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(54) **Title:** NOVEL ALLERGEN

(57) **Abstract:** The present invention relates to a novel allergen from timothy grass (Phleum pratense) pollen, Phl p11 as disclosed in SEQ ID NO 1, and use thereof as a reagent and in a diagnostic kit as well as for immunotherapy.

NOVEL ALLERGEN

Field of the invention

The present invention relates to a novel allergen from timothy grass (*Phleum pratense*) pollen, Phl p11, and use thereof as a reagent and in a diagnostic kit as well as for immunotherapy.

Background of the invention

A hallmark of atopic allergy is the formation of IgE antibodies to proteins present in the sensitizing biological material. Upon contact with the allergen source, these proteins will act to crosslink IgE antibodies present on the surface of mast cells, thereby eliciting the release of inflammation mediators such as histamine. As a result, an allergic reaction occurs (1).

In the industrialized world, up to 10% of the human population shows allergic sensitization to grass pollen, making this one of the most important airborne allergen sources (2). Considerable efforts have been made towards the characterization of pollen allergens from a variety of grass species using biochemical and immunological methods. A number of IgE binding proteins have thus been identified which exhibit conserved structure and serological cross-reactivity between species. Based on these criteria, such immunologically related grass pollen allergens have been assigned to groups designated by numbers. These include group 1, group 2/3, group 4, and group 5 allergens, which are represented in pollen of most grass species (3).

To date, six different allergens from timothy grass (*Phleum pratense*) pollen have been cloned: Phl p 1 (4, 5), Phl p 2 (6), Phl p 5 (7-9), Phl p 6 (10, 11), Phl p 7 (12), Phl p 12 (profilin) (13), and Phl p 13 (14). These allergens have all been produced as recombinant proteins which, by different *in vitro* and *in vivo* activity assays, have been shown to share immunological and allergenic properties with their native counterparts.

Using a panel of four recombinant allergens (rPhl p 1, rPhl p 2, rPhl p 5, and profilin) in serological and skin testing procedures, positive results were obtained in 95% of a large population of grass pollen-allergic individuals (15). Sensitization to allergens such as Phl p 7

(a calcium-binding, two-EF-hand protein) and Phl p 12 occurs in a smaller proportion of grass pollen allergics, but they share IgE epitopes with homologous proteins present in pollen of trees and weeds and can therefore cause immediate-type symptoms in sensitized individuals upon contact with these unrelated allergen sources (12, 16, 17).

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Summary of the invention

The present invention relates to a novel allergen from timothy grass (*Phleum pratense*) pollen, Phl p11. While the absolute majority of grass pollen allergics produce IgE antibodies binding to group 1 and group 5 grass pollen allergens - Phl p 1 and Phl p 5 in the case of timothy grass

10 pollen, a subset of the patients also make IgE antibodies to a variety of other protein components such as profilin, Phl p 7 or Phl p 11. These patients are thought to have a more expansive immunological activity in their allergic disease, putting them at greater risk of developing clinical reactivity to an increasing number of allergen sources (such as weed and tree pollens, cat and dog dander, mites, etc).

15

Profilin and Phl p 7 are highly cross-reactive (vegetables, fruits and pollens of weeds and trees) and not specifically indicative of sensitisation to the grass pollen proteins. Antibody binding structures present on Phl p 11, on the other hand, appear to be specific to grass pollen and may therefore be regarded as a marker of multivalent sensitisation to this particular

20 allergen source, i.e. a marker of enhanced overall propensity to produce IgE antibodies to environmental substances. Recombinant Phl p 11 may be used to identify the subset of patients which are sensitized to polypeptide structures of Phl p 11 and its homologues in pollen of other grass species. About half of all patients that show IgE reactivity to this pollen protein reportedly have their antibodies directed to glycan structures present on the group 11

25 allergen. These glycan structures are cross-reactive in nature and antibodies binding to them may not be informative/diagnostic in relation to grass pollen-specific sensitisation. IgE reactivity to rPhl p 11 may thus be informative with respect to state of immunological diversification of patients' allergic sensitisation to grass pollen, independent of other allergen sources.

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As a defined immunotherapy reagent, recombinant Phl p 11 may be used to treat specifically sensitised patients by way of eliciting a protective or attentuating immune response. The active substance may be a protein comprised of either the natural ("wild-type") polypeptide sequence or a derivative with improved safety or efficacy properties.

Thus, in a first aspect the invention relates to a reagent which may be recombinantly produced or chemically synthesized, Phl p11, comprising the amino aid sequence according to SEQ ID NO 1 of the enclosed sequence listing as well as essentially homologous (such as 75%) and cross-reactive variants and derivatives thereof. These variants have equivalent or similar function with respect to antibody binding. The invention also relates to DNA sequences encoding said amino acid sequence.

In a second aspect, the invention relates to a diagnostic kit comprising the above reagent. The diagnostic kit may also comprise one or more other known Phl allergens, such as Phl p 1, Phl p 2, Phl p 4, Phl p 5a, Phl p 5b, Phl p 6, Phl p 7, Phl p 12 and Phl p 13.

In a third aspect, the invention relates to an immunoassay comprising the following steps:

a) obtaining a patient blood sample from a patient with suspected grass pollen allergy
b) allowing serum or plasma derived from the blood sample to contact the allergen reagent, immobilized on a solid phase or in solution.
c) detecting antibodies bound to the allergen reagent using a specific detection reagent such as an enzyme-conjugated anti-IgE antibody.

The immunoassay may comprise the reagent Phl p11, either natural or recombinantly produced or chemically synthesized. The immunoassay may be in any desired format, such as ELISA.

In a fourth aspect, the invention relates to use of the above reagent or a derivative thereof for production of a drug for immunotherapy ("allergy vaccination") of grass pollen allergic patients showing IgE antibody reactivity to Phl p 11. Preferably, the use is for immunotherapy ("allergy vaccination") of timothy grass pollen allergic patients showing IgE antibody
5 reactivity to Phl p 11.

Detailed description of the invention

FIGURE LEGENDS

10 **FIGURE 1.** Identification of Phl p 11 using SDS-PAGE and immunoblot analysis of *P. pratense* pollen extract. A: Pollen extract was reduced and separated by SDS-PAGE, followed by staining with Coomassie Brilliant Blue. The faint protein band identified as Phl p 11 is marked with an arrow. B: Immunoblot analysis of a duplicate gel, where the binding of one patient's serum IgE antibodies is visualized. Lane 1: molecular weight markers, lane 2: *P.*
15 *pratense* pollen extract.

FIGURE 2. Nucleotide and deduced amino acid sequence of Phl p 11 cDNA. An open reading frame identical to all clones conformed closely with the codon preference derived from all published *P. pratense* genes expressed in pollen. The underlined nucleotide
20 sequences represent primers GSP-1 and GSP-2. Open arrowheads indicate nucleotide differences between the clones that were analyzed; the standard nucleotide ambiguity codes are used at those positions. Homopolymer stretch length variation between the clones is indicated by shading. The sequence shown represents the longest of five analyzed clones, while black arrowheads mark where the other cDNAs ended in a poly-A stretch. The amino
25 acid sequence marked by solid underlining represents the 20 residues which were determined by N-terminal microsequencing of the natural pollen protein. A single site for potential N-linked glycosylation is indicated by dotted underlining.

