

Purification, Structural and Immunological Characterization of a Timothy Grass (*Phleum pratense*) Pollen Allergen, Phl p 4, with Cross-Reactive Potential

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Almost 500 million people worldwide suffer from Type I allergy, a genetically determined immunodisorder which is based on the production of IgE antibodies against *per se* harmless antigens (allergens). Due to their worldwide distribution and heavy pollen production, grasses represent a major allergen source for approximately 40% of allergic patients. We purified Phl p 4, a major timothy grass (*Phleum pratense*) pollen allergen with a molecular mass of 61.3 kDa and a pI of 9.6 to homogeneity. Circular dichroism spectroscopical analysis indicates that Phl p 4 contains a mixed α -helical/ β -pleated secondary structure and, unlike many other allergens, showed no reversible unfolding after thermal denaturation. We show that Phl p 4 is a major allergen which reacts with IgE antibodies of 75% of grass pollen allergic patients (n=150) and induces basophil histamine release as well as immediate type skin reactions in sensitized individuals. Phl p 4-specific IgE from three patients as well as two rabbit-anti Phl p 4 antisera cross-reacted with allergens present in pollen of trees, grasses, weeds as well as plant-derived food. Rabbit antibodies raised against Phl p 4 also inhibited the binding of allergic patients IgE to Phl p 4. Phl p 4 may thus be used for diagnosis and treatment of sensitized allergic patients.

Key words: Allergy/Circular dichroism spectroscopy/Grass pollen allergen/IgE cross-reactivity/Phl p 4.

Introduction

Type I allergy, a genetically determined hypersensitivity disease, represents a major health problem. It affects more than 25% of the population in industrialized countries (Kay, 1997). The symptoms of type I allergy (allergic rhinitis, conjunctivitis, allergic asthma and anaphylactic shock) result from the recognition of *per se* harmless antigens (allergens) by specific IgE antibodies. Allergen exposure induces crosslinking of effector cell (mast cell, basophil)-bound IgE antibodies and the subsequent release of inflammatory mediators (e.g., histamine, leukotrienes) (Beaven, 1993).

Grass pollen belongs to the most potent and frequent allergen source due to the worldwide distribution and heavy pollen production of grasses. It has been estimated that at least 40% of allergic patients are sensitized against grass pollen allergens (Freidhoff *et al.*, 1986). Characterization and standardization of grass pollen allergens by immunochemical, proteinchemical, and, in part, by molecular cloning techniques resulted in the definition of allergen groups which occur as cross-reactive allergens in different grass species (King *et al.*, 1995; Valenta and Kraft, 1996).

Group 1 allergens with a molecular mass of 30 kDa represent targets for IgE antibodies in more than 95% of patients allergic to grass pollens (Johnson, 1965; Laffer *et al.*, 1994). Group 2/3 allergens of 10 to 12 kDa molecular mass are recognized by IgE antibodies of approximately 60% of patients allergic to grass pollen (Ansari *et al.*, 1989; Dolecek *et al.*, 1993). Seventy to 80% of grass pollen allergic patients react with group 5 allergens (Matthiesen, 1991; Vrtala *et al.*, 1993). While group 6 allergens with a molecular mass of 10 kDa are expressed only in few grass species (Matthiesen *et al.*, 1993; Petersen *et al.*, 1995; Vrtala *et al.*, 1999), group 7 allergens represent highly conserved calcium-binding proteins which occur as cross-reactive allergens in pollens of most higher plants (Suphioglu *et al.*, 1997; Smith *et al.*, 1997; Niederberger *et al.*, 1999; Valenta *et al.*, 1999). Group 11 grass pollen allergens show sequence homology with other pollen-specific proteins from trees and weeds (van Ree *et al.*, 1995). Profilins, which represent highly conserved eukaryotic actin-binding proteins constitute the group 12 allergens, the probably most cross-

reactive allergens known to date (Valenta et al., 1991, 1993, 1994).

While many of the grass pollen allergens are well described, relatively little is known about group 4 grass pollen allergens. Group 4 allergens were first described in *Lolium perenne* pollen and named high molecular weight basic allergens (HMBA) (Ekramoddoullah et al., 1983).

Further immunological studies demonstrated the presence of group 4 glycoprotein allergens in several grass species (e.g., ryegrass, Bermuda grass, timothy grass and orchard grass) (Jaggi et al., 1989; Su et al., 1991; Müller et al., 1993; Leduc-Brodard et al., 1996). Crossreactivity of a monoclonal anti-Phl p 4 antibody with antigens in weed pollens indicated that group 4-related aller-

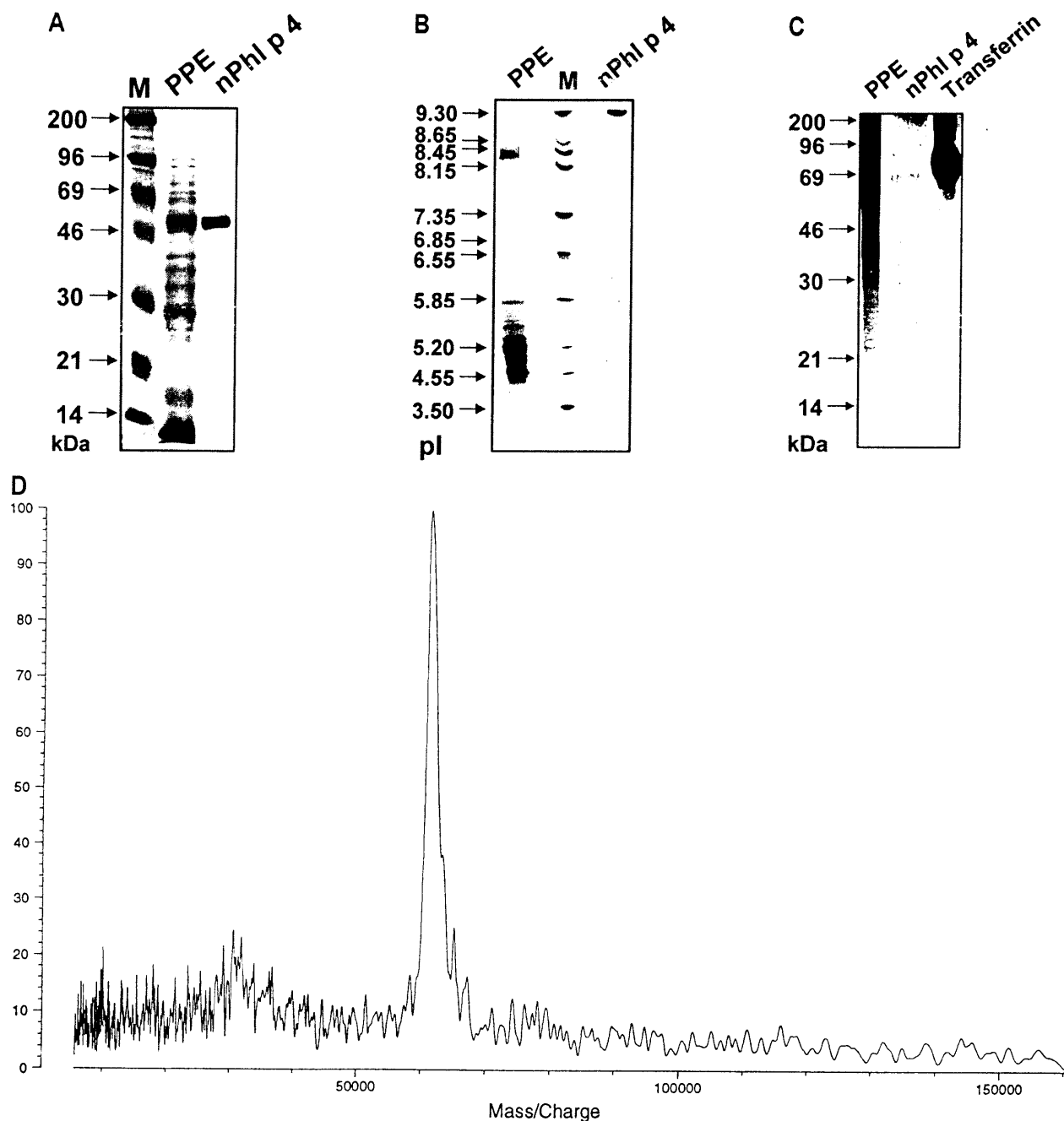


Fig. 1 Characterization of Purified nPhl p 4.

