

Group 13 grass allergens: Structural variability between different grass species and analysis of proteolytic stability

Arnd Petersen, PhD,^a Roland Suck, PhD,^b Susan Hagen, MSc,^b Oliver Cromwell, PhD,^b Helmut Fiebig, PhD,^b and Wolf-Meinhard Becker, PhD^a *Borstel and Reinbek, Germany*

Background: Determination of the allergen composition of an extract is essential for the improvement of hyposensitization therapy. Surprisingly, although grass pollen extracts have been studied intensively for 20 years, a further major allergen, Phl p 13, was detected recently in timothy grass pollen.

Objectives: We sought to determine the occurrence and importance of group 13 allergens in various grass species and to investigate their proteolytic stability.

Methods: The group 13 allergens were determined by means of 2-dimensional PAGE blotting with patient sera and group 13-specific mAbs. The allergens were isolated chromatographically from several pollen extracts and analyzed by means of microsequencing. Cross-reactivity among various grass species was studied by using Western blots and immunoblot inhibition tests. The stability of the allergens was tested under defined extraction conditions.

Results: Group 13 allergens are detectable in all common grasses and show IgE cross-reactivity among them. The allergenic components were identified in the neutral pH range with molecular masses of 50 to 60 kD, and in the case of Phl p 13, maximal binding of the isoforms was observed at 55 kD and at an isoelectric point of 6 to 7.5. Protein sequencing clearly confirms structural identities between different grass species, although individual variations are found. If low-molecular-mass components were depleted by means of gel filtration, a rapid degradation of group 13 allergens was observed. This is in contrast to other pollen allergens described thus far.

Conclusion: Group 13 allergens are widespread and are major allergens in the grasses. Predicted from their primary structures, these allergens are polygalacturonases. This class of enzymes is already known from microorganisms, and these enzymes are recognized as potential inducers of asthma. Our studies indicate that the group 13 allergens show a considerable microheterogeneity and degradation, especially after depletion of low-molecular-mass components. One has to be aware of this pivotal fact when soluble grass pollen extracts are prepared for diagnostics and hyposensitization therapy. (*J Allergy Clin Immunol* 2001;107:856-62.)

Key words: Cross-reactivity, grass pollen, group 13 allergens, IgE binding, immunoblotting, polygalacturonase, timothy grass

Hypersensitivity to grass pollen is the most frequent IgE-mediated allergy.¹ Although the efficacy of hyposensitization therapy is well documented, the mechanism is not understood in detail. During the last decade, numerous allergens have been isolated, cloned, and characterized.² This information provides a basis for preparing better standardized allergens, which in turn provide improved diagnosis and specific immunotherapy.

Concerning grasses, the most important allergens are those of groups 1 and 5, which are found in all temperate grass species and account for IgE reactivity in 95% and 80% of pollen-sensitized individuals, respectively.³ Other groups of grass pollen allergens (eg, groups 2/3 and 4) are recognized by less than 50% of the patients.⁴ The group 6 allergen seems to be exclusive to timothy grass but is recognized by IgE from up to 50% of the patients with grass allergy.⁵ Improved diagnosis through the evaluation of individual sensitization patterns will enable tailor-made extracts to be prepared, which may lead to more effective hyposensitization therapy⁶; that is to say that each patient would only be treated with those components to which he or she showed allergic sensitization, thus avoiding the risk of sensitizing patients to further extract components. A prerequisite for this concept is that all allergens be available.

Recently, we^{7,8} identified a further allergen in timothy grass pollen, which was recognized by more than 50% of the individuals with grass pollen allergy and which was termed, according to the International Union of Immunological Societies committee, Phl p 13. To determine the importance of this allergen, we looked for homologous IgE-reactive components in other grass species and characterized the novel group 13 allergens with respect to their structural variability and proteolytic sensitivity.

METHODS

Preparation of grass pollen extract

Five grams of various grass pollen species (Allergon, Angelholm, Sweden) was incubated in 20 mL of 20 mmol/L TRIS/HCl buffer (pH 8.0). Afterward, the soluble fraction was isolated by means of centrifugation at 17,000g for 30 minutes. The extract was stored at -20°C.

From ^aForschungszentrum Borstel, Borstel; and ^bAllergopharma Joachim Ganzer KG, Reinbek.

Received for publication August 7, 2000; revised December 21, 2000; accepted for publication January 8, 2001.

Reprint requests: Arnd Petersen, PhD, Research Center Borstel, Biochemical and Molecular Allergology, Parkallee 22, D-23845 Borstel, Germany.

Copyright © 2001 by Mosby, Inc

0091-6749/2001 \$35.00 + 0 1/83/114114

doi:10.1067/mai.2001.114114

Abbreviation used
pI: Isoelectric point

Stability testing of extracts

Subsequent to preparation, crude grass pollen extract was separated by means of gel filtration on a G25 column (PD-10; Pharmacia, Freiburg, Germany) from low-molecular-mass components (with a 5-kD cutoff). Depleted and untreated (control) extracts were stored in solution for defined intervals at 4°C. Afterward, the solutions were supplemented with SDS sample buffer⁹ and kept at -20°C until used.

