to the 8 allergen components of *Phleum pratense* (Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 7, Phl p 11, and Phl p 12) included in the most recent version of a commercial allergen test (ImmunoCAP ISAC, Thermo Fisher Diagnostics, Waltham, Massachusetts). A skin test for house dust mite extract also showed a positive reaction in this patient, whereas other allergens relevant in the area where she lived had a negative score (eTable 1).

In addition, the authors studied the serum from another adult patient (number 202) with allergy who had been sensitized to grass according to the skin prick test reaction and had coherent seasonal symptoms. The diagnoses and results from the total in vitro specific IgE determination from these 2 patients are listed in eTable 1.

To identify the putative allergen recognized by the IgE in patient 101, the authors used a previously described approach.³ Briefly, the western blot reaction of sera from patients 101 and

201 to the proteome extracted from 5 different grass pollens was resolved by 2-dimensional gel electrophoresis (details are presented in eMethods). Pollen extract from 5 grasses (*P pratense*, *Lolium italicum*, *Dactylis glomerata*, *Festuca elatior*, and *Poa pratensis*; Lofarma Allergeni, Milan, Italy) was used to increase the possibility of identifying rare reactivity allergens. As depicted in Figure 1, a single-spot reactivity against a protein with an isoelectric point of 5.82 and a molecular weight of approximately 23 kDa resulted from the incubation of serum from patient 101, whereas strong reactivity with several other protein spots resulted from the incubation with the positive reference serum from patient 201, as expected. Notably, the reactivity of the serum from patient 201 included the same 23-kDa spot, although it yielded a weak signal. Based on the reactivity and identified proteins defined in the authors' previous work,³ some of the reactivity observed for patient 201 could be related to different isoforms of Phl p 1 and Phl p 5 allergens, which showed high scores in the ImmunoCAP ISAC assay (eTable 1).

The protein spot corresponding to the serologic reactivity of patient 101 was excised from a preparative gel and analyzed by liquid chromatographic electron spray ionization tandem mass spectrometry for protein identification (eFig 1 and eMethods). Mass spectrometric analysis yielded a unique unequivocal identification of superoxide dismutase (SOD) from *P pratense* (K6Z7E6_PHLPR entry in UniProtKB/TrEMBL database) with a significantly high score (eTable 2).

The recent de novo sequencing of the entire timothy grass pollen transcriptome allowed the identification of 93 previously undescribed proteins from *P pratense*, which were analyzed for T-cell reactivity.⁴ These included “antigen 90” (ORF JAA00190, gene bank GAAJ01000274.1), predicted to code for a previously unidentified timothy grass SOD, which was recognized by T cells from patients allergic to grass.

In the present work, the authors used sequencing to identify 4 unique peptides matching the *P pratense* SOD sequence from the eluted spot. Also, the spot electrophoretic mobility feature (ie, relative mass 22,999 Da) was compatible with that predicted for the SOD sequence (ie, relative mass 22,979 Da).

Plant SOD catalyzes the dismutation of superoxide to molecular oxygen and water, thus working as an antioxidant system against oxidative stress. SOD or SOD homologs have been identified as allergens in different organisms, such as *Hevea brasiliensis*,^{5,6} *Aspergillus fumigatus*,⁷ and *Olea europea*.⁸

The present results indicate that *P pratense* SOD was targeted by the IgE from 1 patient with respiratory symptoms during grass pollen season. This observation adds to the finding by Schulten et al⁴ the notion that this putative allergen is clinically relevant and can stimulate cellular and humoral immunities.