(A) SDS-PAGE analysis of purified natural Phl p 4. Coomassie Blue-stained SDS PAGE containing a molecular mass marker (M), timothy grass pollen (*Phleum pratense*) extract (PPE) and purified natural Phl p 4 (nPhl p 4). Molecular masses are displayed in kDa at the left margin. (B) IEF analysis of purified natural Phl p 4. Coomassie Blue-stained IEF gel containing a standard Iso Gel pI Marker (M), timothy grass pollen extract (PPE) and purified natural Phl p 4 (nPhl p 4). The pI values are indicated on the left margin. (C) Glycan detection. Nitrocellulose-blotted timothy grass pollen extract (PPE), purified natural Phl p 4 (nPhl p 4), and transferrin (Transferrin) were subjected to glycan detection. Molecular masses are displayed in kDa at the left margin. (D) Mass spectrometry analysis of purified nPhl p 4. The mass/charge is shown on the x-axis. The signal intensity is shown on the y-axis as percentage of the most intensive signal obtained in the investigated mass range.

gens may be even present in other higher plant species that are unrelated to grasses (Fischer *et al.*, 1996). Additional support for the importance of group 4 allergens came from immunoblotting studies indicating that the majority of grass pollen allergic patients contained IgE anti-group 4 antibodies (Laffer *et al.*, 1996a).

In the present study we report on the purification, structural and immunological characterization of natural Phl p 4. Folding, secondary structure contents and thermal stability of purified Phl p 4 were studied by circular dichroism (CD) spectroscopy. The isoelectric point of pure Phl p 4 was determined by isoelectric focusing. In addition, we raised rabbit antisera against purified Phl p 4 to investigate the presence of Phl p 4-related allergens in pollens of trees, grasses, weeds as well as in plant-derived food. Using purified Phl p 4 for IgE immunoblot inhibition experiments we characterized plant allergens containing cross-reactive IgE epitopes. The prevalence of IgE recognition of purified Phl p 4 was determined using 150 sera from timothy grass pollen allergic patients and we studied the influence of periodate treatment on IgE recognition of Phl p 4. The allergenic activity of purified Phl p 4 was investigated *in vitro* by basophil histamine release experiments and *in vivo* by skin prick testing.

The fact that Phl p 4 inhibited IgE binding to related allergens in plant-derived food and that a rabbit anti-Phl p 4 antiserum cross-reacted with antigens in tree, grass and weed pollens as well as in fruit and vegetables indicates that Phl p 4 represents a plant allergen with cross-reactive potential. We discuss the usefulness of purified Phl p 4 for the diagnosis and, perhaps, treatment of sensitized allergic patients.

Results

Purified Natural Phl p 4 Represents a Basic Protein with a Molecular Mass of 61.3 kDa

Purified natural Phl p 4 migrated in SDS-PAGE as a single band with an apparent molecular weight in the range of 55–60 kDa (Figure 1A: lane 'nPhl p 4'). Timothy grass pollen extract contained several prominent Coomassie Blue-stained bands in the molecular mass range of group 1/5 allergens (28–35 kDa), group 2/3/6/12 allergens (10–14 kDa) and group 4 allergens (50–60 kDa; Figure 1A: lane 'PPE'). A pI of 9.6 was estimated for purified Phl p 4 by IEF whereas most timothy grass pollen proteins represented acidic proteins (Figure 1B). Purified natural Phl p 4 exhibited a positive reaction in the glycan detection reaction which was however much less pronounced than that of the positive control (transferrin) and timothy grass pollen extract (Figure 1C). The molecular mass of purified Phl p 4 was determined by mass spectroscopy to be 61.3 kDa (Figure 1D). The amino acid composition determined for Phl p 4 is displayed in Table 1. It shows that aspartic acid (10.2%), glycine (8.9%), alanine (8.7%) and valine (8.0%) were the most frequently occurring amino acids.

Table 1 Amino Acid Composition of Purified nPhl p 4.

Amino acid	nPhl p 4
Aspartic acid	10.2%
Threonine	4.5%
Serine	5.8%
Glutamic acid	7.6%
Proline	7.9%
Glycine	8.9%
Alanine	8.7%
Valine	8.0%
Methionine	2.1%
Isoleucine	5.7%
Leucine	7.5%
Tyrosine	5.2%
Phenylalanine	4.8%
Histidine	2.1%
Lysine	6.8%
Tryptophan	0.0%
Arginine	4.2%

Phl p 4 Consists of Mixed α -Helical/ β -Pleated Secondary Structure and Aggregates Irreversibly after Thermal Denaturation

The far-ultraviolet circular dichroism spectrum of purified Phl p 4 indicates that the protein contains a mixed α -helical/ β -pleated secondary structure (Figure 2A). The spectrum recorded at 20°C is characterized by two broad minima at 208 nm and 216 nm (Figure 2A). Thermal unfolding of Phl p 4 was monitored as change in observed ellipticity (θ) at 208 nm (Figure 2B). The unfolding transition of Phl p 4 appears to be monophasic and highly cooperative with a melting point of approximately 52°C. When heated above 70°C the protein aggregates as evident from the far UV CD spectrum recorded at 80°C (Figure 2B) and at 20°C after cooling from 80°C (Figure 2B). The far-UV spectrum recorded at 80°C presents features of a protein aggregate (broad minimum in the range between 200–230 nm) which is retained in the spectrum recorded at 20°C after cooling from 80°C. In addition, the cooling from 80°C monitored at 208 nm yielded a flat curve indicative of unchanged spectral features of aggregated Phl p 4 (Figure 2B). The analysis of a Phl p 4 sample at 4 μ M instead of 10 μ M gave similar results and thus argues against a concentration dependence of the aggregation (data not shown).

Phl p 4 Represents a Major Timothy Grass Pollen Allergen Containing Periodate-Sensitive IgE Epitopes

The prevalence of IgE recognition of Phl p 4 was determined using sera from 150 timothy grass pollen allergic patients. Immunocaps were prepared from purified natural Phl p 4 and recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11, rPhl p 12) to allow quantitative CAP RAST IgE anti-

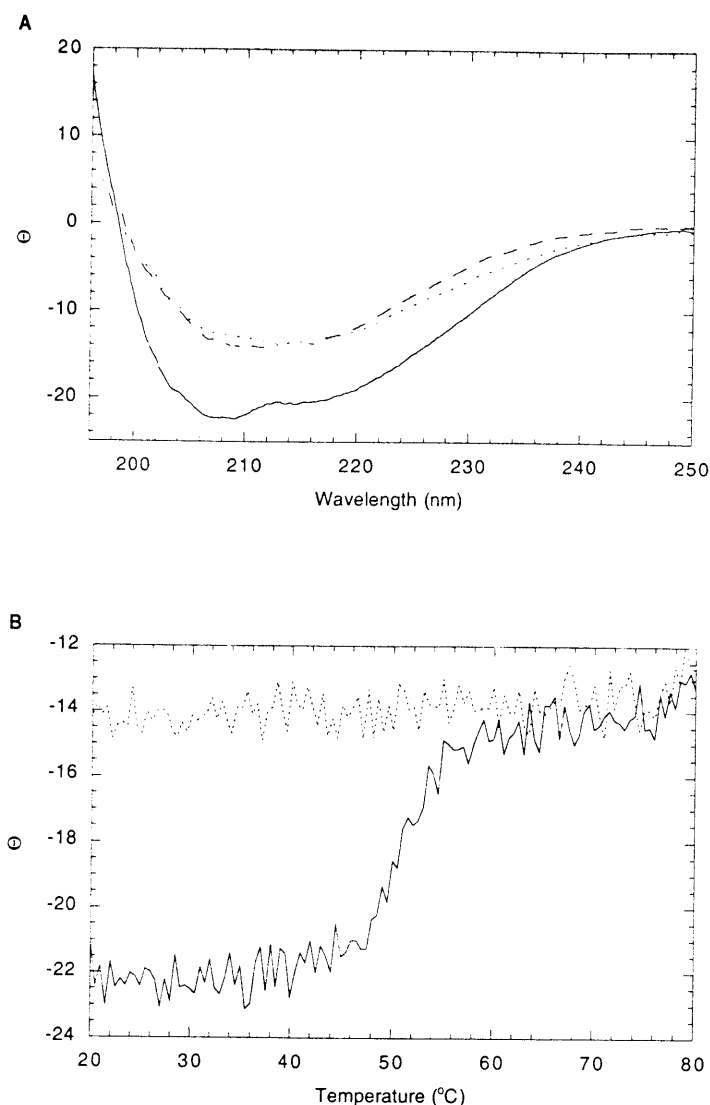


Fig. 2 Far-Ultraviolet Circular Dichroism Analysis of Purified Natural Phl p 4.