FIGURE 3. Multiple amino acid sequence alignment of Phl p 11 and structurally related
30 proteins. Each sequence retrieved from the database is preceded by its accession number: A54002 (*Lolium perenne*), 1815759 (*Phalaris coerulescens*), S31710 (*Oryza sativa*), P33050

(*Zea mays*), 2765366 (*Betula pendula*), P13447 (*Lycopersicon esculentum*), 2832664 and 398899 (*Arabidopsis thaliana*), S43242 and S43244 (*Syringa vulgaris*), 3256212 (*Ligustrum vulgare*), and 926885 (*Olea europaea*). All entries are shown in full, except the *A. thaliana* sequence 2832664 which was truncated to show only the domain aligning with the protein family examined here. Positions marked x indicate unidentified or atypical residues. The third through eighth sequence include a putative N-terminal leader peptide. Hyphens indicate gaps introduced to maximize the number of aligning residues.

FIGURE 4. Analysis of recombinant Phl p 11 expression in *E. coli.* An *E. coli* strain prepared for expression of a MBP-Phl p 11 fusion protein was grown to mid log phase and then subjected to a temperature shift in order to de-repress the expression system. Samples were prepared by boiling pelleted cells in loading buffer containing SDS and β-mercaptoethanol. Lane 1: molecular weight markers, lane 2: preinduction sample, lane 3: postinduction harvest, lane 4: sample of purified protein. Proteins were visualized by Coomassie Brilliant Blue staining.

FIGURE 5. Immunoblot inhibition of IgE binding to immobilized *P. pratense* extract proteins by soluble rPhl p 11. Pollen extract was reduced and separated by SDS-PAGE, and electroblotted onto nitrocellulose membrane. The membrane was incubated with serum samples from two Phl p 11-sensitized grass pollen-allergic subjects (A and B), after preincubation with either BSA (lane 1), rPhl p 11 (lane 2) or MBP (lane 3).

MATERIALS AND METHODS

General reagents, plasmids, oligonucleotides, bacterial strains and antibodies

Salts and buffers were purchased from Sigma (St. Louis, MO) and Fluka (Buchs, Switzerland). Pollen from timothy grass (*Phleum pratense*) was obtained from Pharmacia Allergon AB (Välinge, Sweden). Protein analysis by SDS-PAGE was performed using 4-20% Tris-glycine gels (Novex, San Diego, CA) and for electroblotting Hybond-C Extra membrane (Amersham Life Science, Amersham, UK) was used. For immunoblot analysis of IgE binding, rabbit anti-IgE antiserum (MIAB, Uppsala, Sweden) and horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Life Science) were used, followed by ECL detection

(Amersham Life Science). Preparation of polyadenylated RNA from total RNA and subsequent synthesis of cDNA for RT-PCR were performed using the mRNA Purification Kit and the First-strand cDNA Synthesis Kit, both from Amersham Pharmacia Biotech (Uppsala, Sweden). Plasmids pET-23a(+) and pMAL-c2 were purchased from Novagen (Madison, WI)
5 and New England Biolabs (Beverly, MA), respectively. Restriction endonucleases *Eco*RI, *Hin*dIII, *Nde*I and *Xho*I, as well as *Taq* DNA polmerase and deoxynucleotides were from Amersham Pharmacia Biotech. *Pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA). DNA from PCR and other enzyme reactions was purified using appropriate Wizard kits from Promega (Madison, WI). For solid phase capture of biotinylated PCR
10 products, streptavidin-modified magnetic beads (M-280) from Dynal AS (Skøyen, Norway) were used. For large-scale plasmid preparation, the Plasmid Maxi Kit from Qiagen (Düsseldorf, Germany) was used. Oligonucleotides were obtained from Scandinavian Gene Synthesis (Köping, Sweden). DNA sequencing was performed using the T7 Sequencing Kit from Amersham Pharmacia Biotech and [α-^{35}S]dATP from Amersham Life Science. The *E.*
15 *coli* strains used were XL1-Blue MR (Stratagene) for cloning purposes and BL21 (Novagen) harboring plasmid pT7POL23 (18) for expression. HiTrap Chelating columns (Amersham Pharmacia Biotech) were used for immobilized metal ion affinity chromatography (IMAC). Buffer exchange and size exclusion chromatography of protein preparations were performed using an FPLC system and columns packed with Sephadex G-25 and Superdex 75,
20 respectively (Amersham Pharmacia Biotech). Quantitative serology for the recombinant allergen was established using Pharmacia CAP System (Pharmacia Diagnostics), employing reagents and procedures as recommended by the supplier. For IgE detection in immunoblot inhibition experiments, an ^{125}I-labeled anti-human IgE antibody from Pharmacia Diagnostics was used. Histamine release from isolated granulocytes of allergic and healthy individuals was
25 measured by a radioimmunoassay (Immunotech, Marseille, France). As a positive control for histamine release capacity of cells, the monoclonal anti-IgE antibody E124.2.8 Dε2 (Immunotech) was used. Histamine and sodium chloride solution for skin prick tests were obtained from ALK (Hørsholm, Denmark).

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Patient samples

A total of 188 grass pollen-allergic subjects or serum samples were examined in this study. One hundred and fifty serum samples were from an in-house collection at Pharmacia Diagnostics, selected on the basis of IgE sensitization to *P. pratense*. Thirty-eight subjects were from a Vienna clinic and were characterized by case history indicative of grass pollen allergy, positive RAST result for timothy grass pollen, and positive skin prick test to grass pollen extract. The allergen sensitization profiles of these subjects were established with natural and recombinant timothy grass pollen allergens as described (19). Serum samples from two non-allergic individuals were included for control purposes.

Protein extracts, SDS-PAGE and immunoblot analysis

Phleum pratense pollen was extracted at room temperature for 2 hrs in 5 mL of distilled water per gram of pollen. After centrifugation for 5 min at 13 000 x g, the clear supernatant was divided into small aliquots and stored at -20°C until use. The pollen extract was subjected to reducing SDS-PAGE and either stained with Coomassie Brilliant Blue or electroblotted onto nitrocellulose membrane. Protein blots were blocked for 1 hr at room temperature using either 1% (v/v) Tween-20 in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) or 5% (w/v) defatted dry milk in PBS and then incubated overnight with patient serum diluted five-fold in PBS containing 0.1% Tween-20. After washing in the same buffer, bound IgE was visualized using a rabbit anti-IgE antiserum followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG and ECL detection.

Protein sequencing

An IgE-binding protein band corresponding to Phl p 11 was identified by immunoblotting using an essentially monoreactive serum sample. The band was excised from a Coomassie Brilliant Blue-stained SDS-polyacrylamide gel, homogenized and extracted in 6 M guanidinium hydrochloride. After removal of polyacrylamide fragments by centrifugation, the extracted protein was subjected to 20 cycles of sequencing from the N-terminus using a Hewlett-Packard G1000A instrument.

Cloning and characterization of Phl p 11 cDNA

Polyadenylated RNA was isolated from total RNA of *Phleum pratense* pollen, prepared by the guanidinium isothiocyanate method of Chirgwin *et al.* (20). Phl p 11 cDNA was generated by 3'-RACE, performed essentially according to Frohman (21), and RT-PCR, using
5 cloned *Pfu* DNA polymerase throughout the experiment. All thermocycle reactions were carried out in the following reagent conditions: 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 mg/mL BSA, 0.1% Triton X-100, 10% DMSO, 0.4 mM dNTP and primers at 0.5 μM each.

First-strand cDNA was synthesized from purified poly-A^+ RNA using the primer 5'-CCA
10 GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GC$(T)_{18}$-3' (Q_T). For 3'-RACE, the two nested specific forward primers GSP-1 (5'-CAT TAC ATA TGG ACA AGG GCC CSG GCT TCG TSG TSA C -3') and GSP-2 (5'-CAT GAA TTC GGA CGC GTC TAC TGC GAC-3') were used, together with the two nested universal reverse primers Q_O (5'-CCA GTG AGC AGA GTG ACG-3') and Q_I (5'-GAG GAC TCG AGC TCA AGC-3'). Primers GSP-1
15 and GSP-2 were designed from the N-terminal amino acid sequence of the Phl p 11 protein while primers Q_O and Q_I were identical to adjacent parts of cDNA synthesis primer Q_T.