Patient sera and mAbs

Sera were drawn from individuals with pollinosis to grasses. The mAbs AF 6 and EG 9 were raised in BALB/c mice against crude timothy pollen extract. They recognize allergen grass group 13. For comparison, the antibodies 5H1 and IG 12 were applied directly against groups 4 and 1, respectively.^{10,11}

Dot blotting

Pollen extract and isolated group 13 allergens were diluted in 20 mmol/L NaOH to final concentrations of 5 mg/mL and 100 µg/mL, respectively. One hundred microliters of the protein solutions were dotted onto nitrocellulose membrane by use of a dot blotter (Miniblotter; Biometra, Göttingen, Germany) for 30 minutes. After washing with double-distilled water and blocking with TRIS-buffered saline with 0.5% Tween-20, incubations with the primary antibodies (individual patient sera diluted 1:10 or hybridoma supernatants diluted 1:50) were performed overnight. The determination for binding was done as described for immunologic detection.

Western blotting

Proteins (14 µg/cm) were separated by means of SDS-PAGE (T = 13.5%, C = 4.0%) by using the Laemmli buffer system.⁹ The determinations of molecular mass were achieved by use of the low-range SDS-PAGE standards (14.4-97.4 kD; Biorad, Richmond, Va) or by the "Benchmark Prestained Protein Ladder" (10-170 kD; Gibco-BRL, Gaithersburg, Md). The proteins were subsequently transferred onto nitrocellulose membrane (PROTRAN BA 83; Schleicher & Schüll, Dassel, Germany) by means of semidry blotting for 30 minutes at 2 mA/cm².¹²

Two-dimensional PAGE blotting

Two-dimensional PAGE blotting was done according to the method of Görg et al.¹³ In the first dimension isoelectric focusing was performed in polyacrylamide gel strips (T = 4%, C = 4%; size, 240 × 80 × 0.5 mm³) with an immobilized pH gradient of 3.6 to 9.3 containing 8 mol/L urea and 10 mmol/L dithiothreitol. Two hundred micrograms of timothy grass pollen extract was applied per lane and separated at 3000 V and 2 mA for 7 hours. After incubation for 30 minutes in an equilibration solution containing 6 mol/L urea, 65 mmol/L dithiothreitol, and 260 mmol/L iodoacetamide, SDS-PAGE was carried out in the second dimension in discontinuous gels (T = 8%-13%, C = 4%; size, 240 × 80 × 0.5 mm³) at 200 V and 30 mA. The determinations of isoelectric point (pI) and molecular mass were achieved by use of marker protein test mix 9 (pH range, 3.5-10.5; Serva, Heidelberg, Germany) and low-range SDS-PAGE standards, respectively.

For immunologic studies, we performed thermoblotting¹⁴ and obtained 3 replicas from each gel. For amino acid sequencing, the proteins were electroblotted onto Immobilon P membrane (Millipore, Eschborn, Germany) for 30 minutes at 200 mA by using 10 mmol/L 3-cyclohexylamino-1-propane-sulfonic acid (pH 11.0) as the blotting buffer.¹⁵

Protein staining and immunologic detections

For protein staining, we used 0.1% India ink (Pelikan, Hannover, Germany) on nitrocellulose membranes¹⁶ and 0.1% Coomassie stain in 50% methanol on polyvinylidene difluoride membrane.

Immunodetection of specific IgE and mAbs was accomplished by using alkaline phosphatase-labeled monoclonal mouse anti-human IgE (Allergopharma, Reinbek, Germany) and anti-mouse IgG/IgM (Dianova, Hamburg, Germany), respectively, with nitro-blue tetrazolium chloride and 5-bromo-4-chloro-indolyl phosphate as the substrate.¹⁷

Isolation of group 13 allergens by means of chromatographic separation

The group 13 allergens were isolated from pollen extracts of various grass species, according to the procedure of Suck et al.¹⁸ Briefly, lyophilized pollen extract was reconstituted in starting buffer (1 mol/L [NH₄]₂SO₄, 20 mmol/L TRIS/HCl, and 1 mmol/L EDTA, pH 8.0), and the solution was applied to hydrophobic interaction chromatography (Phenyl Sepharose High Performance, Pharmacia). The flow-through fraction containing Phl p 13 and Phl p 1 was purified by means of gel filtration (Superdex 75, Pharmacia).

Immunoblot inhibition test

Two hundred microliters of patient serum was preincubated with 250 µg of inhibitor (purified Phl p 13 or ovalbumin; Sigma, Deisenhofen, Germany; negative control) in a total volume of 2 mL of TRIS-buffered saline with 0.1% Tween-20 for 1 hour at room temperature. The samples were then used for immunostaining according to the standard procedure.