(A) Far-UV CD spectrum of purified Phl p 4 at 20°C (continuous line), 80°C (dotted line) and 20°C after cooling from 80°C (dashed line). Spectra are expressed as observed ellipticity (Θ , y-axis) over a given range of wavelengths (x-axis).

(B) Thermal unfolding of Phl p 4 followed by circular dichroism. The observed ellipticity (Θ , y-axis) was monitored at 208 nm during heating (solid line) from 20°C to 80°C (x-axis) and cooling from 80°C (dotted line).

body measurements. We found that 73% of the sera contained Phl p 4-specific IgE antibodies. Phl p 4 and rPhl p 1 (87% reactive sera) belonged to the most frequently recognized timothy grass pollen allergens. The frequency of IgE recognition of the other allergens was as follows: rPhl p 2: 47%; rPhl p 5: 67%; rPhl p 6: 57%; rPhl p 7: 11%; rPhl p 11: 27% and rPhl p 12: 21%.

Previous data and glycan detection provided evidence that Phl p 4 may contain carbohydrates (Fischer *et al.*, 1996; Figure 1C). To investigate whether IgE binding to Phl p 4 may be dependent on glycosylation, ELISA plate-bound Phl p 4 was subjected to periodate treatment prior to incubation with patients IgE antibodies (Figure 3). Periodate treatment led to a significant reduction of IgE binding to Phl p 4 in the range of 20%–86%. Serum 1 exhibited a 86% reduction of IgE reactivity to periodate-

treated Phl p 4, whereas sera 4, 5 and sera 2, 3 showed a 50% and 20% reduction of IgE reactivity, respectively. Serum 6 from a non-allergic individual did not display significant IgE reactivity to Phl p 4 (Figure 3). IgE binding to purified non-glycosylated *E. coli*-expressed birch pollen allergens (rBet v 1, rBet v 2) was not affected by periodate treatment when tested under identical conditions as Phl p 4 (data not shown).

Phl p 4 Is a Plant Allergen with Cross-Reactive Potential

Cross-reactivity of Phl p 4 with allergens from other plant species was studied in two types of experiments. First, we investigated whether purified Phl p 4 can inhibit IgE binding of allergic patients to cross-reactive allergens

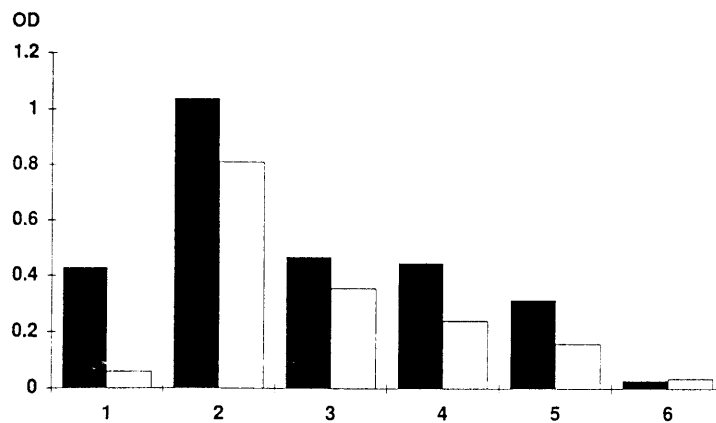


Fig. 3 Influence of Periodate Treatment on IgE Recognition of Natural Phl p 4. Magnitude of IgE reactivity (y-axis: optical density) of sera from 5 grass pollen allergic patients (1–5) and a non-atopic person (6) to ELISA plate-coated Phl p 4 after (white bars) or without (black bars) periodate treatment.

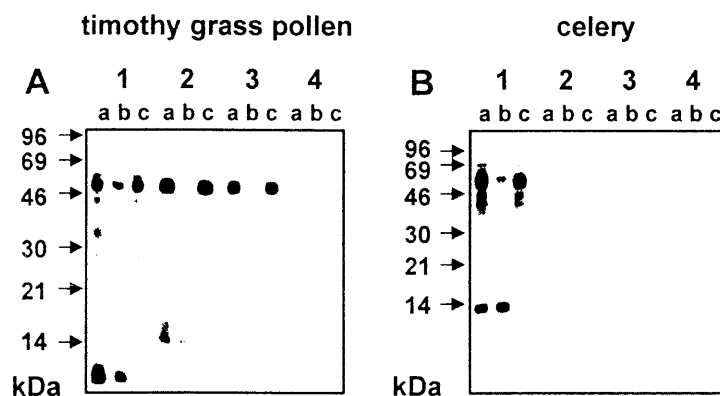


Fig. 4 Purified Natural Phl p 4 Inhibits IgE Binding to Nitrocellulose-Blotted Timothy Grass Pollen (A) and Celery Tuber (B) Extract. Serum samples from a patient with combined grass pollen and celery allergy (panels 1), from two patients with grass pollen allergy (panels 2, 3) and a non-atopic person (panels 4) were preincubated with BSA (lanes a), Phl p 4 (lanes b) or rBet v 2 (lanes c) and exposed to nitrocellulose-blotted timothy grass pollen and celery tuber extract. Bound IgE antibodies were detected with [^{125} I]-labeled anti-human IgE antibodies and visualized by autoradiography. Molecular masses are indicated in kDa at the left margins.

from other plants (Figure 4), and second, we tested if rabbit antibodies raised against purified Phl p 4 cross-react with allergens from other plants (Figure 5A–D).

The IgE inhibition experiments are exemplified for timothy grass pollen and celery extracts using representative Phl p 4-reactive sera (*i.e.*, serum from one patient with combined grass pollen and plant food allergy (Figure 4, panels 1) and sera from two patients with grass pollen but no plant food allergy (Figure 4, panels 2–3). Preincubation of the three sera with Phl p 4 (lanes b) led to a significant reduction (panel 1) or complete (panels 2, 3) abolishment of IgE binding to a band of approximately 55–60 kDa in timothy grass pollen extract (Figure 4A). Serum derived from a patient with combined pollen plant food allergy (Figure 4B, panels 1) displayed IgE reactivity to bands between 40–69 kDa and at 13 kDa in celery extract (Figure 4B, lane a). Preincubation of this serum with Phl p 4 strongly inhibit-

ed IgE binding to the 40–69 kDa components and preadsorption with birch profilin abolished IgE binding to the 13 kDa band (Figure 4A,B; lane c). Serum from a non-atopic individual (Figure 4A,B; panels 4) did not display IgE reactivity to timothy grass pollen or celery extract. These results demonstrate that Phl p 4 contains cross-reactive epitopes as well as grass pollen-specific epitopes. Similar observations (*i.e.* cross-reactive and species-specific recognition) were made when sera from 25 Phl p 4-reactive patients were analyzed (data not shown).