To generate a Phl p 11-enriched template for 3'-RACE, second-strand cDNA was synthesized by 40 cycles of primer extension of biotinylated GSP-1 using first-strand cDNA as template. The cycling profile used was: 95°C/5min, followed by 40 cycles of 95°C/60 sec,
20 58°C/60 sec, 72°C/90 sec. The product of this reaction was then immobilized on streptavidin-modified magnetic beads and washed with 0.1 M NaOH and TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). In the first round of 3'-RACE, a sample of immobilized second-strand cDNA and primers GSP-1 and Q_O were used in the cycling profile: 95°C/5min, followed by 40 cycles of 95°C/1min, 72°C/2 min. One μl of a 20-fold dilution of this reaction was used as template
25 together with primers GSP-2 and Q_I in the second round of 3'-RACE, with the cycling profile: 95°C/5min, followed by 30 cycles of 95°C/60 sec, 58°C/60 sec, 72°C/90 sec. The GSP-2 and Q_I primers were designed to incorporate *Eco*RI and *Hin*dIII sites, respectively, at the ends of the amplification product. After purification and cleavage with these enzymes, the product was cloned between the *Eco*RI and *Hin*dIII sites of pBR322 (22). Five candidate clones were
30 subjected to DNA sequencing, revealing a single open reading frame corresponding to Phl p 11.

Amplification of full-length Phl p 11 coding sequence from immobilized second-strand cDNA was performed using the GSP-1 primer and the reverse primer PP11/R-X (5'-AGT CAC TCG AGT GGC GTC TCG GGG GCG TC-3'), which was based on the 3' end of the Phl p 11 open reading frame. These two primers were designed to incorporate terminal *Nde*I
5 and *Xho*I sites, respectively, in the PCR product. The thermocycling profile used in this reaction was the same as that in round two of the 3'-RACE experiment. The amplification product, purified and digested with *Nde*I and *Xho*I, was cloned between the *Nde*I and *Xho*I sites of a pET-23a(+)-derivative designed for expression of the gene of interest as a fusion to the maltose binding protein (MBP) of *E. coli*. The resulting full-length construct for
10 expression was verified by DNA sequencing.

DNA and amino acid sequence analyses, including translations, protein property predictions and sequence comparisons, were performed using programs of the Wisconsin Package (Genetics Computer Group, Madison, WI.).

15 *Expression and purification of rPhl p 11*

The *P. pratense* allergen was expressed in *E. coli* as a fusion to MBP. Plasmid DNA from one selected clone was introduced into strain BL21 harboring plasmid pT7POL23 which provides T7 RNA polymerase in a stringently controlled, temperature-dependent manner (18). LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, adjusted to pH 7.0 using 1 M
20 NaOH) was inoculated 1:500 with an overnight culture and first grown at 30°C to mid-log phase. The incubation temperature was then raised to 42°C for 1 hr, followed by 4 hrs at 30°C before harvest. Cells were collected by centrifugation at 10 000 x g for 10 min at 4°C and resuspended in 5 mL of buffer A (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 100 mM β-mercaptoethanol, 5 mM imidazole) per gram (fresh weight) of cells. The resuspended cells
25 were ruptured by sonication while kept on ice, followed by centrifugation to remove solid material. Following exchange to buffer B (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5 mM β-mercaptoethanol) containing 5 mM imidazole using Sephadex G-25, the supernatant was loaded onto a Ni^{2+}-charged 5 mL HiTrap Chelating column for IMAC. The column was washed with 20 mM imidazole in buffer B and elution was performed with a 20-250 mM
30 gradient of imidazole in buffer B. Fractions containing the eluted fusion protein were pooled and subjected to a final step of size exclusion chromatography through Superdex 75,

equilibrated with non-reducing buffer, to obtain a homogeneous, unaggregated preparation without visible contamination by *E. coli* proteins. To serve as a negative control in functional studies, MBP alone was expressed from BL21[pT7POL23] cells harboring the expression vector without insert and the protein purified as above, except that buffer B containing 5 mM
5 imidazole was used in place of buffer A at the stage of cell homogenization. The concentration of MBP-Phl p 11 and MBP in the final preparations was determined from their absorbance at 280 nm, using calculated extinction coefficients of 1.30 and 1.47 per mg/mL, respectively.

10 *Assessment of IgE-binding activity of rPhl p 11 using Pharmacia CAP System*

In vitro IgE-binding activity of the purified recombinant allergen was examined in Pharmacia CAP System, an immunoassay system used for IgE antibody detection in clinical diagnosis of atopic allergy. Experimental ImmunoCAP tests were prepared by covalent immobilization of the purified allergen onto activated cellulose at a concentration chosen to
15 achieve an adequate linear measuring range and a background for negative sera adequately below the conventional cut-off value of 0.35 kU_A/L. Negative control tests carrying MBP alone were prepared using the same protein concentration at immobilization. For determination of specific IgE to the whole complement of natural *P. pratense* pollen proteins, the regular pollen extract-based ImmunoCAP test was used. For the purpose of comparison to
20 a previously established recombinant allergen, all serum assays were run in parallel with rPhl p 2 ImmunoCAP tests. Assay controls and calculation of statistical parameters attesting to the quality of the assays were performed using standard assay system routines and software (Pharmacia Diagnostics).

25 *Immunoblot analysis of IgE binding properties of rPhl p 11*

The proportion of timothy grass pollen-specific IgE directed against rPhl p 11 and rPhl p 5 was investigated by a RAST inhibition-based experiment. Serum samples from 10 rPhl p 11-reactive subjects were diluted 1:10 in buffer C (50 mM sodium phosphate pH 7.5, 0.5% (v/v) Tween 20, 0.5% (w/v) BSA, 0.05% (w/v) NaN_3) and preadsorbed overnight at 4°C
30 with either rPhl p 11, MBP (negative control) or rPhl p 5 (positive control), all at a final concentration of 10 μg/mL. To ensure conditions of antigen excess on the solid phase,

approximately 0.2 mg of natural timothy grass pollen protein extract was immobilized to nitrocellulose strips of exactly the same size (0.6 x 3 cm). Strips were blocked by preincubation with buffer C (once for 1 hour and twice for 5 minutes) and then exposed to the preadsorbed sera at 4°C overnight. The following day, strips were washed four times in buffer C and then probed with ^{125}I-labeled anti-human IgE antibody at room temperature overnight. Strips were washed again four times in buffer C and dried. The amount of ^{125}I-labeled anti-human IgE antibody was determined using a gamma counter (Wallac, Turku, Finland). The percentage inhibition of IgE binding after preincubation of sera with rPhl p 5 or rPhl p 11 was calculated as follows: %inhibition = 100-100 x (cpm rPhl p 5/cpm MBP or rPhl p 11/cpm MBP).

The capacity of the recombinant allergen to bind Phl p 11-specific IgE antibodies was studied by IgE immunoblot inhibition experiments (17). Sera from two grass pollen allergic subjects with IgE reactivity to rPhl p 11 were preadsorbed with purified rPhl p 11 at 10 μg/mL serum, or, for control purposes, with an equal concentration of MBP or BSA. Preadsorbed sera were exposed to nitrocellulose-blotted timothy grass pollen proteins separated by SDS-PAGE and bound IgE was detected as described (17).