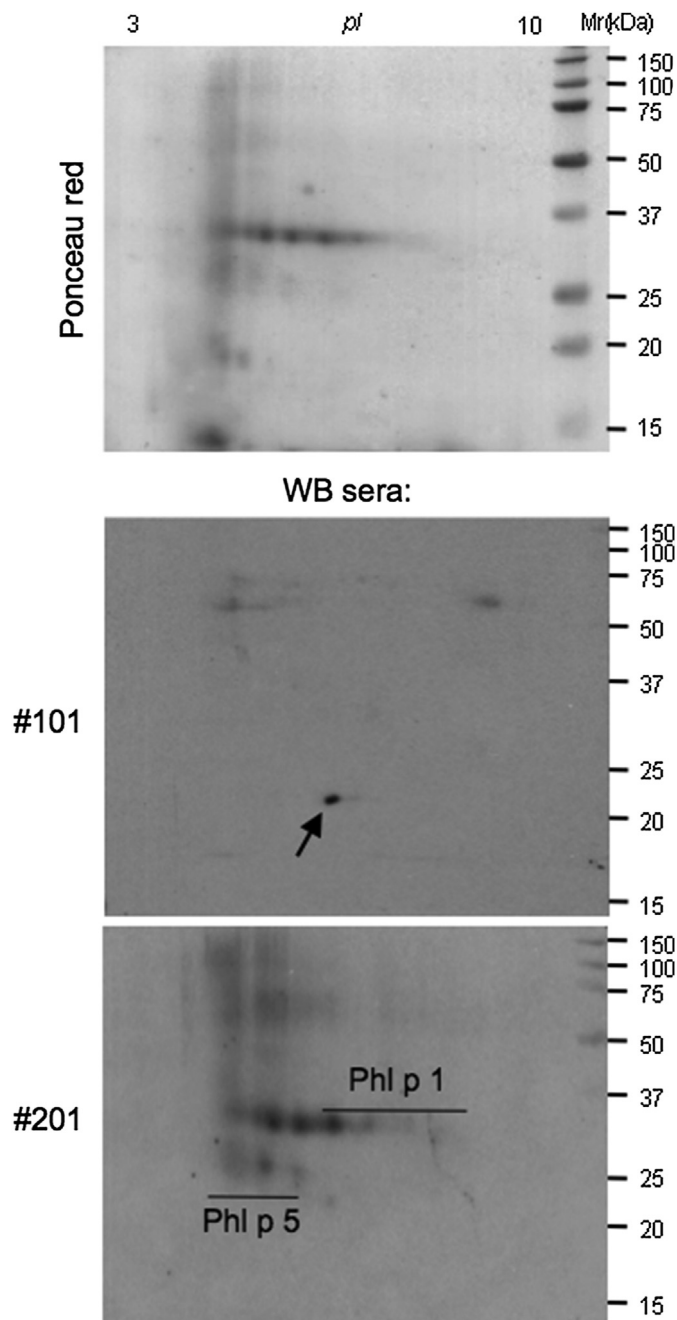


Figure 1. Reactivity of patients' sera to the proteome of grass pollen mix 5 extract resolved by preparative 2-dimensional electrophoresis. (Top) The total protein transferred to nitrocellulose is shown by Ponceau red staining. Western blot (WB) IgE reactivity of sera from patients 101 (middle) and 201 (bottom) is shown. Arrow indicates the protein spot recognized by the serum from patient 101. (Bottom) Reactivity of serum from patient 201 against Phl p 1 and Phl p 5 allergens. Mr, relative mass; pI, isoelectric point.

Notably, patient 101 had a negative score not only for the component-resolved diagnosis but also when the in vitro specific IgE determination was based on grass allergen extracts. The

positive skin prick test reaction to the *P pratense* extract might have arisen from the hypothetical grass SOD allergen being absent or denatured in the ImmunoCAP device when reacted with the entire extract but being maintained in the grass extract of the skin prick test solution.

The cloning and characterization of this new allergen are in progress. Only after this procedure will the actual relevance of *P pratense* SOD as a new grass allergen in the population sensitized to grass pollen be determined. In single patients in whom the component-resolved diagnosis does not support the clinical evidence, a proteomic approach is a powerful complementary diagnostic tool to identify new allergens.

Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jana.2013.12.015>.

Antonio Conti, PhD*

Massimo Alessio, PhD*

Mariasabina Pesca, PhD*

Armando Soldarini, PhD†

Daniela Breda, BA‡

Paolo D. Pigatto, MD§

Samuele E. Burastero, MD‡

*Proteome Biochemistry

‡Cellular and Molecular Allergy

San Raffaele Scientific Institute

Milan, Italy

†Diagnostica e Ricerca San Raffaele SpA

Milan, Italy

§Department of Biosciences for Health

Dermatological Clinic

IRCCS Galeazzi Hospital

University of Milan

Milan, Italy

burastero.samuele@hsr.it

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eMethods

Two-Dimensional Electrophoresis

A grass pollen protein extract was obtained from Lofarma Allergeni. This was an “in-house” reference preparation of a mixture of pollens from 5 different plants (*Phleum pratense*, *Lolium italicum*, *Dactylis glomerata*, *Festuca elatior*, and *Poa pratensis*) and was used as starting material for skin prick test reagents and for preparations. Proteins were extracted by acetone precipitation and resuspended in a buffer containing 8 mol/L of urea, 4% (w/v) 3-((3-cholamidopropyl)-dimethylammonium)-1-propanesulfonate, 65 mmol/L of dithiothreitol, 0.2% (v/v) immobilized Ph gradient buffer (pH 3–10 NL; GE-Healthcare, Milan, Italy), and 0.05% (w/v) bromophenol blue.