In the second type of cross-reactivity experiments we used two rabbit antisera which were raised against purified Phl p 4 to test for cross-reactivity with various pollen and plant food extracts. Figure 5A demonstrates the specificity of the anti-Phl p 4 antisera which reacted exclusively with Phl p 4 but not with other important timothy grass pollen (Phl p 1, Phl p 2, Phl p 5, Phl p 6, Phl p 13) or cross-reactive

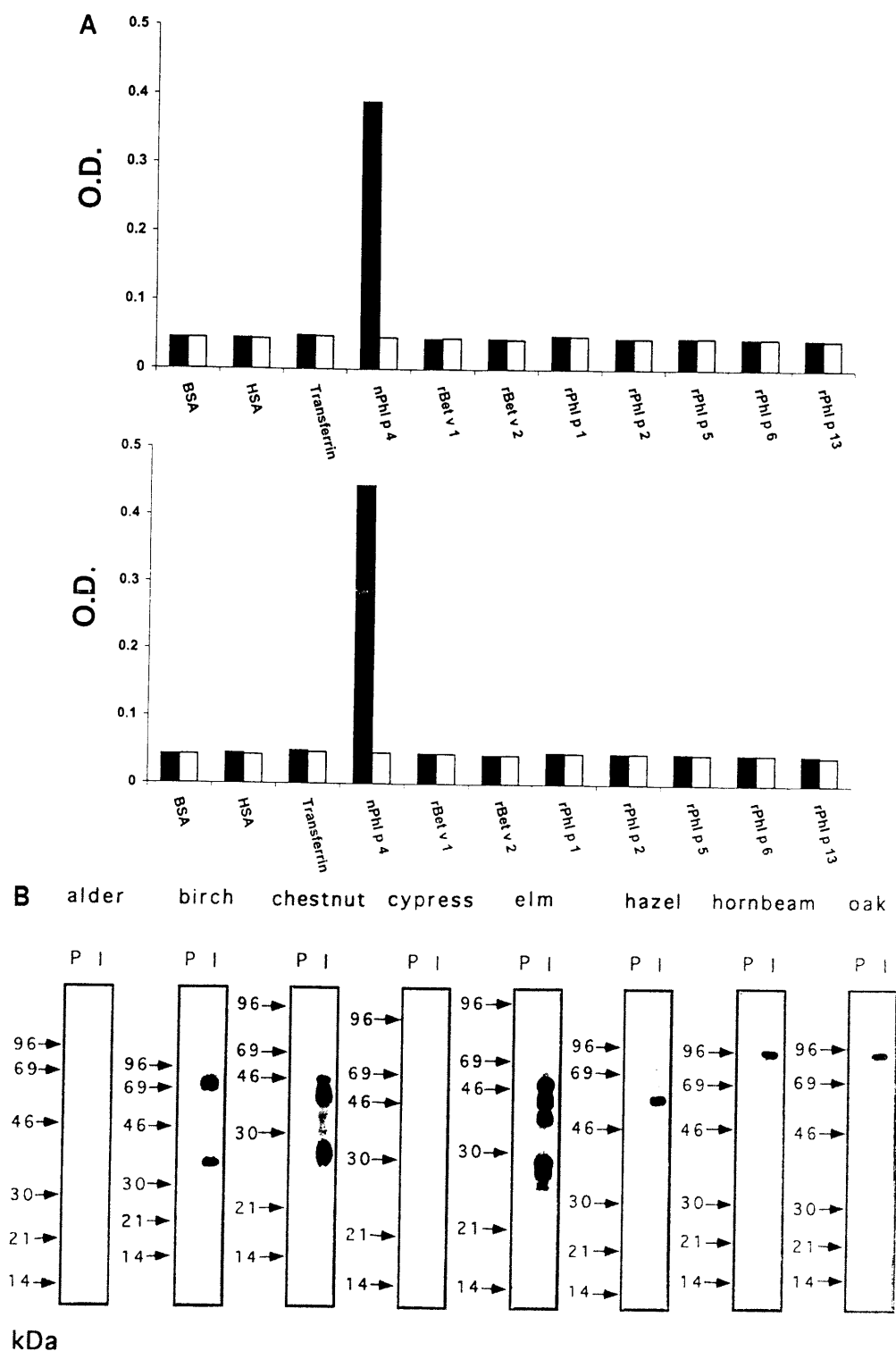


Fig. 5 Detection of Phl p 4-Cross-Reactive Epitopes in Plant Pollen and Food.

(A) Specificity of rabbit anti-nPhl p 4 antisera. ELISA plate-coated allergens from timothy grass pollen (nPhl p 4, rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6, rPhl p 13), birch pollen (rBet v 1, rBet v 2: birch profilin) as well as control proteins (BSA: bovine serum albumin, HSA: human serum albumin, transferrin) were exposed to the rabbit anti-nPhl p 4 antisera (black bars) and the corresponding preimmune sera (white bars). The upper part of the figure displays results obtained for the rabbit antiserum used in this study and the lower part shows the results for a second anti-Phl p 4 antiserum from another rabbit. The OD (optical density) values displayed at the y-axis correspond to the amount of bound antibodies.

(B-D) Cross-reactivity of a rabbit anti-nPhl p 4 antiserum with antigens in plant pollen and plant-derived food. A rabbit antiserum raised against purified nPhl p 4 (lanes I) and the corresponding preimmune serum (lanes P) was incubated with nitrocellulose-blotted pollen extracts (panel B: alder, birch, chestnut, cypress, elm, hazel, hornbeam, oak; panel C: olive, plane, poplar, timothy grass, mugwort, ragweed) and plant food extracts (panel D: apple, carrot, celery, hazelnut, kiwi, peach). Bound rabbit antibodies were detected with an [^{125} I]-labeled donkey anti-rabbit Ig antiserum and visualized by autoradiography. Molecular masses are indicated on the left margins.

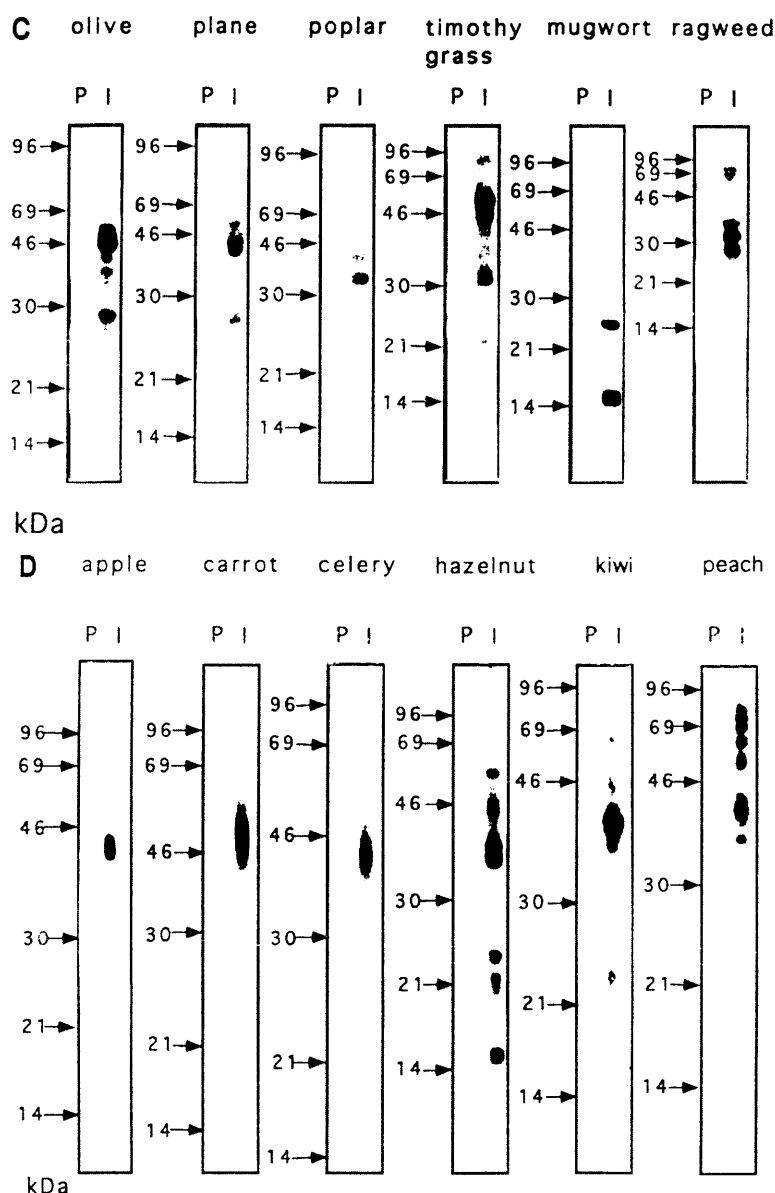


Fig. 5 (continued)

birch pollen (Bet v 1, Bet v 2: birch profilin) allergens (Figure 5A, black bars). Neither of the two Phl p 4-specific antisera reacted with bovine or human serum albumin or with a glycosylated control protein (transferrin). In this context it is noteworthy that although Phl p 4 and Phl p 13 represent important timothy grass pollen allergens with almost identical molecular weight (Suck *et al.*, 2000b), the purification protocol developed in our study delivered pure Phl p 4 which did not give rise to antibodies against Phl p 13. No significant reactivity of the rabbits preimmune serum with any of the allergens was noted (Figure 5A, white bars).