Histamine release experiments

Granulocytes were isolated by dextran sedimentation of heparinized blood samples (23, 24) from two grass pollen allergic and one non-allergic individuals. Aliquots of washed cells were incubated with a range of concentrations (0.001 μg/mL, 0.01 μg/mL, 0.1 μg/mL, 1 μg/mL) of purified rPhl p 11, MBP, and a monoclonal anti-IgE antibody. Histamine released in the supernatant was measured by radioimmunoassay. Total histamine was determined after freeze-thawing of cells. Results were displayed as mean values of triplicate determinations and represent the percentage of total histamine.

Skin testing

After informed consent was obtained from two grass pollen-allergic and four non-allergic individuals, skin prick tests were performed on their forearms as described (25). Individuals were pricked with 20 μl aliquots of solutions containing different concentrations (0.1 μg/mL, 1 μg/mL, 10 μg/mL, 100 μg/mL) of purified rPhl p 11 and MBP, and with timothy grass

pollen extract, histamine and sodium chloride. The skin reactions were recorded 20 minutes after sample application by photography and by transferring a ballpoint pen-tracing of the wheal area to paper using adhesive tape. Mean wheal diameters (Dm) were determined as follows: Dm=0.5x(D1+D2) where D1 and D2 represent the largest longitudinal and transverse
5 diameters in mm, respectively.

RESULTS

Immunochemical detection, isolation and protein sequencing of natural Phl p 11

Immunoblot analysis of serum from a grass pollen-allergic subject, which lacked IgE
10 antibodies to all purified or recombinant allergens from *P. pratense* currently available (rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, and rPhl p 12), revealed predominant IgE-binding to a single protein band at approximately 20 kDa. One faint band in Coomassie-stained SDS-PAGE aligned perfectly with the IgE-reactive band in the immunoblot analysis (Fig. 1), although a more abundant protein of slightly smaller size could not unambiguously
15 be ruled out. Protein from both these bands was extracted separately and a portion of each applied to nitrocellulose membrane for dot-blot analysis. Incubation with the reactive serum and subsequent IgE detection allowed a positive identification of the band of slightly higher MW as the target for IgE antibodies present in the serum sample (not shown). The extracted protein was subjected to N-terminal sequencing and the following 20-residue determination
20 was obtained: DKGPGFVVTGRVYCDPCRAG. A database search for homologous sequences revealed an exact match to the rye grass allergen Lol p 11, previously purified and amino acid sequenced by van Ree *et al.* (26).

cDNA cloning and sequence analysis of Phl p 11

25 After back-translation of the N-terminal amino acid sequence into DNA, using the codon preference seen in other genes expressed in *P. pratense* pollen, two nested forward PCR primers (GSP-1 and GSP-2) were designed for use in 3'-RACE and RT-PCR. First-strand cDNA was synthesized from a poly-A^+ RNA preparation, using a universal oligo-dT primer carrying terminal target sequence for two nested reverse PCR primers, Q_O and Q_T, to be used
30 in subsequent steps of amplification. Specifically enriched second-strand cDNA, generated by 40 cycles of primer extension of GSP-1 on first-strand cDNA, was used as template in the first

round of 3'-RACE, carried out with primers GSP-1 and Q_O. In a second round, 1/1000 of the first round reaction was used as template together with primers GSP-2 and Q_I. Analysis of this reaction by agarose gel electrophoresis revealed two distinct bands of similar intensity, approximately 700 and 800 bp in size (not shown). The use of raised annealing temperature
5 did not change the appearance of the second round 3'-RACE product. The double-band product was therefore tentatively considered genuine and specific. The product was cloned and transformants harboring inserts matching both fragment sizes were identified and analyzed by DNA sequencing. All five clones examined contained inserts of nearly identical sequence and it appeared that the difference in size between the two bands seen after the
10 second round of 3'-RACE was due to alternative sites for priming of cDNA synthesis (Fig. 2), possibly as a result of heterogeneity in the site of transcript polyadenylation. All clones contained an identical open reading frame with a codon usage that agreed well with that of previously known genes expressed in *P. pratense* pollen. Beyond the observed stop codon, none of the three forward reading frames displayed codons that fulfilled this criterion. In order
15 to obtain a cDNA encoding the full-length polypeptide, an RT-PCR reaction was performed using forward primer GSP-1 and reverse primer PP11/R-X, the latter designed from the 3' end of the open reading frame. The product of this reaction, which appeared as a single band in agarose gel electrophoresis, was cloned in an expression vector and its sequence confirmed.

The open reading frame of the cDNA defined a polypeptide of 143 amino acid residues
20 with a calculated isoelectric point of 4.8, a molecular mass of 15.8 kDa and one potential site for N-linked glycosylation (Fig. 2). A similarity search through the databases available at NCBI (www.ncbi.nlm.nih.gov) identified pollen proteins from a range of mono- and dicotyledonous plant species with sequence homology to the polypeptide deduced from the cDNA sequence. These included *Lolium perenne* (rye grass), *Phalaris coerulescens* (canary
25 grass), *Oryza sativa* (rice), *Zea mays* (maize), *Betula pendula* (birch), *Arabidopsis thaliana, Lycopersicon esculentum* (tomato), *Olea europaea* (olive), *Syringa vulgaris* (lilac) and *Ligustrum vulgare* (privet). The level of amino acid sequence identity within this family of pollen proteins ranged from 32% to 95% and an alignment, displaying secondary structure predictions and conserved features, is shown in Fig. 3. From the sequence comparisons it is
30 clear that the *P. pratense* allergen is a counterpart of the *L. perenne* allergen Lol p 11 and should therefore be designated Phl p 11.

The most prominent difference in primary structure observed between Phl p 11 and Lol p 11 (sequence accession No. A54002) was a stretch of nine additional amino acid residues (-DLRDAPETP) at the C-terminus of Phl p 11, equivalent to a 1.0 kDa increment in molecular mass. In comparison to the *L. perenne* homologue, the Phl p 11 sequence contained a total of six amino acid substitutions, four of which were non-conservative (D42N, K56G, D57L, K83T). At position 103, which was not determined in the case of Lol p 11, an asparagine residue was present in the Phl p 11 sequence. The two homologues showed conservation of one potential site for N-linked glycosylation (residue 24) and six cysteine residues.

As previously shown by van Ree *et al.* (26), group 11 grass pollen allergens are structurally related to the soybean trypsin inhibitor and may therefore present antigenic structures similar to proteins belonging to this family. Very recently, structurally related allergens from English plantain, *Plantago lanceolata*, (Pla l 1) and goosefoot, *Chenopodium album*, (Che a 1) were reported (27, 28).

The discrepancy between the observed apparent MW of the native Phl p 11 allergen by SDS-PAGE and the MW calculated from the deduced amino acid sequence is presumably explained by post-translational modification of the native allergen. In support of this is the report by van Ree *et al.* (26), where the homologous *L. perenne* protein was shown to carry N-linked glycosylation amounting to approximately 8% of the total molecular mass, and the conservation of the corresponding glycan attachment site in the amino acid sequence of Phl p 11.

Expression in Escherichia coli and purification of rPhl p 11

With the aim of allergenic and serological characterization of the Phl p 11 allergen, the protein was expressed in *E. coli* and purified to homogeneity. Because of poor solubility when the allergen was initially expressed with an N-terminal hexahistidine tag as the only engineered addition, we chose instead to produce it as a fusion to the *E. coli* maltose binding protein as a means to aid solubility. After preparing a construct where transcription of the fusion was under control of the T7 promoter, using *E. coli* XL1-Blue as a cloning host, the plasmid was transferred to strain BL21 harboring plasmid pT7POL23. In this binary system

the construct is quiescent at 30°C and recombinant protein expression induced by a temperature shift to 42°C (Fig. 4).