Amino acid sequencing

After blotting, the polyvinylidene difluoride membrane was washed with double-distilled water, stained with 0.1% Coomassie stain (Serva) in 50% methanol, destained in 50% methanol, and air-dried. Protein bands were excised, and microsequencing was performed by using a 476 A protein sequencer with online PTH amino acid analyzer (PE Biosystems, Weiterstadt, Germany).

To obtain internal sequence data from Zea m 13, we digested the allergen with endoproteinase Lys-C (Roche, Mannheim, Germany) at an enzyme/substrate ratio of 1:20 in a 25 mmol/L TRIS buffer (pH 8.5) at 37°C overnight. Fragments of Zea m 13 were isolated by means of Western blotting for protein sequence determination.

Computer analyses

Sequence data were analyzed on GeneWorks and PCGENE software (Intelligenetics, Geel, Belgium). To screen for sequence homology, we used the Swiss Prot Data Library (European Molecular Biology Laboratory). Sequence alignment was performed by using DNASIS (Pharmacia).

RESULTS

Identification of Phl p 13 by means of 2-dimensional electrophoresis

To characterize Phl p 13, a fresh extract of timothy grass pollen was separated under reducing conditions by means of 2-dimensional PAGE immunoblotting. Phl p 13 was identified by using patient IgE and the mAbs AF 6 and EG 9.

As detectable in Fig 1, B, mAb AF 6 recognizes components of 55 kD, especially in the pI range of 6 to 7.5 (6.5, 6.7, 6.9, 7.2, and 7.4). There is also weak binding to proteins of similar size but with pIs of 4.8 to 5.2 and 8 to 9. EG 9 showed the same pattern (data not shown). These

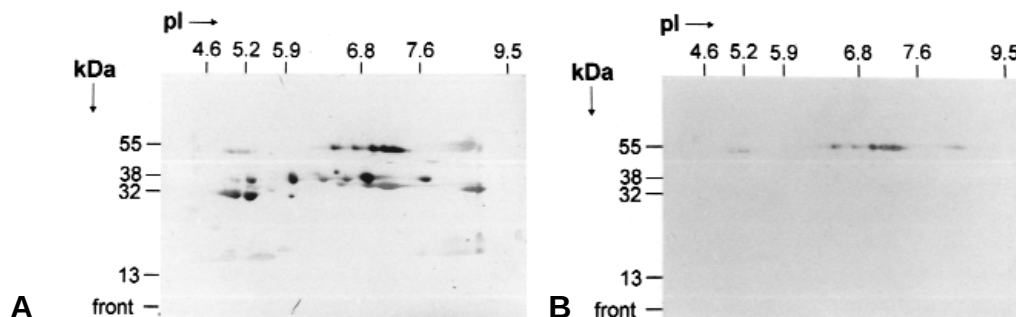


FIG 1. Two-dimensional PAGE immunoblotting for the identification of group 13 allergens in timothy grass pollen (*Phleum pratense*). IgE-reactive components were identified by using patient pooled IgE (A) and the group 13-specific mAb AF 6 (B).

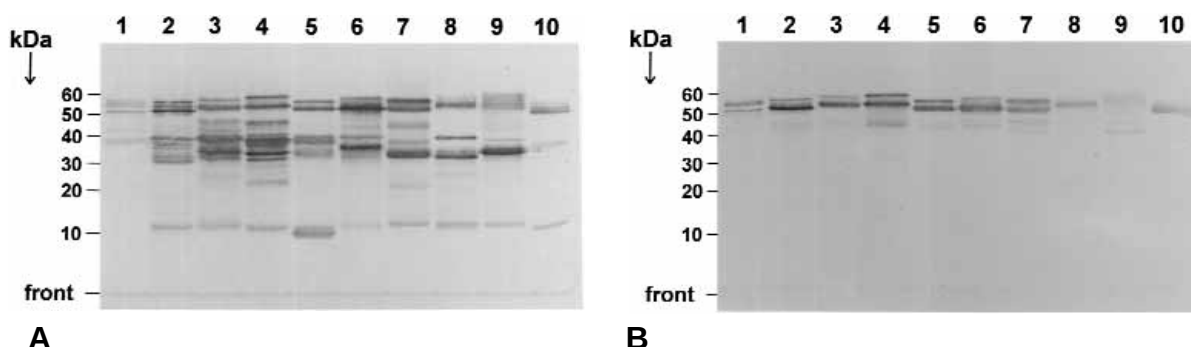


FIG 2. Identification of group 13 allergens in various grass pollen species by means of Western blotting. Group 13 allergens were identified by using patient pooled IgE (A) and by using the specific mAb AF 6 (B). Grass species are as follows: *Cynodon dactylon*; *Dactylis glomerata*; *Festuca pratensis*; *Lolium perenne*; *Phleum pratense*; *Poa pratensis*; *Secale cereale*; *Holcus lanatus*; *Hordeum vulgare*; and *Zea mays*.

components are allergens, as identified by use of patient IgE (Fig 1, A). Microsequencing of the isoforms confirmed all the spots as Phl p 13. No cross-reactivity was identified to group 4 allergens, as determined by use of mAb 5H1 (data not shown). Thus Phl p 13 covers a wide spectrum with broad and diffuse spots in the neutral pH.