As exemplified for one of the two rabbit anti-Phl p 4 antisera, we found that the immune sera (lanes I) but not the preimmune sera (lanes P) cross-reacted with several bands in nitrocellulose-blotted pollen extracts from unrelated trees (alder, birch, chestnut, cypress, elm, hazel, hornbeam, oak, olive, plane, poplar), weeds (mugwort, ragweed) as well as plant-derived food (apple, carrot,

celery, hazelnut, kiwi, peach) (Figure 5B-D). Although raised against the pure 61.3 kDa Phl p 4 preparation, we noted that the rabbit antiserum reacted with several bands between 21–96 kDa in timothy grass pollen extract and cross-reacted with components between 14–96 kDa in some of the plant extracts investigated (Figure 5B-D). These results confirm that Phl p 4-homologous proteins/allergens are expressed in mature pollen of a variety of botanically unrelated higher plants, including monocotyledonic and dicotyledonic species, as well as in some plant-derived foods, mainly fruits.

Rabbit Anti-Phl p 4 Antibodies Specifically Inhibit the Binding of Allergic Patients' IgE to Phl p 4

Whether rabbit anti-Phl p 4 antibodies bind to or close to epitopes recognized by IgE antibodies of allergic patients was studied by IgE immunoblot inhibition experiments. Ni-

trocellulose-blotted Phl p 4 and, for control purposes, an immunologically unrelated grass pollen allergen, the major timothy grass pollen allergen, Phl p 2, were preincubated with rabbit anti-Phl p 4 antibodies and, for control purposes, with rabbit preimmune antibodies. Subsequently, blotted allergens were exposed to patients IgE antibodies. We found that rabbit anti-Phl p 4 (Figure 6, lanes I) but not the preimmune antibodies (Figure 6, lanes P) inhibited IgE binding of three out of the four patients (Figure 6, 1–3) to Phl p 4 but not to the unrelated allergen, Phl p 2. IgE binding of patient 4 to Phl p 4 was not significantly inhibited by the

rabbit anti-Phl p 4 antibodies. Serum from a non-allergic patient (Figure 6, panel 5; negative control) did not exhibit IgE reactivity to any of the two blotted allergens.

Phl p 4 Induces Dose-Dependent Histamine Release and Immediate Type Skin Reactions in Sensitized Allergic Patients

The allergenic activity of Phl p 4-specific IgE antibodies was investigated by basophil histamine release as well as skin test experiments (Figure 7A, B, C; Table 2). Incubation

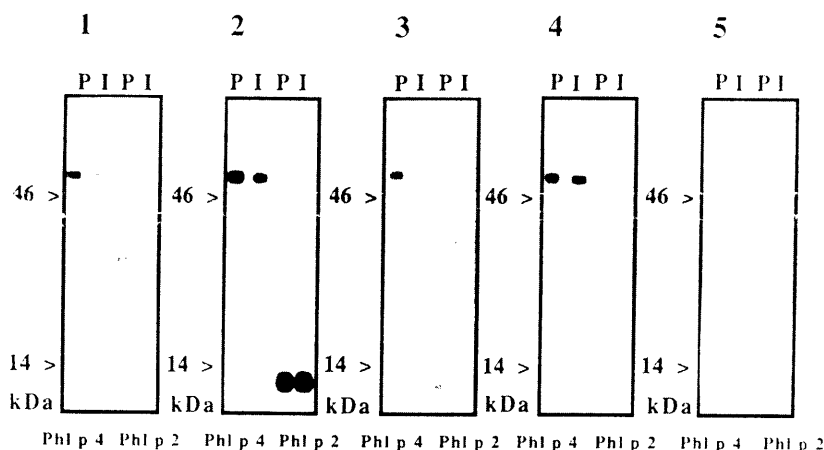


Fig. 6 A Rabbit Anti-Phl p 4 Antiserum Inhibits Allergic Patients IgE Binding to Phl p 4.

Nitrocellulose strips containing nPhl p 4 and rPhl p 2 were preincubated either with the preimmune serum (P) or the immune serum (I) of a Phl p 4-immunized rabbit. Strips were then exposed to sera from grass pollen allergic patients (1–4) or to serum from a non-atopic person (5).

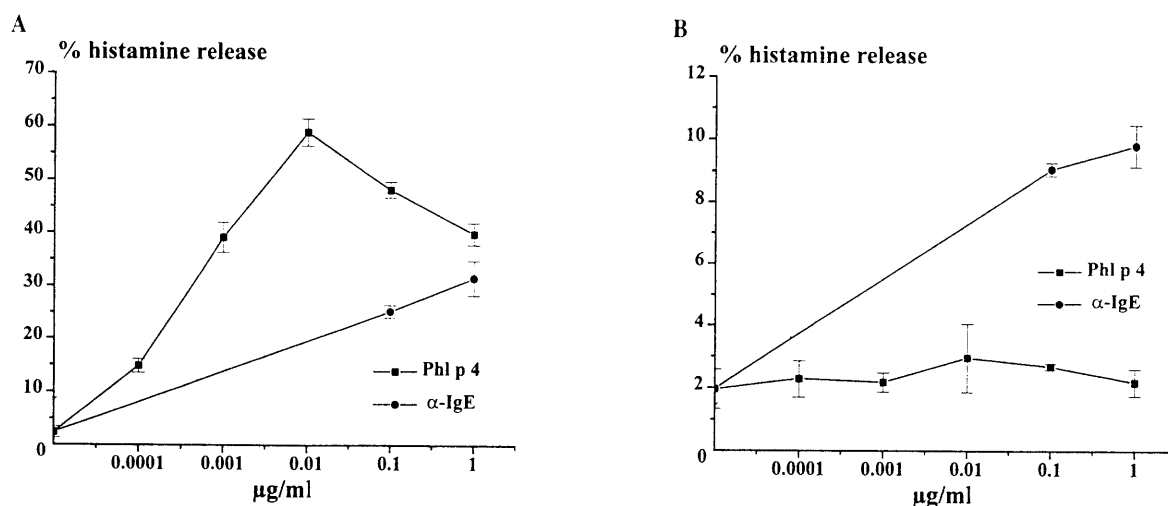


Fig. 7 Biological Activity of nPhl p 4.

(A, B) Purified natural Phl p 4 induces specific basophil histamine release. Granulocytes from a grass pollen allergic patient containing Phl p 4-specific IgE (A) and from a non-atopic individual (B) were incubated with various concentrations of purified natural Phl p 4 (■) or a monoclonal anti-human IgE antibody (●) (x-axis). The percentage of histamine release is displayed on the y-axis. Results represent mean values of triplicate determinations.

(C) Purified nPhl p 4 induces immediate type skin reactions in sensitized patients. Two grass pollen allergic patients containing Phl p 4-specific IgE antibodies (A, B) and a non-atopic individual (C) were skin prick tested with various concentrations of purified nPhl p 4, with timothy grass pollen extract, sodium chloride (NaCl) and histamine (Hist). The wheal areas were surrounded with a ball point pen.