Using this strain for expression, accumulation of MBP-Phl p 11 to approximately 10% of total cellular protein was obtained, as estimated from Coomassie-stained SDS-PAGE (Fig. 4).
5 Analysis of fractionated cellular material revealed that approximately half of the fusion protein was present in the soluble phase (not shown). The proportion of soluble protein tended to be higher when the culture had been returned to 30°C after a period of induction at 42°C, as opposed to being kept at 42°C until harvest (not shown). In order to minimize aggregation of the soluble fusion protein, post-harvest processing was performed under reducing conditions.
10 After buffer exchange to lower the concentration of reductant in the cleared cell extract, the protein was subjected to a first step of purification by IMAC. While the eluted material appeared as a single distinct band of the expected size on reducing SDS-PAGE, analytical gel filtration indicated the presence of different aggregation forms in addition to the monomer. A step of size exclusion chromatography using Superdex 75 was therefore added to the
15 purification process. The final preparation appeared monomeric by analytical gel filtration and free of contaminating bacterial proteins by SDS-PAGE. It appeared stable and no formation of aggregates was observed upon storage at –20°C. The final yield of purified protein was 12 mg per liter of bacterial culture, or 1.7 mg per gram of cell pellet (fresh weight).

20 *Analysis of antibody recognition of rPhl p 11*

To examine the IgE antibody binding capacity of the recombinant allergen and investigate the frequency and magnitude of Phl p 11-specific IgE sensitization among grass pollen-allergics, serological tests were prepared for use in Pharmacia CAP System. As a control for antibody binding to the MBP part of the fusion protein, tests carrying MBP alone were
25 prepared and used in parallel. Upon analysis of serum samples of 184 grass pollen-sensitized subjects using these tests, 59 (32%) of them were found to contain specific IgE reactivity to the recombinant allergen (Table II). The average level of IgE to rPhl p 11 in the specifically reactive sera was 16 kU_A/L, as compared to 79 kU_A/L of IgE to natural extract of *P. pratense* pollen. Thus, it appears that on average among these subjects, approximately 20% of the IgE
30 reactivity to *P. pratense* pollen allergens was directed to rPhl p11.

In two of the sera that showed a positive result with the rPhl p 11 test, there was also an apparent binding of IgE to MBP alone. For one of these sera the IgE determination was in fact higher with the MBP test, and this serum was therefore regarded as lacking IgE to rPhl p 11. For the other serum, the contribution by MBP to the IgE binding by the fusion protein was only about 1%, which was considered insignificant. In total, only four sera of all 184 tested (2%) showed detectable IgE binding to MBP alone, indicating that MBP may be a suitable fusion partner for recombinant allergen production in instances when a soluble non-fusion protein cannot be efficiently produced in *E. coli.*

For the purpose of comparison, the 184 serum samples were also tested with an assay specific for a previously established major grass pollen allergen, rPhl p 2. IgE antibody reactivity directed to this allergen was found in 103 (56%) of all tested subjects, with an average IgE level of 11.4 kU_A/L. Binding to rPhl p 2 would thereby account for approximately 15% of the total level of IgE to whole, natural extract of *P. pratense* pollen in this subset of sera, which was 74 kU_A/L on average. In summary, the serological analysis shows that the *E. coli*-expressed rPhl p 11 has significant and specific IgE antibody binding capacity, comparable in frequency and magnitude to that of rPhl p 2.

Inhibition of IgE binding to natural grass pollen extract by soluble rPhl p 11

To compare in a more direct way the IgE binding characteristics of recombinant and natural Phl p 11, an immunoblot inhibition experiment was performed. In this analysis, competition for IgE binding to immobilized natural allergen by soluble rPhl p 11 would be visualized as attenuation of IgE binding to immobilized natural Phl p 11 after preincubation of patient serum with the recombinant allergen. As a control for unspecific inhibition, both serum samples used were preincubated with BSA and MBP in parallel with the rPhl p 11 pretreatment. While the control proteins had no visible effect on IgE binding to extract proteins, as compared to preincubation with buffer (not shown), pretreatment of the serum samples with rPhl p 11 almost completely abolished the autoradiography signal at 20 kDa molecular weight (Fig. 5). The result demonstrated that the recombinant protein shared epitopes for human IgE antibodies with natural Phl p 11.

The contribution of Phl p 11 to the total IgE binding activity of pollen proteins was further examined by dot blot inhibition experiments in which rPhl p 5, an allergen known for

its high IgE binding capacity (7), was used for comparison. Equal amounts of pollen protein extract were spotted onto identical pieces of nitrocellulose membrane and exposed to patients' sera that had been preincubated with either rPhl p 11, rPhl p 5 or MBP. From serological analyses, these sera were known to contain IgE to both Phl p 11 and Phl p 5, but not to MBP. As controls, buffer incubation and serum from one non-allergic individual were used. After washing, membrane-bound IgE was determined radiometrically and the inhibition effects of rPhl p 11 and rPhl p 5 were calculated in relation to the MBP-pretreated samples. The results of the experiment are shown in Table III. On average rPhl p 11 was found to inhibit 25% of the IgE binding to pollen extract, which corresponds to the quantitative serological data shown in Table II, while rPhl p 5 caused an average inhibition of 55%. We conclude that Phl p 11 accounts for a relevant proportion of timothy grass pollen-specific IgE antibodies, although smaller than Phl p 5.

rPhl p 11 induces basophil histamine release and immediate skin reaction

In an experiment on basophils from a high-level Phl p 11-sensitized allergic individual, rPhl p 11 induced dose-dependent release of histamine, demonstrating its capacity to productively cross-link cell surface-bound IgE antibodies. Limited histamine release occurred from cells of a low-grade Phl p 11-sensitized subject and none from cells of a non-allergic upon incubation with rPhl p 11. (Table 1). Evidence of specific biological activity of rPhl p 11 *in vivo* was obtained from skin test experiments. In two sensitized subjects, dose-dependent wheal reactions resulted from challenge with a dilution series of the allergen while no reaction occurred in four non-allergic controls tested. (Table 2). The rPhl p 11 fusion partner MBP alone gave rise to no reaction in neither these experiments. Hence, rPhl p 11 exhibited biological activity which fulfilled criteria of specificity.

Table 1. Specific induction of histamine release from basofils by rPhl p 11[a]

	Patient A			Patient B			Patient C		
Conc (µg/ml)	MBP	MBP-rPhl p 11	anti-IgE	MBP	MBP-rPhl p 11	Anti-IgE	MBP	MBP-rPhl p 11	anti-IgE
0	6.5	6.7	6.6	6.5	6.5	6.5	3.5	3.5	3.5
0.001	5.8	16	11.3	5.5	5.5	8.25	3.5	3.5	8.25
0.01	5.2	29	16	5.5	5.8	10	3.4	3.5	14
0.1	4.9	34	28.5	5.4	7.8	23	3.5	3.8	43
1	4.7	36.5	34	5.3	7.8	13	3.5	3.5	55

5 a) Release of histamine is expressed as percentage of the cells' total content of histamine.

Table 2. Skin prick tests with rPhl p 11[b]

	MBP-rPhl p 11				MBP	Pollen extract	Histamine
Subject	100 µg/ml	10 µg/ml	1 µg/ml	0,1 µg/ml	all conc		
Allergic No. 1	5.5	2.5	-	-	-	9.0	3.6
Allergic No. 2	8.0	5.5	4.0	-	-	10.5	7.0
Non-allergic No. 1	-	-	-	-	-	-	8.0
Non-allergic No. 2	-	-	-	-	-	-	7.5
Non-allergic No. 3	-	-	-	-	-	-	4.9
Non-allergic No. 4	-	-	-	-	-	-	8.5

10

b) Two allergics and four non-allergics were skin tested with rPhl p 11 at different concentrations, with timothy grass pollen extract and histamine. The diameter of the resulting wheal reactions were determined and are presented in mm.