Identification of group 13 allergens in common grass species

Pollens from 10 frequently occurring grass species were analyzed by using Western blotting to investigate the occurrence of group 13 allergens.

As shown in Fig 2, all the temperate grass species tested reveal protein bands of 50 to 60 kD that were identified with mAb AF 6 (Fig 2, B) as group 13 allergens. The binding patterns varied in intensity and showed very faint bands in the case of barley pollen (No. 9). In addition to the main bands, components of 40 kD and greater were detected, which were recognized by patient IgE and by mAb AF 6, indicating that they are fragments of group 13 allergens.

For further investigations, extracts of *Dactylis glomerata*, *Festuca pratensis*, *Lolium perenne*, *Poa pratensis*, and *Zea mays* obtained by means of the specific chro-

matographic separation procedure and the isolated components were used for microsequencing.

Fig 3 depicts a comparison of the protein sequences. Except for maize, the N-termini were determinable from the complete proteins. To circumvent the blocking in the case of the *Zea mays* sample, we cleaved the particular protein with endoproteinase Lys-C and isolated a 35-kD fragment, which revealed the sequence indicated in Fig 4. This part showed complete identity with a polygalacturonase of maize PG1 (accession number G1129940) cloned by Niogret et al,¹⁹ and thus PG1 is a synonym for Zea m 13. We introduced the deduced N-terminus of that protein in Fig 3. The glutamic acid at position 1 tends to form a pyroglutamic acid, which is not accessible to Edman degradation.

To determine the allergenicity of group 13 in other grass species, a panel of 24 sera obtained from patients allergic to grass pollen were screened for their IgE reactivity to group 13 allergens, as shown in Fig 5.

Comparable with Phl p 13, the group 13 components of the other grass species were also detected as allergens. The IgE prevalence varied for Fes p 13, with 18 (75%) of 24 sera, and for Zea m 13, with 10 (42%) of 24 sera.

		1				5				10				
Phl p 13		G	K	K	E	E	K	K	E	E	K	K	E	S
Dac g 13		K	D	K	E	E	K	K	D	E	K	K	E	S
Fes p 13		K	D	K	E	E	K	K	D	E	K	K	E	G
Lol p 13		K	D	K	E	E	K	K	D/E	E	K	K	E	D
Poa p 13	K	G	K	D	A	E	K	K	E	E	K	K	D	E
Zea m 13							E	K	E	E	S	K	G	I

FIG 3. N-terminal sequences of grass group 13. Consensus motifs are highlighted. Numbers correspond to the amino acid residues of Phl p 13. Because Zea m 13 is blocked at the N-terminus, the sequence was deduced from an internal fragment, which completely fits to PG1, a polygalacturonase from maize.¹⁹ Grass group 13 allergens of the following species were analyzed: Phl p 13 (*Phleum pratense*), Dac g 13 (*Dactylis glomerata*), Fes p 13 (*Festuca pratensis*), Lol p 13 (*Lolium perenne*), Poa p 13 (*Poa pratensis*), and Zea m 13 (*Zea mays*).

			10	20	30	40	50
Phl p 13	1	<u>GKKEEKKEEK</u>	<u>KESGDAASGA</u>	DGTYDITKLG	AKPDGKTDCT	KEVEEAWASA	
Zea m 13	1	<u>E-KEES</u>	<u>KGIDAKASGP</u>	GGSFDTITKLG	ASNGKTDST	KAVQEAWASA	
			60	70	80	90	100
Phl p 13	51	CGGTGKNTIV	IPKGDFLTGP	LNFTGPCKGD	SVTIKLDGNL	LSSNDLAKYK	
Zea m 13	47	<u>CGGTGKQITL</u>	IPKGDFLVGQ	LNFTGPCKGD	-VTIQVDGNL	LATTDLSQYK	
			110	120	130	140	150
Phl p 13	101	--ANWIEIMR	IKKLTITGKG	TLDGQGKAVW	GKNSCAKNYN	CKILPNTLVL	
Zea m 13	97	DHGNWIEILR	VDNLVITGKG	NLDGQGPAVW	SKNSCTKKYD	CKILPNLSLV	
			160	170	180	190	200
Phl p 13	151	DFCDDALIEG	ITLLNAKFFH	MNIYECKGVT	VKDVTITAPG	DSPNTDGIHI	
Zea m 13	147	DFVNNGEVSG	VTLLNSKFFH	MNMYRCKDML	IKDVTITAPG	DSPNTDGIHM	
			210	220	230	240	250
Phl p 13	201	GDSSKVTITD	TTIGTGDDCI	SIGPGSTGLN	ITGVTCTGPGH	GISVGSLLGRY	
Zea m 13	197	GDSSGITITN	TVIGVGDDCI	SIGPGTSKVN	ITGVTCTGPGH	GISIGSLLGRY	
			260	270	280	290	300
Phl p 13	251	KDEKDVTDIT	VKNVCLKKST	NGLRIKSYED	AKSPLTASKL	TYENVKMEDV	
Zea m 13	247	KDEKDVTDIN	VKDCTLKKT	FGVRIKAYED	AASVLTVSKI	HYENIKMEDS	
			310	320	330	340	350
Phl p 13	301	<u>GYPIIIDQKY</u>	CPNKICTSKG	DSARVTVKDV	TFRNITGTSS	TPEAVSLLCS	
Zea m 13	297	ANPIFIDMKY	CPNKLCTANG	-ASKVTVKDV	TFKNITGTSS	TPEAVSLLCT	
			360	370	380	390	
Phl p 13	351	DKQPCNGVTM	NDVKIEYSGT	NNKTMAVCTN	AKVTAKGVSE	ANTCAA	
Zea m 13	347	AKVPCTGVMT	DDVNVEYSGT	NNKTMAICTN	AKGSTKGCLK	ELACF	