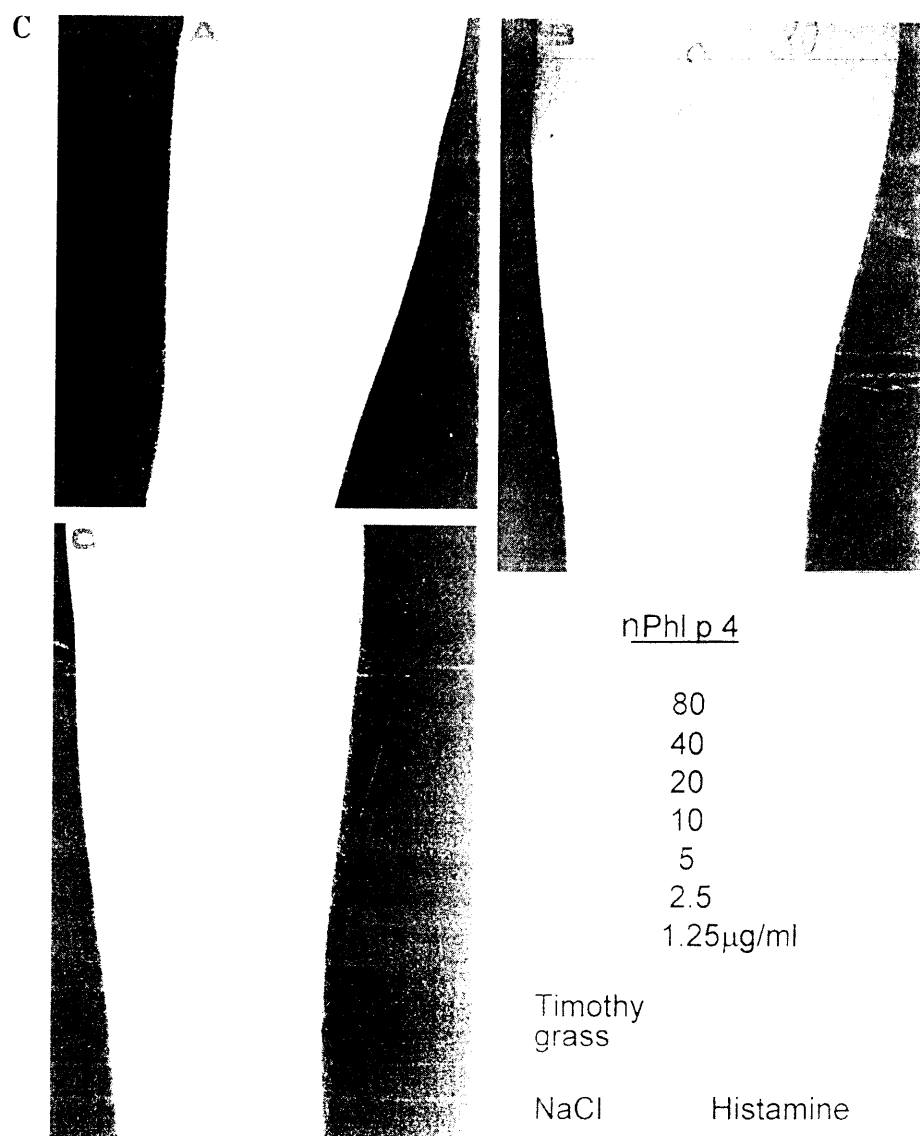


Fig. 7 (continued)

Table 2 Induction of Immediate Type Skin Reactions by Purified nPhl p 4.

Individual	Mean wheal diameter (mm)									
	nPhl p 4 concentration							Timothy	NaCl	Histamine
	80 $\mu\text{g}/\mu\text{l}$	40 $\mu\text{g}/\mu\text{l}$	20 $\mu\text{g}/\mu\text{l}$	10 $\mu\text{g}/\mu\text{l}$	5 $\mu\text{g}/\mu\text{l}$	2.5 $\mu\text{g}/\mu\text{l}$	1.25 $\mu\text{g}/\mu\text{l}$			
1	5.0	5.0	5.0	4.5	2.0	0	0	7.0	0	5.0
2	8.5	7.5	5.5	4.5	2.5	0	0	12.5	0	5.5
3	0	0	0	0	0	0	0	0	0	5.5

of granulocytes from a grass pollen allergic patient containing Phl p 4-specific IgE antibodies with Phl p 4 induced dose-dependent histamine release starting at 10^{-4} $\mu\text{g}/\text{ml}$ (Figure 7A). No histamine release was observed when granulocytes from a non-atopic individual were exposed to Phl p 4 (Figure 7B). Anti-human IgE antibodies induced basophil histamine release from basophils of the atopic as well as non-atopic individual (Figure 7A, B).

Next we performed skin test experiments in two additional patients containing Phl p 4-specific IgE antibodies and in another non-atopic person (Figure 7C; Table 2). In both Phl p 4-sensitized allergic patients, purified Phl p 4 elicited significant immediate skin reactions already at a concentration of 5 $\mu\text{g}/\text{ml}$. No immediate type skin reactions were induced by Phl p 4 in the non-atopic person up to a concentration of 80 $\mu\text{g}/\text{ml}$. Timothy grass pollen ex-

tract induced skin reactions in the two grass pollen allergic patients but not in the non-atopic person. Histamine induced immediate skin reactions in all three individuals tested (Figure 7C, Table 2).

Discussion

We presented here the purification and characterization of a major timothy grass pollen allergen, Phl p 4, with cross-reactive potential. Using conventional chromatography we were able to purify milligram amounts of Phl p 4 from timothy grass pollen extracts. The purification procedure developed allowed to obtain pure Phl p 4 without Phl p 13, a timothy grass pollen allergen with a molecular weight similar to Phl p 4 (Suck *et al.*, 2000b). Purified Phl p 4 represents a 61.3 kDa glycoprotein with a characteristic basic pI of approximately 9.6. The basic pI represents one of the characteristic features of group 4 allergens which have been detected in many grass species (Ekramoddoullah *et al.*, 1983; Su *et al.*, 1991; Leduc-Brodard *et al.*, 1996). Purified Phl p 4 was recognized by the majority of grass pollen allergic patients and therefore represents a major grass pollen allergen. We previously noted that Phl p 4 may contain carbohydrates and attributed the proteins remarkable resistance against proteolytic digestion to a protective role of the protein-bound carbohydrate moieties (Fischer *et al.*, 1996). Here we showed that Phl p 4 yields a weak positivity in the glycan detection reaction and that periodate treatment reduces the IgE binding capacity of Phl p 4, suggesting that carbohydrates either play a role regarding the correct assembly of peptide IgE epitopes or, as has been proposed by other authors, themselves may represent target structures for IgE antibodies (Mari *et al.*, 1999). In this context it is noteworthy that the purified 61.3 kDa Phl p 4 allergen inhibited IgE binding to several bands ranging between 25–60 kDa in timothy grass pollen extract and in the range of 40–69 kDa in celery extract. We also found that rabbit anti-Phl p 4 antisera cross-reacted with several bands in pollen and plant food extracts. There are several explanations for these observations: first, it is possible that glycosylation or other posttranslational modifications may influence the electrophoretic mobility of Phl p 4. The immunoreactive bands that were smaller than the purified Phl p 4 molecule (61.3 kDa) may represent degradation products or protein subunits. Alternatively, it is possible that the Phl p 4 molecule contains cross-reactive carbohydrate epitopes which inhibit IgE binding to other glycoproteins and can give rise to rabbit antibodies which cross-react with carbohydrate moieties on other glycoproteins.

Regardless whether the peptide backbone or the attached carbohydrate moieties of Phl p 4 represent the epitopes recognized by the cross-reactive IgE and rabbit antibodies, our data indicate that purified Phl p 4 contains cross-reactive epitopes. This assumption is supported by two types of experiments: first, we found that purified Phl p 4 could inhibit IgE binding to related aller-

gens in other plant species as exemplified for a celery allergen of comparable molecular weight. Second, we demonstrate that rabbit anti-Phl p 4 antisera which inhibited binding of allergic patients IgE to Phl p 4, also cross-reacted with antigens in pollen extracts from a wide range of botanically unrelated plants (trees, weeds) as well as with antigens from a variety of plant-derived food (vegetables, fruits). Our contention that the cross-reactivity of the rabbit anti-Phl p 4 antiserum used for all experiments in this study is primarily due to Phl p 4-derived epitopes is supported by our finding that the antiserum did not react with other highly immunogenic and/or abundant timothy grass pollen allergens (e.g., Phl p 1, Phl p 2, Phl p 5, Phl p 6, Phl p 13), highly cross-reactive birch pollen allergens (Bet v 1, Bet v 2) or other control proteins (BSA, HSA, transferrin) (Figure 5A, upper part). A second, independently raised anti-Phl p 4 antiserum exhibited a similar reactivity profile (Figure 5A, lower part).

