

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

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Summary

Background More than 70% of the patients allergic to grass pollen exhibit IgE-reactivity against the high molecular mass fraction between 50 and 60 kDa of timothy grass pollen extracts. One allergen from this fraction is Phl p 4 that has been described as a basic glycoprotein. A new 55/60 kDa allergen, Phl p 13, has recently been purified and characterized at the cDNA level.

Objective The relative importance of the two high molecular mass allergens has been characterized with respect to their IgE-binding frequency and capacity.

Methods Both high molecular mass allergens were biochemically purified and subjected to nitrocellulose strips. About 306 sera obtained from subjects allergic to grass pollens were used to determine specific IgE-binding frequency to Phl p 4 and Phl p 13. IgE-binding of allergens was quantified by ELISA measurements. Pre-adsorption of sera with purified allergens and subsequent incubation of nitrocellulose-blotted timothy grass pollen extract was performed to determine whether or not Phl p 4 and Phl p 13 represent the whole high molecular mass allergen fraction. Proteolytic stability of both allergens was investigated by addition of protease Glu-C.

Results More than 50% of 300 patients displayed IgE-binding with both allergens. Clear differences concerning the immunological properties of Phl p 4 and Phl p 13 were confirmed by individual IgE reactivities. Quantification of specific IgE for both allergens revealed comparable values. For complete inhibition of IgE-binding in the high molecular mass range preincubation of sera with both allergens was necessary. Interestingly, inhibition of strong reacting sera with Phl p 13 eliminated not only reactivity of the 55/60 kDa double band, but in addition a 'background smear'. Whilst undenatured Phl p 4 was resistant to proteolytic digestion with Glu-C, native Phl p 13 was degraded rapidly.

Conclusion Phl p 4 and Phl p 13 are immunologically different and must both be considered as major allergens. They are judged to be important candidates for potential recombinant therapeutics that may provide a basis for improved immunotherapy.

Keywords: *Phleum pratense*, timothy grass pollen, Phl p 4, Phl p 13, major allergen

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Introduction

Timothy grass pollen extracts are complex mixtures containing various allergenic components. Various allergens of *Phleum pratense* have been described in detail, including

the major allergens Phl p 1 [1] and Phl p 5 [2]. These allergens exhibit molecular masses between 25 and 35 kDa. In addition, smaller timothy grass pollen allergens with molecular masses around 12 kDa have been investigated at the sequence level, for instance Phl p 2 [3] and Phl p 6 [4]. In contrast, little is known about the allergens with molecular sizes between 50 and 60 kDa, although 75% of grass allergic patients produce IgE specifically directed against these

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components [5]. One component designated as Phl p 4 has been described as a basic glycoprotein with a molecular mass of 55 kDa [6,7]. Monoclonal antibodies directed against this allergen have been generated [6], thus facilitating its detection and quantification [8]. Group 4 allergens were first described in *Lolium perenne* [9] and also characterized in other grass species, such as and *Dactylis glomerata* [10]. The high molecular mass allergen BG-60 from *Cynodon dactylon* shares at least size and isoelectric point with group 4 [11]. Leduc-Brodard *et al.* [10] have used mAbs to demonstrate cross-reactivity between group 4 allergens from various sources.

Recently, another timothy pollen allergen with a similar molecular size as Phl p 4 has been purified [12] and characterized at the cDNA level (EMBL accession no. AJ238848). Since this new allergen, designated Phl p 13, exhibited different biochemical properties compared with Phl p 4, it was assumed that at least two different groups of allergens are present in the high molecular mass fraction of timothy grass pollen extract.

In order to estimate the importance of both high molecular allergens, they were purified, subjected to immunoblotting and analysed with 306 different sera derived from subjects allergic to grass pollens. Specific IgE was determined to evaluate of the binding capacities of each allergen. Investigations were also conducted to determine whether these two allergens alone were enable to effect complete inhibition of IgE-binding in the high molecular mass range.

Materials and methods

Purification of timothy grass pollen allergens

The purification of Phl p 1, group 2/3 and Phl p 13 was performed as described previously [12]. Briefly, a timothy grass pollen extract was separated by hydrophobic interaction chromatography (HIC) using phenyl sepharose HP (Pharmacia Biotech, Freiburg, Germany). The flow-through fraction was subsequently concentrated with ultrafiltration devices Centriprep-10 (Millipore, Eschborn, Germany). Proteins of the flow-through fraction were finally purified using gel filtration with Superdex 75 (Pharmacia Biotech).