FIG 4. Comparison of the Phl p 13 and Zea m 13¹⁹ sequences. Underlined amino acid residues were determined by means of protein sequencing. Sequence identities are indicated by bold letters, and the polygalacturonase consensus motifs are printed in italics.

To study the cross-reactivity of group 13 components in different species, we performed an immunoblot inhibition test. Because groups 4 and 13 have similar molecular masses, we separated the group 13 allergens by using SDS-PAGE blotting and analyzed the cross-reactivity by inhibition of a patient's serum with preincubation with soluble Phl p 13.

As shown in Fig 6, the Phl p 13-depleted serum did not bind to Phl p 13 and Zea m 13 on the nitrocellulose membrane and less to Lol p 13 and Poa p 13. The use of similar ovalbumin concentrations as a control showed similar binding compared with untreated serum. Thus the group 13 allergens are composed of similar IgE-reactive epitopes.

Analysis of the proteolytic stability of Phl p 13

Our previous experiments (Figs 1, 2, and 6) had shown multiple bands/spots of the group 13 allergens, indicating a microheterogeneity, fragmentation, or both. For further studies, we tested the stability of these grass pollen allergens under defined conditions for extract production and storage. The removal of low-molecular-mass components with gel filtration (G25 column) caused a dramatic decrease in grass group 13.

Fig 7, A, demonstrates a high degree of similarity in the qualitative and quantitative protein contents between the extracts that had been treated in different ways. The

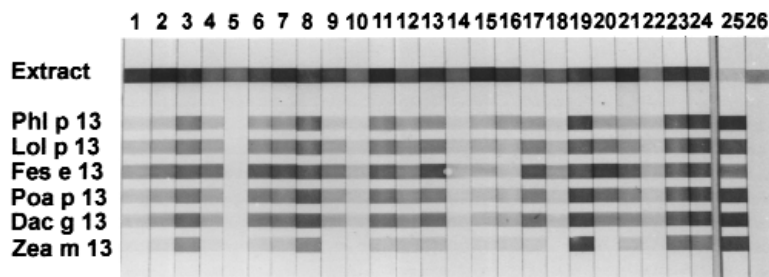


FIG 5. Determination of IgE reactivity to group 13 allergens of several grass species by means of dot blotting. Individual patient sera (1-24) were tested for IgE reactivity to crude timothy pollen extract and to group 13 allergens isolated from timothy grass (Phl p 13), ryegrass (Lol p 13), meadow fescue (Fes e 13), Kentucky blue grass (Poa p 13), cocksfoot (Dac g 13), and maize pollen (Zea m 13). Samples 25 and 26 were incubated with mAbs directed against group 13 (AF 6) and group 4 (5H1), respectively, and served as controls.

immunodetection with patient IgE (Fig 7, B) and with mAb AF 6 (Fig 7, C) identified a complete degradation of Phl p 13 allergen from the gel-filtrated extract after storage for 96 hours. Storage of the extract at 4°C without gel filtration caused little effect on the generation of cleavage products. This result is in contrast with that for Phl p 4, which was not affected by either treatment (Fig 7, D). Beside the fact that Phl p 13 can be easily cleaved by endoproteases (eg, Lys-C, which is used to obtain internal fragments of Zea m 13 for protein sequencing, and Glu-C),⁸ it reveals microheterogeneity not only in isoforms differing in their pI but also tends to degrade into fragments under natural conditions. The rapid fragmentation of group 13 allergens is especially observed after depletion of the low-molecular-mass fraction.

DISCUSSION

Phl p 13 has recently been identified as a major allergen.⁸ Our results demonstrate that homologous allergenic molecules of the group 13 allergens exist in all grasses investigated thus far, and thus it is more prominent than the group 4 allergens, which exhibit very similar molecular weights and which have been characterized previously.²⁰ The present results indicate that group 13 is completely different. The especially high resistance of group 4 allergens to enzymatic degradation (eg, by trypsin)²¹ is in contrast to that of the sensitive group 13 allergens.