15

DISCUSSION

Grass pollens belong to the most frequently sensitizing and potent allergen sources. They contain a number of allergenic molecules, several of which have been identified and characterized in the recent past (3). In the present invention we report the identification,
20 cloning and recombinant production of a novel *P. pratense* pollen allergen which adds new important epitopes to the growing panel of recombinant grass pollen allergens (29).

Despite the fact that the group 11 grass pollen allergens are glycoproteins (26) and contain several cysteine residues, we were able to produce soluble, monomeric and immunologically active rPhl p 11 allergen by utilizing MBP as a fusion partner for expression in *E. coli*. Extensive serological characterization of IgE reactivity to rPhl p 11 was carried out using a quantitative assay system where allergen is covalently immobilized onto activated cellulose. Using the rPhl p 11-specific tests, we found that about one third of all grass pollen sensitized subjects analyzed (n=184) contained serum IgE antibodies binding to rPhl p 11 and that the magnitude of binding corresponded to a significant proportion of grass pollen-specific IgE antibodies in these subjects.

Evidence supporting the authenticity of epitope presentation by rPhl p 11 was obtained from immunoblot inhibition experiments, where natural grass pollen proteins were attached on solid phase and rPhl p 11 used as fluid-phase inhibitor.
Specific and extensive inhibition of IgE binding to the natural allergen occurred in both of two patient sera examined, demonstrating that rPhl p 11 could compete with natural Phl p 11 for IgE antibody binding. Taken together, the serological data show that immunoreactive rPhl p 11 can be produced using *E. coli* expression and that the recombinant protein shares epitopes for IgE antibodies with the natural allergen. Based on the results obtained, it is clear that rPhl p 11 represents an important addition to the panel of recombinant grass pollen allergens useful for *in vitro* diagnosis of grass pollen allergy.

Relevant to this discussion is the immunological analysis of chemically deglycosylated natural Lol p 11 reported by van Ree *et al.* (26), which suggested the involvement of carbohydrate structures in the IgE binding properties of this allergen. Thus, we cannot exclude that a qualitative difference in allergenic properties exists between natural Phl p 11 and the recombinant molecule described in this paper and that expression of Phl p 11 in a glycosylated form, using a eukaryotic host, could yield a recombinant allergen with different IgE binding characteristics. On the other hand, in view of recent notions that glycan epitopes may not be efficient elicitors of IgE-mediated reactions or informative in relation to clinical allergy manifestation (30-34), it is possible that an unmodified recombinant allergen expressed in *E. coli* is more useful for diagnostic purposes.

Despite the significant sequence homology among the members of the widely represented (grasses, trees and weeds) group of allergens exemplified by Phl p 11 in timothy grass and Ole e 1 in olive tree pollen, little cross-reactivity for IgE antibodies appears to exist between them. In a preliminary analysis, we have been unable to detect cross-reaction between rPhl p 11 and Ole e 1 (Niederberger, Valenta & Lidholm, unpublished data) and the results of recent studies on other members of this allergen family (27, 28) are in agreement with this observation.

One important implication of the apparent lack of significant cross-reactivity between rPhl p 11 and other members of this allergen family is that they are useful as diagnostic markers to more precisely identify the primary sensitizer of allergic individuals, as compared to natural extracts or cross-reactive components such as profilin, two-EF-hand allergens or Bet v 1 homologues. Thus, a preferential IgE recognition of Phl p 11, in relation to other members of this allergen family, may suggest a primary sensitization by grass pollen rather than another allergen source containing cross-reactive components. The use of selected recombinant allergens in this way may provide information useful for advice on allergen avoidance and adequate selection of allergen extract for specific immunotherapy treatment.

In conclusion, the present invention concerns cDNA cloning and recombinant production of an IgE-reactive and biologically active group 11 grass pollen allergen. Recombinant Phl p 11 can be used to identify group 11 allergen sensitization in patients and for specific immunotherapy.

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CLAIMS

1. A reagent, Phl p11, comprising the amino acid sequence according to SEQ ID NO 1 or variants or derivatives or fragments thereof with equivalent or similar function with respect to antibody binding

2. A reagent according to claim 1 which is recombinantly produced or chemically synthesized

3. A diagnostic kit comprising the reagent according to claim 1.

4. A diagnostic kit according to claim 3 also comprising one or more of other known Phl allergens, such as Phl p 1, Phl p 2, Phl p 4, Phl p 5a, Phl p 5b, Phl p 6, Phl p 7, Phl p 12 and Phl p 13.

5. An immunoassay comprising the following steps:
   a) obtaining a blood sample from a patient with suspected grass pollen allergy
   b) allowing serum or plasma derived from the blood sample to contact the allergen reagent according to claim 1, immobilized on a solid phase or in solution.
   c) detecting antibodies bound to the allergen reagent using a specific detection reagent such as an enzyme-conjugated anti-IgE antibody.

6. An immunoassay according to claim 5 where the allergen is recombinantly produced or chemically synthesized

7. Use of the reagent according to claim 1 or 2 for production of a drug for immunotherapy ("allergy vaccination") of grass pollen allergic patients showing IgE antibody reactivity to Phl p 11.

8. Use according to claim 7 for production of a drug for immunotherapy ("allergy vaccination") of timothy grass pollen allergic patients showing IgE antibody reactivity to Phl p 11.