Although we found that Phl p 4 contains also non-cross-reactive epitopes our data suggest that it may play a role as a cross-reactive allergen in the pathogenesis of the oral allergy syndrome, a complex of symptoms denoting the simultaneous occurrence of food allergy in patients suffering from pollen allergy (Ortolani *et al.*, 1988). In this context it is of note that in contrast to other cross-reactive plant allergens (e.g., Bet v 1, Bet v 2) which refold after thermal denaturation, Phl p 4 remained aggregated (Ebner *et al.*, 1995; Laffer *et al.*, 1996b; Mittermann *et al.*, 1998). Assuming that Phl p 4-homologous food allergens behave in a similar way it is possible that these molecules belong to those thermolabile food allergens which reportedly lose IgE reactivity after extensive cooking (Vieths *et al.*, 1997). So far two cross-reactive pollen allergens could be identified as elicitors of the oral allergy syndrome: they comprise the major birch pollen allergen, Bet v 1, a possible pathogenesis-related protein which reportedly exhibits RNase activity (Breiteneder *et al.*, 1989; Bufe *et al.*, 1996) and plant profilins, representing ubiquitous actin-binding proteins (Valenta *et al.*, 1991, 1999). Due to their conserved biological function both allergens can occur simultaneously in pollen and somatic plant tissues and thus can be responsible for allergic reactions in pollen allergic individuals when they ingest plant-derived food (Valenta *et al.*, 1996). Similar as described for Bet v 1 and profilin-mediated cross-reactivities we found that Phl p 4 contained more IgE epitopes than the homologous plant food proteins: Not all Phl p 4-reactive sera cross-reacted with plant-derived food (e.g. celery) indicating that these patients were primarily sensitized against grass pollens. The fact that Phl p 4 contains cross-reactive as well as monocot-specific epitopes may also be the reason why certain monoclonal antibodies raised against grass group 4 allergens cross-reacted only with monocot group 4 allergens but not with tree- or weed pollen-derived homologues (Leduc-Brodard *et al.*, 1996) whereas other group 4-specific antibodies cross-reacted with homologous allergens in weed pollens (e.g. ragweed; Fischer *et al.*, 1996).

Phl p 4 is clearly different from a recently characterized timothy grass pollen allergen, Phl p 13, which exhibits a similar molecular weight in SDS-PAGE (Suck *et al.*, 2000a) for several reasons: first, unlike Phl p 13 (pI 6–7.5), it exhibits a basic pI of approximately 9.6 (Petersen *et al.*, 2001). Second, the amino acid composition determined for Phl p 4 is different from that of Phl p 13 and third, both rabbit anti-Phl p 4 antisera did not cross-react with Phl p 13 (Figure 5A).

Our contention that purified Phl p 4 may be used for allergy diagnosis is supported by the fact that it was possible to prepare sufficient amounts of Phl p 4 to prepare immunocap tests for *in vitro* allergy diagnosis. Furthermore, Phl p 4 bound specifically IgE antibodies in other IgE assays, elicited specific and dose-dependent histamine release as well as immediate type skin reactions in sensitized patients. Phl p 4 may thus be used for component-resolved *in vitro* as well as *in vivo* diagnosis of grass pollen allergy (Valenta *et al.*, 1999). IgE competition experiments with purified Phl p 4 may allow to identify patients who suffer from combined grass pollen-food allergy due to cross-sensitization to Phl p 4-related allergens. Precise diagnosis of sensitization to Phl p 4 may then represent the basis for patient-tailored specific immunotherapy with the purified allergen.

Materials and Methods

Characterization of Allergic Patients, Antibodies, Allergen Extracts

Patients allergic to grass pollen were characterized by case history indicative of seasonal grass pollinosis, determination of serum IgE antibodies specific for timothy grass pollen extract (RAST, Pharmacia) and recombinant timothy grass pollen allergens as well as by skin prick testing (Niederberger *et al.*, 1998). Antisera against Phl p 4 were produced by immunization of two rabbits with the purified Phl p 4 protein. A total of four subcutaneous and intramuscular injections were administered over a period of 13 weeks, the first comprising 300 µg of protein in Freund's complete adjuvant and the remaining with 100 µg of protein in Freund's incomplete adjuvant. The two rabbits used were selected from a larger number based on the absence of detectable antibody reactivity to grass pollen prior to immunization. Immunizations were carried out in Sweden under an ethical review board approval. The specificity of the rabbit anti-Phl p 4 antisera was confirmed by demonstrating lack of reactivity to purified timothy grass pollen, birch pollen allergens and other control proteins (BSA, HSA, transferrin) by ELISA. nPhl p 4, recombinant Phl p 1, rPhl p 2, rPhl p 5, rPhl p 6 (Vrtala *et al.*, 1996, 1999) and rPhl p 13 (Swoboda and Valenta, unpublished) from timothy grass pollen as well as rBet v 1 and rBet v 2 from birch pollen (BIOMAY, Vienna, Austria) were coated to ELISA plates (Nunc, Roskilde, Denmark) in PBS at a concentration of 1 µg/ml overnight at 4 °C. Plates were washed and blocked as described (Vrtala *et al.*, 1996) and exposed to the rabbit anti-Phl p 4 antiserum and, for control purposes, to the rabbits preimmune serum, both diluted 1:100 in TBST, 0.5% (w/v) BSA overnight at 4 °C. After washing, bound rabbit antibodies were detected with an alkaline phosphatase-conjugated goat anti-rabbit Ig antiserum (Dianova, Hamburg, Germany) diluted 1:1000 in TBST, 0.5% (w/v) BSA. After washing, the colour reaction was started

with alkaline phosphatase substrate (Sigma, St. Louis, USA) and the optical densities corresponding to the amount of bound antibodies were determined in an ELISA reader (Dynatech, Denkendorf, Germany). Results were measured in duplicates and are expressed as means with less than 5% error.

Pure pollens from alder (*Alnus glutinosa*), birch (*Betula verrucosa*), chestnut (*Aesculus hippocastaneum*), cypress (*Cypripedium sempervirens*), elm (*Ulmus campestris*), hazel (*Corylus avellana*), hornbeam (*Carpinus betulus*), oak (*Quercus robur*), olea (*Olea europaea*), plane (*Platanus acerifolia*), poplar (*Populus alba*), timothy grass (*Phleum pratense*), mugwort (*Artemisia vulgaris*) and ragweed (*Ambrosia elatior*) were obtained from Allergon AB (Välinge, Sweden) and stored at 4 °C until use. Fresh apples, carrots, celery, hazelnuts, kiwis and peach were bought from the local market. Aqueous extracts from pollens were prepared by homogenizing pollens in water containing 5 mM PMSF using an ultraturrax (IKA, Stauffen, Germany) for two minutes. The extracts were centrifuged at 5000 g in an SS34 rotor (Sorvall, DuPont) for 30 minutes at 4 °C (Sorvall, RC5B) to remove insoluble particles. The protein contents in the supernatants were determined using a BCA kit (Pierce, Rockford, IL) and the extracts were stored at –20 °C until use. Plant food extracts were prepared by homogenizing 8.4 grams fresh material in 20 ml SDS-PAGE sample buffer (Laemmli, 1970) using an ultraturrax. The homogenates were extracted by boiling for 10 minutes and insoluble particles were removed by centrifugation as described for the aqueous pollen extracts. Protein contents and quality of extracts were analyzed by SDS-PAGE and Coomassie Blue staining (Bradford, 1976; Fling, 1986).

Purification of Phl p 4

Natural Phl p 4 was purified from aqueous timothy grass pollen extract using a combination of ion exchange chromatography and size exclusion chromatography. Two hundred g of timothy grass pollen (Allergon AB) was suspended in 2 liters of distilled water and agitated 2 hours at room temperature. Coarse debris was removed by 10 min centrifugation at 2000 g at 4 °C and the supernatant carefully decanted into fresh centrifuge bottles. The extract was then clarified by centrifugation for 15 min at 35 000 g at 4 °C, followed by passage of the supernatant through an MF-Millipore Type RA 1.2 µm filter. Purification of Phl p 4 was performed using three chromatographic steps. First, the clear extract was loaded onto a XK50/20 column (143 ml bed volume) containing SP Sepharose C-50 (Pharmacia Biotech, Uppsala, Sweden) at a rate of 5 ml/min. The column was washed with 500 ml 50 mM NaPO₄, pH 7.0. The elution was done with a linear gradient of 0–0.5 M NaCl in 50 mM NaPO₄, pH 7.0, over two column volumes. Phl p 4 was found to elute in a peak centered at 180 mM NaCl in the gradient. Fractions which according to SDS-PAGE analysis contained Phl p 4 were pooled and the volume was reduced to 1/4 using an Amicon cell (PM-30 membrane). Size exclusion chromatography was performed using Superdex 75 µg in XK50/100 column (1760 ml bed volume) (Pharmacia Biotech). The column was equilibrated with 0.15 M NaCl and the concentrated pool was loaded at 10 ml/min. Elution was carried out with 0.15 M NaCl at the same flow rate. Two major elution peaks resulted and analysis by SDS-PAGE revealed that Phl p 4 was present at the slower side of the high molecular weight peak. Fractions containing Phl p 4 were pooled and concentrated 10 fold using an Amicon cell (PM-30 membrane). In order to remove last impurities, the size exclusion chromatography step was repeated identically.