The instability of allergens may lead to problems in diagnosis and possibly hyposensitization therapy. Allergen extracts (eg, from house dust mites and cats) are dialyzed after the extraction procedure to deplete low-molecular-mass irritants, such as histamine. As shown for Phl p 13, this step would probably cause the complete loss of a major allergen. Therefore it is essential to perform stability tests on each important allergen of an extract.

In comparison with complete allergens, the IgE reactivity to allergenic fragments is generally decreased because epitopes, especially conformational (discontinuous) ones, are lost. On the other hand, Burks et al²² identified 23 linear epitopes in the peanut allergen Ara h 1, and Bufer et al²³ showed an increase in histamine release of the Phl p 5b fragments, which is in contrast to the complete molecules.

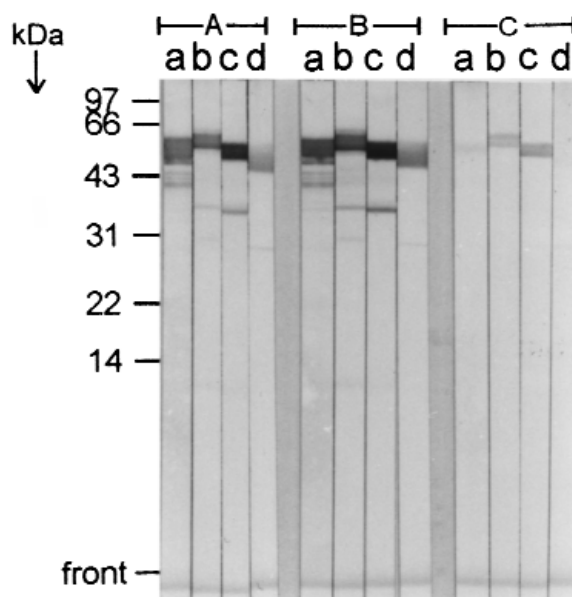


FIG 6. Immunoblot inhibition test to determine the cross-reactivity between the group 13 allergens of different grass species. Patient serum (diluted 1:10) was incubated with inhibitors (A, none; B, ovalbumin; and C, Phl p 13) at a concentration of 125 µg/mL. Binding of the preincubated serum was tested on the following: a, Phl p 13; b, Lol p 13; c, Poa p 13; and d, Zea m 13.

The degradation of allergens by enzymes in extracts has been shown several times. For example, in house dust mite extracts, a decrease of IgE reactivity was observed after storage. This is because the important allergens are cysteine and serine proteases and cause degradation of the extracts.²⁴ In the case of Phl p 13, the dialysis or gel-filtration step seems to be very crucial. It is possible that a low-molecular-weight component (eg, a specific inhibitor) might be depleted, thus allowing degradation.

Presumably, the instability of group 13 allergens is the reason that they have not been identified earlier. An IgE-reactive protein smear without defined protein spots in 2-dimensional PAGE immunoblotting in the 55-kD region covering a wide neutral pH range was already observed but was thought to be due to aggregation of other aller-