Phl p 4 was purified according to the procedures of Haavik *et al.* [6] and Fischer *et al.* [7] with some modifications. A timothy grass pollen extract obtained from 5 g pollens was subjected to G-25 gel filtration in phosphate buffer (20 mM, pH 7.0). The first peak was collected and applied to a 16/10 cation exchange column packed with Source 30S (Pharmacia Biotech) equilibrated with phosphate buffer. Elution of bound proteins was achieved using running buffer supplemented with 1 M NaCl. In order to

remove trace amounts of Phl p 13, eluted proteins were subsequently adjusted to 20 mM Tris-HCl pH 8.0, 1 M $(\text{NH}_4)_2\text{SO}_4$ and subjected to hydrophobic interaction chromatography. After elution of Phl p 4 with distilled water, final purification was performed using size exclusion chromatography with Superdex 75.

SDS-PAGE and immunoblotting

Proteins were separated by SDS-PAGE (120 × 80 × 0.8 mm) with the buffer system of Laemmli [13]. After electrophoresis, proteins were transferred onto a supported nitrocellulose membrane (Sartorius, Göttingen, Germany) by semidry blotting for 30 min at 2 mA/cm². Immobilized proteins were either stained using india ink (Pelikan, Hannover, Germany) and afterwards AuroDye (Janssen Biochimica, Beerse, Belgium) [14] or incubated with mAbs or serum.

In order to screen multiple purified allergens with sera derived from allergic subjects, a Miniblot MN45 apparatus (Biometra, Göttingen, Germany) was used to apply allergens onto nitrocellulose membranes. Purified allergens (0.1 µg/µL), which were dissolved in 20 mM NaOH, were applied to the conduits of the apparatus and incubated for 30 min. After washing the conduits with distilled water, the membranes were blocked using Tris-buffered saline with 0.05% (v/v) Tween 20. For immunodetection, strips from the membranes (allergen strips) were incubated with sera or mAbs overnight.

Immunodetection and inhibition experiments

Prior to immunodetection, membranes were blocked by treatment for 30 min with Tris-buffered saline (TBS, pH 7.4) supplemented with 0.05% Tween 20. Incubation with 1:20 diluted sera derived from allergic subjects or mAbs was carried out overnight. Identification of Phl p 4 was performed using monoclonal antibodies 5H1 and 3C4 [7], of group 1 allergens by mab 9C12 [15] and group 5 allergens using mab 1D11C8 [16,17]. After washing, bound antibodies were detected with alkaline phosphatase conjugated monoclonal mouse antihuman IgE (Allergopharma, Reinbek, Germany) or goat antimouse IgG/M with alkaline phosphatase (Dianova, Hamburg, Germany). The binding patterns were visualized by a substrate solution consisting of NBT/BCIP (Life Technologies) in 100 mM Tris-HCl-buffered saline solution (pH 9.5).

Inhibition experiments were conducted by adding 75 ng allergen per µL undiluted serum. After incubation for 30 min under gentle agitation, sera were diluted 1:20 in TBST and subsequently used for probing of nitrocellulose blotstrips carrying timothy grass pollen extract separated in a 12.5% SDS-PAGE.

Determination of allergen-specific IgE

Allergen-specific IgE levels were determined by EAST measurements with a specific 'IgE ELISA RV' kit (Allergopharma, Reinbek, Germany) containing alkaline phosphatase conjugated monoclonal mouse antihuman IgE antibodies. Experimental allergen discs carrying purified allergens were prepared according to the method of Ceska *et al.* [18]. In order to determine a value of kU/L reference sera and discs, which were included in the kit, were used to generate a standard curve. Results are displayed as means of duplicate determinations.

Proteolytic digestion of purified allergens

Lyophilized proteins were reconstituted in 25 mM Na₂HPO₄, pH 7.3 at a concentration of 0.5 mg/mL. Proteolysis was performed by the addition of 0.25 µg Glu-C sequencing grade (Promega, Heidelberg, Germany) to 5 µg allergen and subsequent incubation at 37 °C for 2 h. The reactions were stopped by addition of reducing sodium dodecylsulphate sample buffer and boiling for 5 min

Results

In order to investigate the relevance of the high molecular weight allergen group between 50 000 and 60 000 of timothy grass pollens, the two known members Phl p 4 and Phl p 13 were biochemically purified. Phl p 4 was purified on the basis of its basic pI by ion exchange chromatography, whilst Phl p 13 was obtained after hydrophobic interaction chromatography in the flow-through fraction. In addition, Phl p 1 and group Phl p 2/3 were purified for control purposes. SDS-PAGE separation demonstrated that the allergens were purified to near homogeneity (Fig. 1).