5

Fig. 1

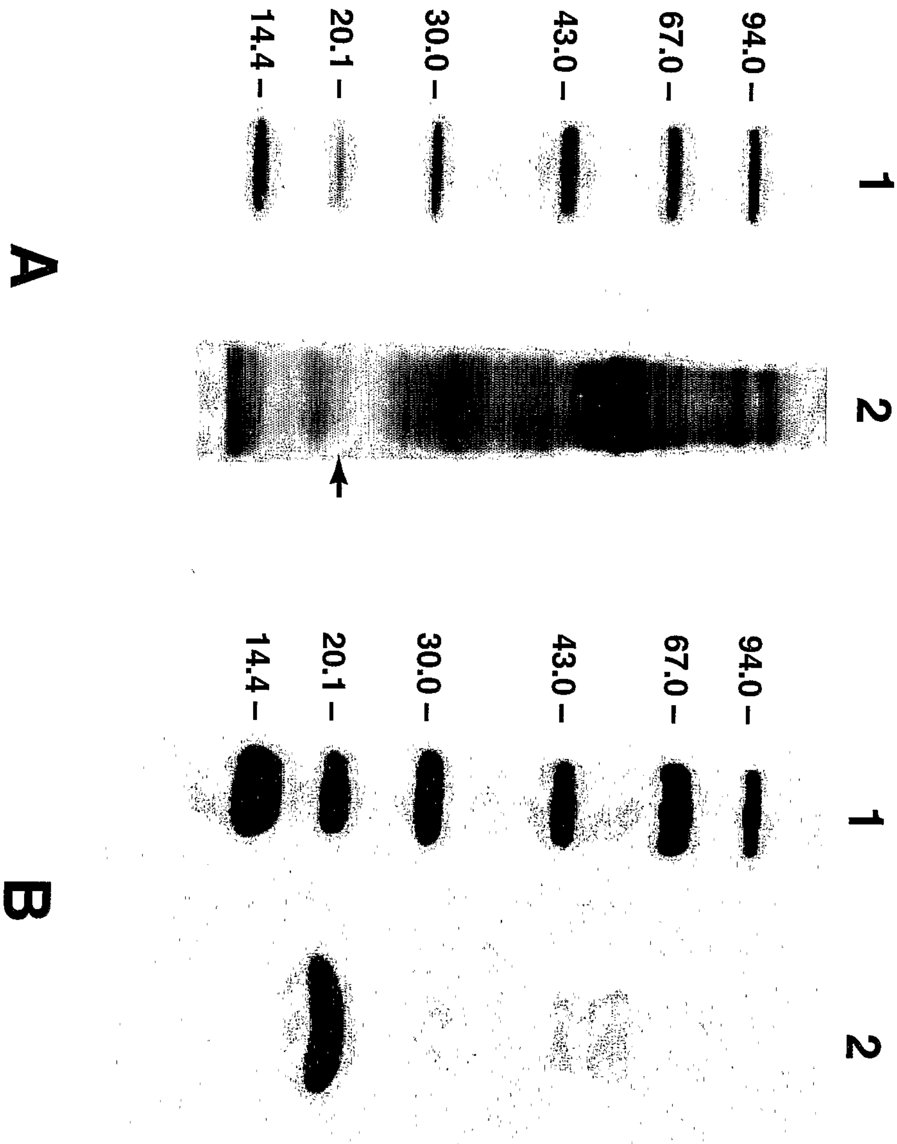

Fig. 2

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Asp Lys Gly Pro Gly Phe Val Val Thr Gly Arg Val Tyr Cys Asp    15
GAC AAG GGC CCG GGC TTC GTG GTC ACG GGA CGC GTC TAC TGC GAC    45

Pro Cys Arg Ala Gly Phe Glu Thr Asn Val Ser His Asn Val Gln    30
CCC TGC CGC GCC GGC TTC GAG ACC AAC GTC TCC CAC AAC GTC CAA    90

Gly Ala Thr Val Ala Val Asp Cys Arg Pro Phe Asn Gly Gly Glu    45
GGG GCG ACC GTG GCG GTG GAC TGC CGG CCG TTC AAC GGC GGC GAG   135

Ser Lys Leu Lys Ala Glu Ala Thr Thr Asp Gly Leu Gly Trp Tyr    60
AGC AAG CTC AAG GCG GAG GCG ACG ACG GAC GGT CTG GGC TGG TAC   180

Lys Ile Glu Ile Asp Gln Asp His Gln Glu Glu Ile Cys Glu Val    75
AAG ATC GAG ATC GAC CAG GAC CAC CAG GAG GAG ATC TGC GAG GTG   225

Val Leu Ala Lys Ser Pro Asp Thr Thr Cys Ser Glu Ile Glu Glu    90
GTG CTG GCC AAG AGC CCC GAC ACG ACG TGC TCC GAG ATC GAG GAG   270

Phe Arg Asp Arg Ala Arg Val Pro Leu Thr Ser Asn Asn Gly Ile   105
TTC CGC GAC CGC GCC CGC GTC CCG CTC ACC AGC AAC AAC GGC ATC   315

Lys Gln Gln Gly Ile Arg Tyr Ala Asn Pro Ile Ala Phe Phe Arg   120
AAG CAG CAG GGC ATC CGC TAC GCC AAC CCC ATC GCA TTC TTC CGC   360

Lys Glu Pro Leu Lys Glu Cys Gly Gly Ile Leu Gln Ala Tyr Asp   135
AAG GAG CCG CTC AAG GAG TGC GGC GGC ATC CTC CAG GCC TAC GAC   405

Leu Arg Asp Ala Pro Glu Thr Pro *                             143
CTC AGG GAC GCC CCC GAG ACG CCA TGA AGC CCC ACA CCA GCA CGA   450

CGT ACC ACC TAT AGT TAC TTG CCG CCG GCC GAG ACG ATG TTA CCT   495

                                ▽
CTG CGA GCC GCT GCC GGA GAG GAR ATG ACA ACC TTT TAA TGG GCC   540

                            ▽
TCA CGT GCG CCT TAA TAT TCR CGT CCT GCT TTC TCT TTT ATT CAT   585

                        ▽
GTT ATT GTC TTC CTG TYC TCT AAT TAT TTA CGT GTT GAC CTA TAT   630

                                            ▼
GTG AGC TAG TTC CAA GGA TCT GTT CTA TGT GTA ATA AGA GAA CAC   675

        ▽   ▼                                              ▽
AAA TAT TTS GTA CGT GCA TAT CCG ATG TAT ATC CTC TTT TCG GGS   720

        ▽▽  ▽                                          ▽
AAA AAA AAW AYT CTG ATG TAT ATC CTC TGG ACA CAA ATT AAR TGG   765

        ▼           ▽ ▼
CCA GCT AAT GAA TTS AGT ACT (A)n                             803+

▽
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Fig. 3

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ruler      1                                                           60
Phl p11                                  DK--GPGFVVTGRVYCDPCRAGFETNVSHNVQG
A54002                                   DK--GPGFVVTGRVYCDPCRAGFETNVSHNVEG
1815759    MASLR--ALSVIAVAVVLFALADTAVATK--APDYVVQGRVYCDRCRAGFETNVTEYIKG
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P33050     MASVPAPATTTAAVILCLCVVLSCAAADDPNLPDYVIQGRVYCDTCRAGFVTNVTEYIAG
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3256212                            EDVPQPPV---SQFYIQGQVYCDTCRARFITELSEFIPG
926885                             EDVPQPPV---SQFYIQGQVYCDTCRAGFITELSEFIPG

ruler      61                                                         120
Phl p11    ATVAVDCRPFNGGESKLKAEATTDGLGWYKIEIDQDHQEEICEVVLAKSPDTTCSEIEEF
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2832664    AKVKLECRSRTNGTVTLTKEAVTDKTGSYRMEVTGDHEEEVCELVLVESPDSGCSDVSKE
398899     AVVRLECKDRRTMELTYSHEARTDSTGSYKILVNEDHDEQFCDAMLVRSSQLRCSNVSPG
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3256212    AGVRLQCKDGENGKITFTEVGYTRAEGLYSMLIERDHKNEFCEITLLSSSRKDCDEIPIE
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ruler      121                                                    175
Phl p11    ---RDRARVPLTSNNGIKQQGIRYANPIAFFRKEPLKECGGILQAYDLRDAPETP
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1815759    ---RDRAPVLLTRNVGISDN-LRLANPLGYLKDVPLPVCGDLLKMFKLADDDDDQ
S31710     ---RDRARVLLTRNVGICDN-LRLANPLGYLKDYHCPS-AALLKQFDLADDDNE
P33050     ---RDRAGVLLTRNVGISDS-LRPANPLGYFKDVPLPVCAALLKQLDSDDDDDQ
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3256212    GWVKPSLKFMLNTVNG----TTRTINPLGFFKKEALPKCPQVFNKLGMYPPNM
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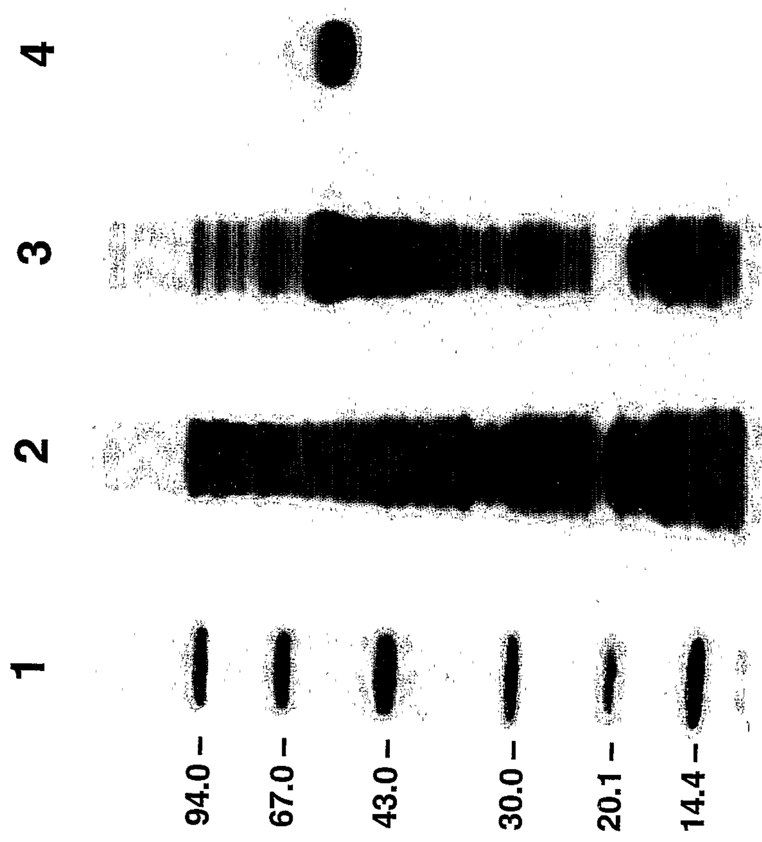