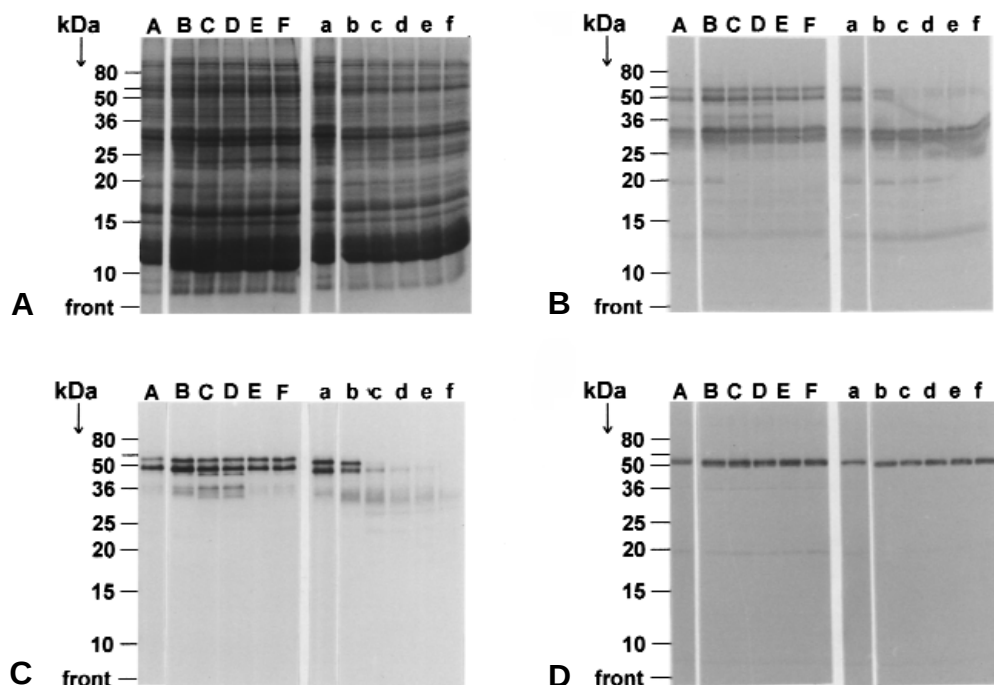


FIG 7. Stability of timothy grass pollen allergens in aqueous extracts. After extraction, crude extract solution was stored at 4°C for defined periods of time (A-F) or was passed over a G25 column to deplete low-molecular-mass components (a-f) before storage in 20 mmol/L TRIS buffer (pH 8.0). Analysis of the extracts was performed by using Western blotting. Storage times were 0 (A and a), 4 (B and b), 24 (C and c), 48 (D and d), 72 (E and e), and 96 (F and f) hours. Coomassie-stained gel (A) and immunoblotting with patient IgE (B), anti-Phl p 13 (mAb AF 6; (C), and anti-Phl p 4 (mAb 5H1; (D) are shown.

gens.^{14,25} Indeed, dimers of Phl p 5b²² and Phl p 12⁶ were identified with similar size. By use of the group 13 mAbs, we were able to identify the components, and the separation procedure allowed us to isolate large amounts of these sensitive proteins. The comparison of the IgE reactivity of individual patient sera to various grass group 13 allergens (Fig 3) and the results of the immunoblot inhibition test (Fig 4) clearly indicate that the group 13 allergens are important allergens that bear similar IgE-reactive epitopes.

Protein sequencing confirms the sequence homology among the group 13 allergens, although the identity is less conserved compared with that of group 1 allergens. It is remarkable that the N-termini of all group 13 species bear conserved motifs formed by glutamic acid and lysine residues. This causes an extremely hydrophilic part, which might be involved in the protein function, to access polygalacturonic acids. Although Zea m 13¹⁹ shows the most differences to the N-terminus of Phl p 13, it reveals a complete sequence identity of 67%. Both allergens completely match as regard the 4 predicted N-glycosylation sites and also 14 cysteine residues; Phl p 13 contains 16 cysteines, whereas Zea m 13 bears 15 cysteines. Thus it is likely that both proteins have very similar conformations as a result of disulfide bridges.

From the protein sequences, Phl p 13 and Zea m 13 (PG1) are predicted as polygalacturonases. According to

the homology, this is characteristic for the grass group 13 allergens. Polygalacturonases have an important enzymatic role to lyse the cell walls of the stylus tissue so that the pollen tube can reach the ovule for fertilization.²⁷ In human subjects several carbohydrate-cleaving enzymes from microorganisms, such as α -amylases, xylanases, and polygalacturonases from *Aspergillus* species,^{28,29} are known as potent allergens. Namba et al³⁰ characterized the pollen allergen Cry j 2 from the Japanese cedar (*Cryptomeria japonica*), which causes pollinosis in Japan. This allergen is also predicted as a polygalacturonase and shows 34% sequence identity to Phl p 13. The polygalacturonase from *Aspergillus* species³¹ shows 26% sequence identity. Our studies demonstrate for the first time that such enzymes are also common allergens in the grasses. Thus these carbohydrate-degrading enzymes may represent a further kind of panallergen, not in the sense of cross-reactivity but in terms of function. Investigations are necessary to establish whether these allergens are functionally active enzymes. Furthermore, the substrate specificity has to be determined because in the case of Cry j 2, only methylesterified polygalacturonic acids serve as substrates.³² These studies will help to answer the question as to whether the physiologic function of an allergen contributes or even induces its allergenicity.

We thank Mrs Marisa Böttger for excellent technical assistance.