Laser desorption mass spectra of Phl p 4 were acquired in a linear mode with a time-of-flight Compact MALDI II instrument (Kratos, Manchester, UK), operating at 20 kV acceleration volt-

age and equipped with a nitrogen UV laser (337 nm, pulse duration 3 ns) (PiCHEM, Graz, Austria).

The amino acid composition of Phl p 4 was determined with a Beckman Analyzer (Beckman Instruments, Inc., Fullerton, USA) (W. M. Keck Foundation Biotechnology Resource Laboratory, New Haven, USA).

SDS-PAGE, Isoelectric Focusing (IEF), Glycan Detection

Allergen extracts were separated by 10% SDS-PAGE (Fling, 1986). A Rainbow Marker (Amersham, Buckinghamshire, UK) was used as molecular weight standard. Isoelectric focusing was performed using a Multiphor II electrophoresis and Ampholine PAGplate (pH 3.5–9.5) as recommended by the manufacturer (Pharmacia Biotech). Six hundred µg of timothy grass pollen extract and 20 µg purified nPhl p 4 were subjected to IEF using an Marker with a pH range from pH 3.5–9.3 (Pharmacia Biotech) and, in certain experiments, a marker with a pH range from 3–10.2 (FMC BioProducts, USA) as standards.

Glycan detection of nitrocellulose- (Schleicher & Schuell, Dassel, Germany) blotted proteins was carried out with a DIG-Glycan detection kit (Boehringer, Mannheim, Germany) using transferrin as a positive control exactly according to the manufacturer's protocol.

Circular Dichroism Analysis of Purified Natural Phl p 4

Circular dichroism (CD) spectra were recorded on a Jasco J-710 spectropolarimeter fitted with a Jasco PTC-348WI peltier-type temperature control system and interfaced with a Fisons HAAKE GH water. The instrument was calibrated with a 0.1% aqueous solution of d-10-camphor-sulphonic acid. Results were expressed as observed ellipticity (Θ) at a given wavelength. Far ultraviolet CD spectra were recorded at 20°C, 80°C and 20°C after cooling from 80°C. Spectra were recorded with 0.1 nm resolution and resulted from averaging 10 scans. The final spectra were corrected by subtracting the corresponding base line spectrum obtained under identical conditions. Thermal denaturation of nPhl p 4 was monitored using a 2 mm cuvette (Hellma), by recording the observed ellipticity at a fixed wavelength (208 nm) while heating at 50°C/h with a computer-controlled circulating water bath. The reversibility of the unfolding process was checked by measuring the restoration of the CD signal after cooling (50°C/h) to the starting temperature (20°C). All measurements were performed in MilliQ water, pH 7.2 at a protein concentration of 10 µM, using a 2 mm path-length quartz cuvette (Hellma).

Immunoblotting, IgE Immunoblot Inhibition Experiments

Allergen extracts were separated by preparative SDS-PAGE and blotted onto nitrocellulose (Schleicher & Schuell) (Towbin *et al.*, 1979). Nitrocellulose strips were blocked twice for 5 minutes and once for 30 minutes in buffer A (50 mM sodium phosphate, pH 7.5, 0.5% v/v Tween-20, 0.5% w/v BSA, 0.05% w/v NaN₃). After blocking, strips were reacted overnight at 4°C with human sera diluted 1:10 in buffer A, the rabbit anti-Phl p 4 antiserum or the corresponding rabbits preimmune serum diluted 1:2000 in buffer A. Membranes were washed in buffer A and bound rabbit antibodies were detected with a [¹²⁵I]-labeled donkey anti-rabbit Ig antiserum (Amersham). Bound human IgE antibodies were detected with [¹²⁵I]-labeled anti-human IgE antibodies (Pharmacia). IgE immunoblot inhibition experiments were performed by preincubating one ml aliquots of 1:5 in buffer A diluted sera overnight at 4°C with 5 µg nPhl p 4, or with 5 µg rBet v 2 or, for control purposes, with 5 µg BSA. Buffer A was added to the preadsorbed sera to yield 2 ml of a 1:10 dilution of which one ml was exposed

to nitrocellulose-blotted timothy grass pollen and 1 ml to celery extract, respectively. Bound IgE was detected with 1:10 diluted [¹²⁵I]-labeled anti-human IgE antibodies (Pharmacia).

IgE Immunoblot Inhibition Experiments with Rabbit Antisera

Purified Phl p 4 and for control purposes, an immunologically unrelated timothy grass pollen allergen (recombinant Phl p 2: 10 kDa) were blotted onto nitrocellulose (1 µg allergen/cm) (Schleicher & Schuell). Nitrocellulose strips containing the purified allergens were then preincubated either with a rabbit anti-Phl p 4 antiserum or, for control purposes, with the corresponding preimmune serum, diluted 1:2000 in buffer A at 4°C overnight. Strips were then washed 3 times in buffer A for 20 minutes and exposed to 1:10 in buffer A diluted sera from grass pollen allergic patients or, for control purposes, with serum from a non-atopic person overnight at 4°C. Nitrocellulose strips were washed again in buffer A and bound IgE antibodies were detected with [¹²⁵I]-labeled anti-human IgE antibodies (Pharmacia) and visualized by autoradiography.

ELISA Experiments, Periodate Treatment, Immunocaps

ELISA plates were coated with 100 µl/well of purified nPhl p 4 (10 µg/ml in PBS, pH 6.8) overnight at 4°C. Plates were then washed twice with 200 µl Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBST) per well and blocked with 200 µl TBST containing 1% BSA for 2 h at room temperature to block non-specific protein binding sites. Wells were then incubated with 100 µl/well of 50 mM NaAc (pH 4.5) with and without 10 mM sodium m-periodate (Sigma) for 1 h at room temperature and then washed five times with 200 µl TBST. Wells were then incubated with 100 µl of sera from Phl p 4 allergic patients and from a non-atopic individual (diluted 1:5 in TBST containing 1% BSA) in duplicates overnight at 4°C. Bound IgE antibodies were detected with an alkaline phosphatase-conjugated monoclonal anti-human IgE antibody (Pharmingen, San Diego, USA) as described (Niederberger *et al.*, 1998). The optical density corresponding to the amount of bound IgE antibodies was determined in an ELISA reader (Dynatech, Denkendorf, Germany) and is displayed as mean of duplicate determinations with an error of <5%.

Experimental immunoCAP tests were prepared by covalent immobilization of purified Phl p 4 onto activated cellulose at a concentration chosen to achieve an adequate linear measuring range and a background for negative sera adequately below the conventional cut-off value of 0.35 kUA/L. 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). Quantitative CAP RAST IgE determinations were performed on a UNICAP instrument (Pharmacia). Serum samples containing specific IgE values of >0.35 kUA/L were considered positive.

Histamine Release Assays and Skin Prick Testing

Heparinized blood samples were obtained from a grass pollen allergic patient containing IgE antibodies to Phl p 4 and from a non-atopic individual by venipuncture after informed consent had been given. Granulocytes were prepared by dextran sedimentation and incubated with various doses of purified nPhl p 4 as well as with anti-human IgE antibodies as previously described (Valent *et al.*, 1989). Histamine released in the cell-free supernatant was measured by radioimmunoassay (Immunotech, Marseille, France). Total histamine was determined after freeze thawing of cells. Results are displayed as mean values of triplicate determinations.

Skin prick tests were performed on the individuals forearms as described (Vrtala *et al.*, 1997). Individuals were pricked with 20 μ l aliquots of PBS containing different concentrations (1 μ g/ml, 5 μ g/ml, 10 μ g/ml, 20 μ g/ml, 40 μ g/ml, 80 μ g/ml) of purified Phl p 4. For control purposes, histamine, NaCl and timothy grass pollen extract (ALK, Horsholm, Denmark) were used. The skin reactions were recorded 20 min after testing by photography and by transferring the ball point pen-surrounded wheal area with a scotch tape to paper. The mean wheal diameter (DM, in mm) was determined as follows: $DM = (D1 + D2)/2$. D1 and D2 represent the maximal longitudinal and transversal diameters in mm, respectively.

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