After applying a timothy grass pollen extract and purified allergens onto nitrocellulose membranes, strips were incubated with sera derived from patients allergic to grass pollens and developed with mouse anti human-IgE mAb conjugated with alkaline phosphatase (Fig. 2). For the application of allergens different solutions were investigated, such as Tris-buffered saline and 20 mM NaOH. In both cases identical results were obtained regarding IgE-reaction patterns, but it was found that most efficient binding of allergens and consequently enhanced immunostaining occurred by using NaOH (data not shown).

Human serum albumin (HSA) was used as a negative control. Interestingly, the patients' sera revealed highly differentiated reaction patterns. In each case IgE-binding was observed with the timothy grass pollen extract, whereas reaction with purified allergens varied in nearly every possible combination. In the cases of the high molecular

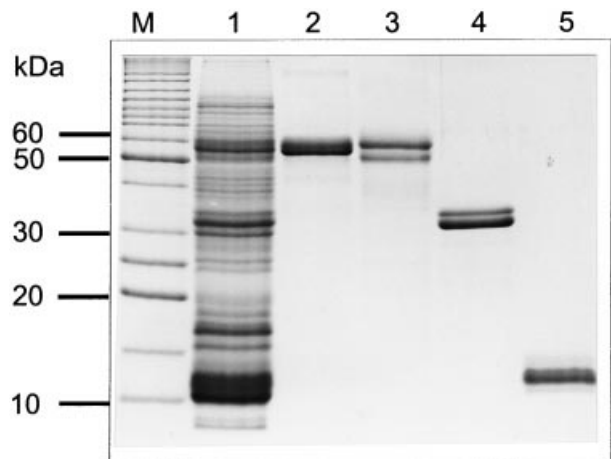


Fig. 1. Coomassie-stained SDS-PAGE of purified allergens showing separated molecular weight marker (M), timothy grass pollen extract (1), Phl p 4 (2), Phl p 13 (3), Phl p 1 (4) and group Phl p 2/3 (5).

mass allergens Phl p 4 and Phl p 13, IgE-binding was detected either for none, one or for both proteins, showing clear differences at the polyclonal IgE-level. The allergens are clearly immunologically distinct. Table 1 contains the IgE-reactivity of 306 sera investigated by allergen strips. Since the allergens exhibited more than 50% IgE-binding, they all meet the requirements for classification as major allergens, though the value for the high molecular mass allergens Phl p 4 and Phl p 13 was found to be only slightly above this arbitrary level. As expected, Phl p 1 displayed the highest IgE-binding frequency of about 95% [19]. The frequency obtained for group 2/3 also correlates with those reported in literature [20,21].

Allergen-specific IgE from eight sera was determined and a sufficient correlation between immunostaining of allergen strips and ELISA quantification was found throughout (Fig. 3).

Serum IgE for Phl p 4 was determined in the range of 0.3–15.5 kU/L, whereas specific IgE for Phl p 13 varied

Table 1. IgE-binding frequency of purified timothy grass pollen allergens

Allergen	IgE-reactive sera (n = 306)	% of sera tested
Phl p 4	170	55.6
Phl p 13	154	50.3
Phl p 1	293	95.8
Phl p 2/3	183	59.8