Fig. 4

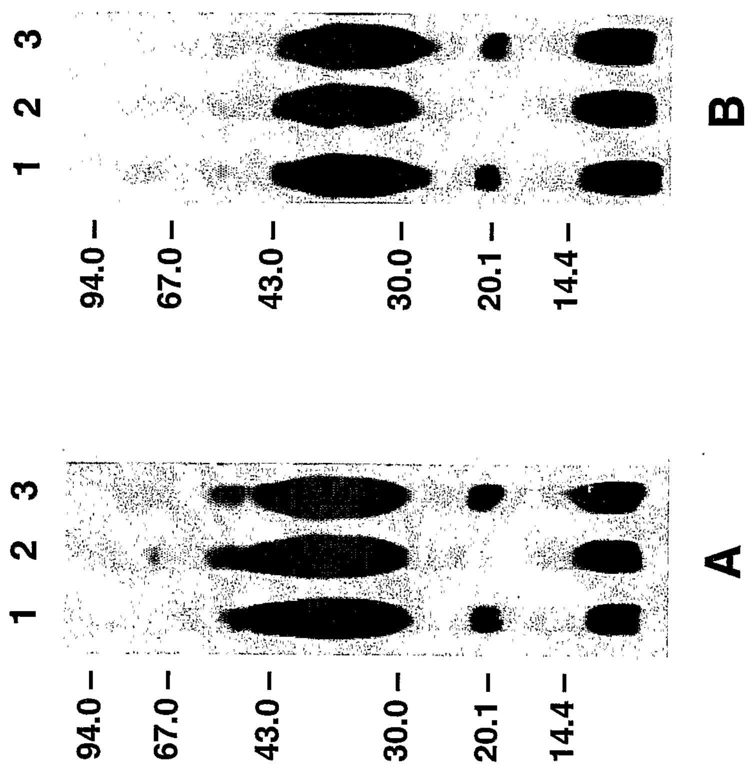

Fig. 5

SEQUENCE LISTING

<110> Pharmacia Diagnostics AB

<120> Novel allergen

<130> P05497SE

<160> 1

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<210> 1

<211> 143

<212> PRT

<213> Phleum pratense

<400> 1

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Thr Val Ala Val Asp Cys Arg Pro Phe Asn Gly Gly Glu Ser Lys Leu
        35                  40                  45

Lys Ala Glu Ala Thr Thr Asp Gly Leu Gly Trp Tyr Lys Ile Glu Ile
    50                  55                  60

Asp Gln Asp His Gln Glu Glu Ile Cys Glu Val Val Leu Ala Lys Ser
65                  70                  75                  80

Pro Asp Thr Thr Cys Ser Glu Ile Glu Glu Phe Arg Asp Arg Ala Arg
                85                  90                  95

Val Pro Leu Thr Ser Asn Asn Gly Ile Lys Gln Gln Gly Ile Arg Tyr
            100                 105                 110

Ala Asn Pro Ile Ala Phe Phe Arg Lys Glu Pro Leu Lys Glu Cys Gly
        115                 120                 125

Gly Ile Leu Gln Ala Tyr Asp Leu Arg Asp Ala Pro Glu Thr Pro
    130                 135                 140
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 03/00499

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 14/415, A61K 39/36, A61P 37/08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K, A61K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI DATA, EPO-INTERNAL, PAJ, MEDLINE, CHEM.ABS.DATA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Allergy, Volume 56, 2001, R. E. Rossi et al: "Measurement of IgE antibodies against purified grass-pollen allergens (Phl p 1,2,3,4,5,6,7,11, and 12) in sera of patients allergic to grass pollen", pages 1180-1185, see the entire document	1-6
Y		4
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X	Database GENESEQ [Online], Accession no. AAY25604, 30 September 1999, retrieved from EBI, 94,8% identity in 134 aa overlap with SEQ.ID.NO. 1 & WO 9934826 A1	1-3,7-8
Y		4
	--	

[X] Further documents are listed in the continuation of Box C.   [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 June 2003	03 -07- 2003
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer IDA CHRISTENSEN/BS Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 03/00499

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9534578 A1 (PHARMACIA AB), 21 December 1995 (21.12.95), the claims	1-8

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE03/00499

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1 (partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see next sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Present claim 1 relates to a very large number of possible amino acid sequences (and thus a large number of reagents), due to the wording "or variants or derivatives or fragments thereof with equivalent or similar function with respect to antibody binding" (the antibody specificity not mentioned). Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of said amino acid sequences. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been focused on reagents comprising the amino acid sequence of SEQ ID NO 1 and functional variants thereof.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/06/03

International application No.

PCT/SE 03/00499

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9534578 A1	21/12/95	AU 2759895 A	05/01/96
		EP 0763059 A	19/03/97
		JP 10501417 T	10/02/98
		SE 9402089 D	00/00/00
		US 6008340 A	28/12/99
		US 2002052490 A	02/05/02

patsnap

专利名称(译)	新型过敏原		
公开(公告)号	[EP1487869A1](EP1487869A1)	公开(公告)日	2004-12-22
申请号	EP2003745064	申请日	2003-03-26
[标]申请(专利权)人(译)	PHARMACIA诊断		
申请(专利权)人(译)	PHARMACIA诊断AB		
当前申请(专利权)人(译)	PHARMACIA诊断AB		
[标]发明人	MARKNELL DEWITT ASA NIEDERBERGER VERENA VIENNA GENERAL HOSPITAL LEHTONEN PIRJO SPITZAUER SUSANNE C O VIENNA GENERAL HOSPITAL SPERR WOLFGANG VIENNA GENERAL HOSPITAL VALENT PETER VIENNA GENERAL HOSPITAL VALENTA RUDOLF VIENNA GENERAL HOSPITAL LIDHOLM JONAS		
发明人	MARKNELL DEWITT, ASA NIEDERBERGER, VERENA,VIENNA GENERAL HOSPITAL LEHTONEN, PIRJO SPITZAUER, SUSANNE,C/O VIENNA GENERAL HOSPITAL SPERR, WOLFGANG,VIENNA GENERAL HOSPITAL VALENT, PETER,VIENNA GENERAL HOSPITAL VALENTA, RUDOLF,VIENNA GENERAL HOSPITAL LIDHOLM, JONAS		
IPC分类号	G01N33/53 A61K39/00 A61K39/36 A61P37/08 C07K14/415		
CPC分类号	A61K39/00 C07K14/415		
代理机构(译)	LINDGREN安德斯		
优先权	0200946 2002-03-27 SE		
其他公开文献	EP1487869B1		
外部链接	Espacenet		

摘要(译)

本发明涉及来自毛茛属草（Phleum pratense）花粉的新型过敏原，如SEQ ID NO 1中公开的Phl p11，及其作为试剂和诊断试剂盒以及用于免疫疗法的用途。