REFERENCES

- Freidhoff LR, Ehrlich-Kautzky E, Grant JH, Meyers DA, Marsh DG. A study of the human immune response to *Lolium perenne* (Rye) pollen and its components, Lol p I and Lol p II (Rye I and Rye II). *J Allergy Clin Immunol* 1986;78:1190-201.
- Stewart GA, Thompson PJ. The biochemistry of common aeroallergens. *Clin Exp Allergy* 1996;26:1020-44.
- Laffer S, Duchene M, Reimtz I, Susani M, Mannhalter C, Kraft D, et al. Common IgE-epitopes of recombinant Phl p I, the major timothy grass pollen allergen and natural group I grass pollen isoallergens. *Mol Immunol* 1996;33:417-26.
- Matthiesen F, Löwenstein H. Group V allergens in grass pollens. II. Investigation of group V allergens in pollen from 10 grasses. *Clin Exp Allergy* 1991;21:309-20.
- Petersen A, Bufe A, Schlaak M, Becker WM. Characterization on the major allergen group VI in timothy grass pollen (Phl p 6). I. Immunological and biochemical studies. *Int Arch Allergy Immunol* 1995;108:49-54.
- Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Valenta R. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin Exp Allergy* 1999;29:896-904.
- Suck R, Petersen A, Hagen S, Cromwell O, Becker WM, Fiebig H. Complementary DNA cloning and expression of a novel high molecular weight allergen Phl p 13 from timothy grass pollen (*Phleum pratense*). *Clin Exp Allergy* 2000;30:324-32.
- Suck R, Hagen S, Cromwell O, Fiebig H. The high molecular mass allergen fraction of timothy grass pollen (*Phleum pratense*) between 50-60 kDa is comprised of two major allergens: Phl p 4 and Phl p 13. *Clin Exp Allergy* 2000;30:1395-402.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
- Fahlbusch B, Müller WD, Rudeschko O, Jäger L, Cromwell O, Fiebig H. Detection and quantification of group 4 allergens in grass pollen extracts using monoclonal antibodies. *Clin Exp Allergy* 1998;28:799-807.
- Petersen A, Schramm G, Bufe A, Schlaak M, Becker WM. Structural investigations of the major allergen Phl p I on the cDNA and protein level. *J Allergy Clin Immunol* 1995;95:987-94.
- Kyhse-Andersen J. Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* 1984;10:203-9.
- Görg A, Postel W, Günther S. Two-dimensional electrophoresis: the current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 1988;9:531-46.
- Petersen A, Becker WM, Schlaak M. Characterization of isoforms of the major allergen Phl p V by two-dimensional immunoblotting and microsequencing. *Int Arch Allergy Immunol* 1992;98:105-9.
- Matsudaira P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* 1987;262:10035-8.
- Hancock K, Tsang VCW. India ink staining of proteins of nitrocellulose paper. *Anal Biochem* 1983;133:157-62.
- Leary JJ, Brigati DJ, Ward DC. Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridised to DNA or RNA immobilized on nitrocellulose: bio-blots. *Proc Natl Acad Sci U S A* 1983;80:4045-9.
- Suck R, Hagen S, Cromwell O, Fiebig H. Rapid and efficient purification of *Phleum pratense* major allergens Phl p 1 and group Phl p 2/3 using a two-step procedure. *J Immunol Methods* 1999;229:73-80.
- Niogret MF, Dubald M, Mandaron P, Mache R. Characterization of pollen polygalacturonase encoded by several cDNA clones in maize. *Plant Mol Biol* 1991;17:1155-64.
- Ekrasmoddullah AK, Kisil FT, Sehon AH. Immunochemical characterization of a high molecular weight basic allergen (HMBA) of rye grass (*Lolium perenne*) pollen. *Mol Immunol* 1983;20:465-73.
- Fischer S, Grote M, Fahlbusch B, Müller WD, Kraft D, Valenta R. Characterization of Phl p 4, a major timothy grass (*Phleum pratense*) pollen allergen. *J Allergy Clin Immunol* 1996;98:189-98.
- Burks AW, Shin D, Cockrell G, Stanley JS, Helm R, Bannon GA. Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicillin protein and a major allergen in peanut hypersensitivity. *Eur J Biochem* 1997;245:334-9.
- Bufe A, Gehlhar K, Schramm G, Schlaak M, Becker WM. Allergenic activity of a major grass pollen allergen is elevated in the presence of nasal secretion. *Am J Respir Crit Care Med* 1998;157:1269-76.
- Soldatova LN, Paupore EJ, Burk SH, Pastor RW, Slater JE. The stability of house dust mite allergens in glycerinated extracts. *J Allergy Clin Immunol* 2000;105:482-8.
- Matthiesen F, Löwenstein H. Group V allergens in grass pollen. I. Purification and characterization of group V allergen from *Phleum pratense* pollen, Phl p V. *Clin Exp Allergy* 1991;21:297-307.
- Petersen A, Schlaak M, Becker WM. Post-translational modifications of allergens and their influence on IgE reactivity. In: Oehling AK, Huerta Lopez JG, eds. Progress in allergy and clinical immunology. Vol. 4. Seattle: Hogrefe & Huber Publishers; 1997. p. 139-43.
- Hadfield KA, Bennett AB. Polygalacturonases: many genes in search of a function. *Plant Physiol* 1998;117:337-43.
- Sander I, Raulf-Heimsoth M, Siethoff C, Lohaus C, Meyer HE, Baur X. Allergy to *Aspergillus*-derived enzymes in the baking industry: identification of beta-xylosidase from *Aspergillus niger* as a new allergen (Asp n 14). *J Allergy Clin Immunol* 1998;102:256-64.
- Sen D, Wiley K, Williams JG. Occupational asthma in fruit salad processing. *Clin Exp Allergy* 1998;28:363-7.
- Namba M, Kurose M, Torigoe K, Hino K, Taniguchi Y, Fukuda S, et al. Molecular cloning of the second major allergen, Cry j II, from Japanese cedar pollen. *FEBS Lett* 1994;353:124-8.
- Bussink HJ, Kester HC, Visser J. Molecular cloning, nucleotide sequence and expression of the gene encoding prepropolygalacturonase II of *Aspergillus niger*. *FEBS Lett* 1990;273:127-30.
- Ohtsuki T, Taniguchi Y, Kohno K, Fukuda S, Usui M, Kurimoto M. Cry j 2, a major allergen of Japanese cedar pollen, shows polymethylgalacturonase activity. *Allergy* 1995;50:483-8.