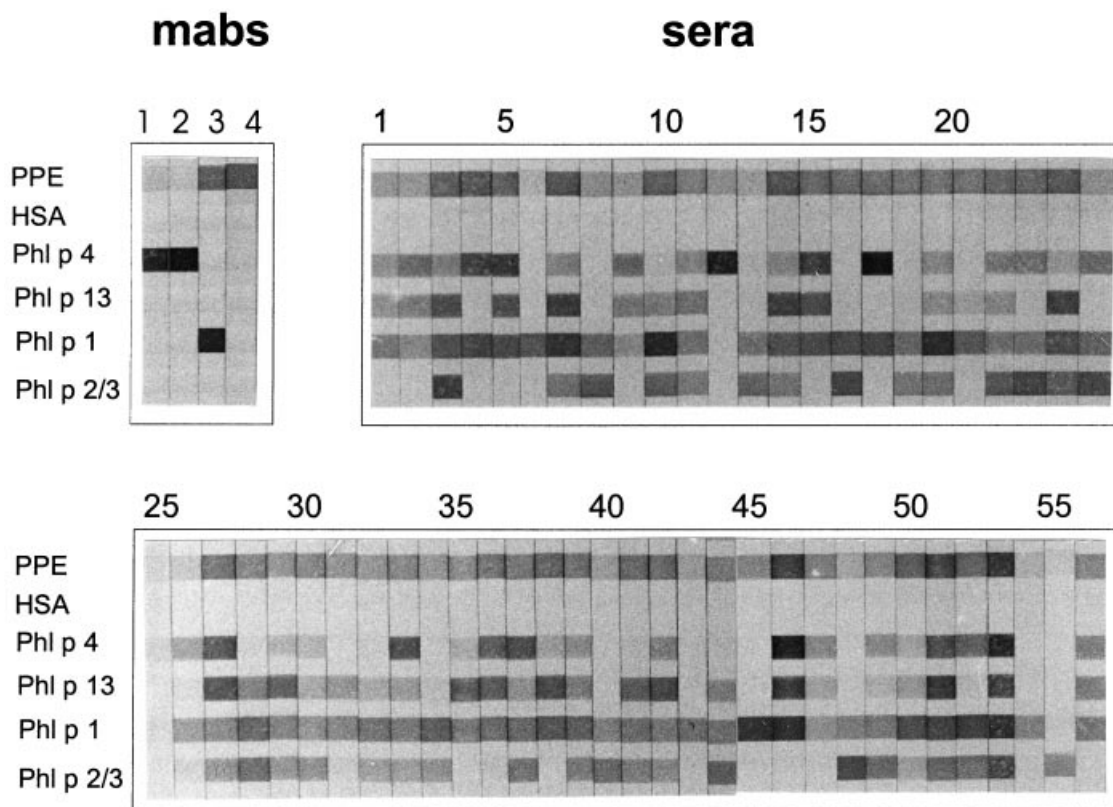


Fig. 2. Detection of allergen-specific IgE from patients allergic to grass pollens. Strips of a nitrocellulose membrane carrying timothy grass pollen extract (PPE), human serum albumin (HSA), Phl p 4, Phl p 13, Phl p 1 and group Phl p 2/3 were either incubated with mabs (1,2 = specific for group 4; 3 = specific for group 1; 4 = specific for group 5) or sera (1–56).

between 0.5 and 17.5 kU/l, which represents the upper limit. Regarding Phl p 13, in some cases the staining of the allergen was very faint or absent whilst the amount of specific IgE detected by the ELISA system revealed relatively high values, as for sera 52 and 48. If allowance is made for this observation then the percentage of sera recognized as reacting with Phl p 13 would even be higher.

In order to determine if Phl p 4 and Phl p 13 together account for the whole high molecular mass allergen fraction, inhibition experiments were performed. A serum previously investigated by allergen strip analysis (Fig. 2, no. 47) was pre-adsorbed with either Phl p 4 (Fig. 4, lane 2), Phl p 13 (lane 3) or both allergens (lane 4) and applied to blot strips carrying separated timothy grass pollen extract. Compared with the IgE reaction patterns without pre-adsorption (Fig. 4, lane 1) inhibition with Phl p 4 led to disappearance of a strongly reacting band corresponding to the protein detected by the Phl p 4 specific mab 3C4 (Fig. 4, lane 5). The intensity of the other bands detected including Phl p 1 and Phl p 5 remained unaltered. In contrast, inhibition of the sera with Phl p 13 resulted not only in an elimination of

slightly diffuse bands in the range between 50 and 60 kDa, but also to a clearance of the background. Surprisingly, pre-adsorption with Phl p 13 also effected a decrease in IgE-reactivity with protein bands with apparent molecular masses between 90 and 110 kDa. As expected, no effect was observed on the reactivity of Phl p 4. Using both allergens for pre-adsorption, complete reaction of allergen bands in the range of 50–60 kDa as well as a 'background smear' produced by smaller bands was eliminated, but detection of allergens between 25 and 35 kDa, corresponding to Phl p 1 and Phl p 5, was unchanged.

It was considered that a possible explanation for the dissimilar activities of Phl p 4 and Phl p 13 could be related to differing susceptibility to proteolysis. In order to investigate this possibility, both allergens were incubated with the protease Gluc-C (Fig. 5). As expected, no effect was observed in the case of Phl p 4, which is known to be trypsin resistant [7]. In contrast, Phl p 13 was completely degraded into smaller fragments covering the range from 50 to 10 kDa. Therefore susceptibility to proteolysis was identified as a distinguishing feature between the two allergens.