Two-dimensional electrophoresis (2DE) was performed as previously described.¹ Briefly, samples corresponding to 210 mg of total protein (measured by Protein Quantitation Assay, Bio-Rad Laboratories, Hercules, California) were applied to 7-cm IPG strips (pH 3–10 NL; GE-Healthcare) by in-gel rehydration at 18°C. Focusing was performed with an IPGphor system (GE-Healthcare) at 50 mA maximum per IPG strip with a gradient voltage (maximum 5,000 V), for a total of 25 kVh. Strips were equilibrated for 15 minutes in 50 mmol/L of Tris-HCl buffer (pH 8.8) containing 6 mol/L of urea, 30% (v/v) glycerol, 2% (v/v) sodium dodecyl sulfate, and 2% (w/v) dithiothreitol and then for 15 minutes in the same buffer after replacing dithiothreitol with 2.5% (w/v) iodoacetamide. Then, the strips were transferred onto a 10% to 20% gradient or 12% sodium dodecyl sulfate polyacrylamide electrophoretic gels for the second-dimension separation. Preparative 2DE gels for protein spot excision (see below) were generated by loading 150 mg of total protein.

Western Blot Analysis

Proteins resolved by 2DE were electronically transferred onto nitrocellulose membranes and incubated overnight with patient sera (1:4 working dilution). Immunoreactivity was shown by incubation with goat antihuman IgE conjugated with horseradish peroxidase (Sigma-Aldrich, St. Louis, Missouri), followed by electrochemiluminescence (GE-Healthcare) reaction and film exposure. Images were acquired using a Molecular Dynamics Personal SI Laser Densitometer (GE-Healthcare) and 2DE protein patterns were analyzed using Progenesis P240 software (NonLinear Dynamics, Newcastle, United Kingdom). Relative mass was estimated by comparison with relative mass reference markers (Precision,

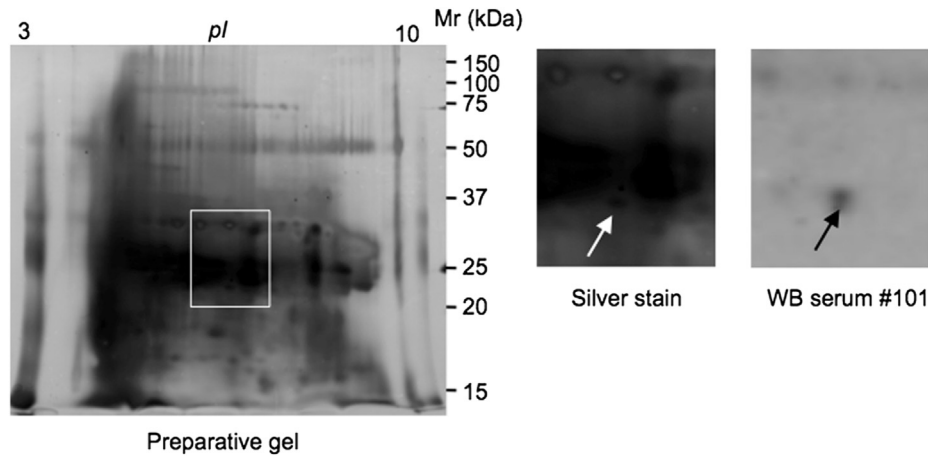
Bio-Rad) and isoelectric point values were assigned to the detected spots according to GE-Healthcare guidelines.

Protein Identification by Liquid Chromatographic Electron Spray Ionization Tandem Mass Spectrometric Analysis

Gels were stained with mass spectrometric compatible silver staining and images were acquired at high resolution using the Molecular Dynamics Personal SI Laser Densitometer (GE-Healthcare) and 2DE protein patterns were analyzed using Progenesis P240 software (NonLinear Dynamics). The spot of interest was excised from gels, reduced, alkylated, and in-gel digested overnight with bovine trypsin (Roche Diagnostics Corp, Basel, Switzerland) as previously described.² Five microliters of digested sample, acidified up to 1% formic acid, was injected in a capillary chromatographic system (Agilent 1100 Series, Santa Clara, California) equipped with a Nano Pump (Agilent). Peptide separation occurred in a homemade RP C18 nano column. The eluting peptides were ionized by a nanoESI online source and analyzed on an API QStar PULSAR (AB-Sciex, Toronto, Ontario, Canada) mass spectrometer. Full-scan mass spectra ranging from *m/z* 350 to 1,600 were collected, and for each mass spectrometric spectrum, the 2 most intense doubly and triply charged ion peaks were selected for fragmentation. Tandem mass spectrometric data files from each chromatographic run were combined and converted to MASCOT generic files using MASCOT.dll 2.2.07 through Analyst QS 1.1 (AB Sciex). All tandem mass spectrometric samples were analyzed using the MASCOT engine to search the UniProt_viri_cp_viri_2013_07 database. Peptide mass tolerances of 200 ppm and 0.3 Da were used for precursor and fragment ions, respectively. The other search parameters were 2 allowed missing cleavages, oxidation of methionine as a variable modification, and carbamid methylation as a fixed modification.

eReferences

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eFigure 1. (Left) The proteome profile of grass pollen mix 5 extract resolved by preparative 2-dimensional electrophoresis after silver staining is displayed. Rectangle indicates region containing the protein spot that was reactive with serum from patient 101. (Right) Enlarged regions depict the spot excised from the preparative gel for mass spectrometric analysis (silver stain; white arrow) and the corresponding sera reactivity (western blot [WB] of serum from patient 101; black arrow). Mr, relative mass; pI, isoelectric point.

eTable 1
Pattern of grass sensitization of patients^a

Patient 101	40-y-old white woman
Diagnosis ^b	intermittent moderate to severe rhinitis in spring for >5 y; never received immunotherapy
Prick test result ^c	<i>Phleum pratense</i> , +++; <i>Dermatophagoides</i> mix, +++ (results for other allergens skin prick tests were negative)
Total IgE (IU/L) ^d	76.3
Grass-specific IgE measured with extract-based assay (kU/L) ^e	<i>Phleum pratense</i> <0.10, <i>Dactylis glomerata</i> <0.10
Grass-specific IgE in CRD-based assay (ISU) ^f	Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 7, Phl p 11, Phl p 12: all <0.30 ISU
House dust mite–specific IgE in CRD-based assay (ISU) ^f	Der p 1 2.98, Der f 1 3.31, Der p 2 4.21, Der f 2 7.53 (other ISAC-tested allergen components <0.30)
Patient 102	35-y-old white woman
Diagnosis ^b	intermittent moderate to severe rhinitis; intermittent controlled asthma in spring for >5 y; never received immunotherapy
Prick test result ^c	<i>Phleum pratense</i> +++
Total IgE (IU/L) ^d	126.3
Grass-specific IgE measured with extract-based assay (kU/L) ^e	<i>Phleum pratense</i> 7.09, <i>Dactylis glomerata</i> 10.02
Grass-specific IgE in CRD-based assay (ISU) ^f	Phl p 1 12.56, Phl p 2 <0.1, Phl p 4 1.80, Phl p 5 4.95, Phl p 6 2.21, Phl p 7 24.74, Phl p 11 2.33, Phl p 12 1.28

Abbreviations: CRD, complement resolved diagnosis; ISU, ISAC standardized units.

^aSensitization to inhalant allergens was established in these patients with the following panel: *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* (50% mix), *Phleum pratense*, *Betula verrucosa*, *Alternaria alternata*, *Parietaria officinalis*, *Felis domesticus*, *Canis familiaris*, *Ambrosia artemisiifolia*, and *Artemisia absinthium*.

^bAn expert allergist (P.D.P.) diagnosed and staged the rhinitis according to guidelines from the Allergic Rhinitis and its Impact on Asthma.³

^cSensitization to inhalant allergens was established with a panel of commercial extracts for skin prick testing (ALK Abellø, Linate, Italy) appropriate to the area where the patients were living. Results were expressed according to the European Academy of Allergy and Clinical Immunology⁴; positive and negative controls were included.

^dTotal IgE was measured with ImmunoCAP total IgE (Thermo Fisher Diagnostics, Waltham, Massachusetts).

^eSpecific IgE was measured with extract-based assays in ImmunoCAP (Thermo Fisher Diagnostics).

^fThe component-resolved diagnosis-based assay used in this case was the ImmunoCAP ISAC (version with 112 allergen components; Thermo Fisher Diagnostics). Patient 101 was tested twice, with virtually identical results.

eTable 2

Protein identified by liquid chromatographic electron spray ionization tandem mass spectrometric analysis

Accession number ^a	Protein name	MW (Da)		pI		MASCOT score ^b	Matched peptides	Peptide
		Observed value	Theoretical value	Observed value	Theoretical value			
K6Z7E6	superoxide dismutase, <i>Phleum pratense</i>	22999	22979	5.82	8.93	155	4	KALEQLDAAVSKG KGDASAVVQLQGAIKF KLSVETTANQDPLVTKG KKLSVETTANQDPLVTKG

Abbreviations: MW, molecular weight; pI, isoelectric point.

^aUniProt_viri_cp_viri_2013_07 database.^bProbability-based score used by MASCOT program.