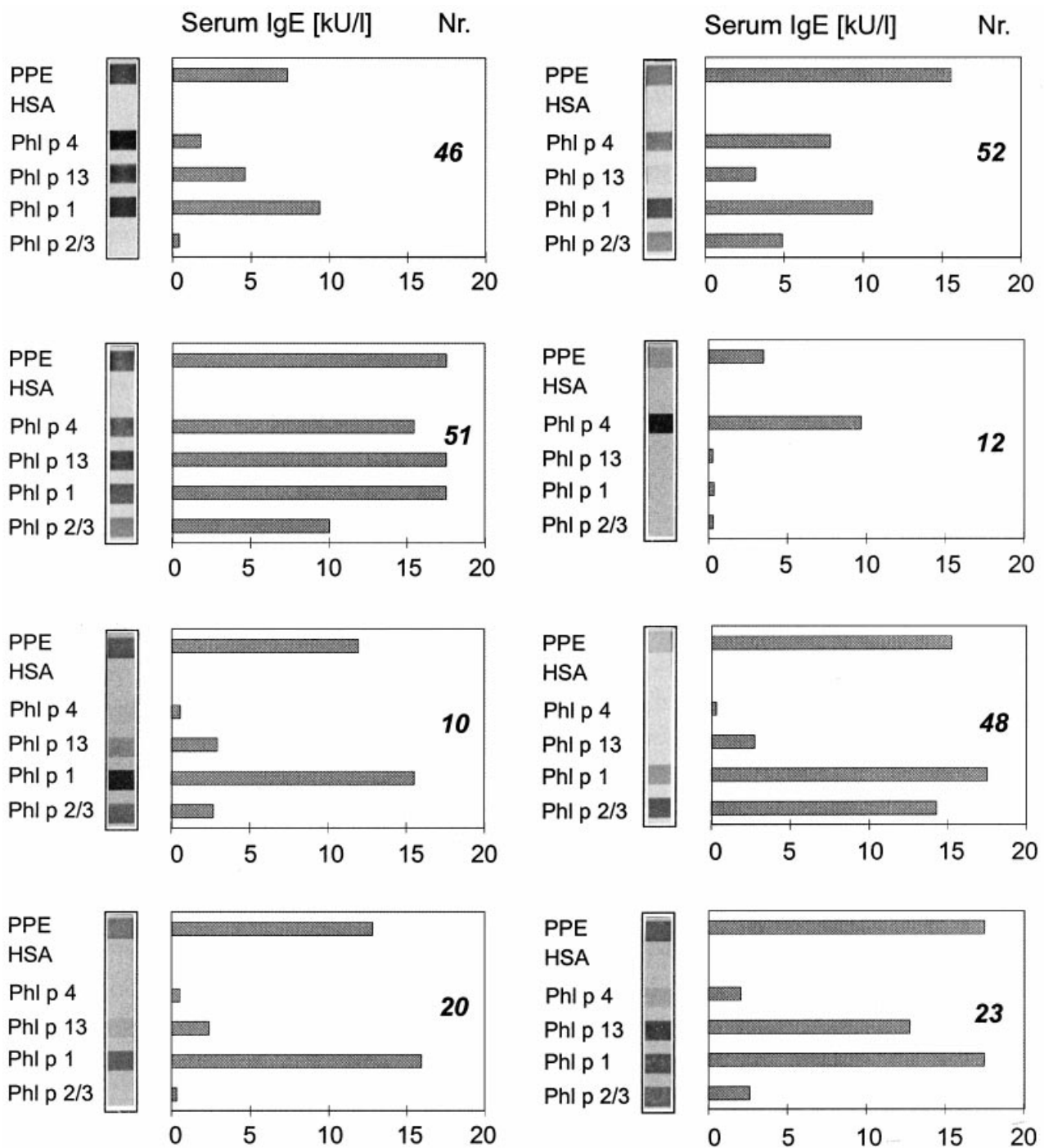


Fig. 3. Quantification of allergen-specific IgE. Sera from eight grass pollen allergic patients previously characterized in Fig. 2 were analysed for grass pollen allergens by ELISA measurements. Values below 0.35 KU/L indicate lack of detectable specific IgE. For comparison, allergen strips are displayed on the left.

Discussion

In previous studies a new high molecular allergen Phl p 13 with an apparent molecular mass of 55–60 kDa has been

biochemically purified and the corresponding cDNA (EMBL accession no. AJ238848) expressed in bacteria. As demonstrated by reactivity with monoclonal antibodies, this allergen was different from another high molecular

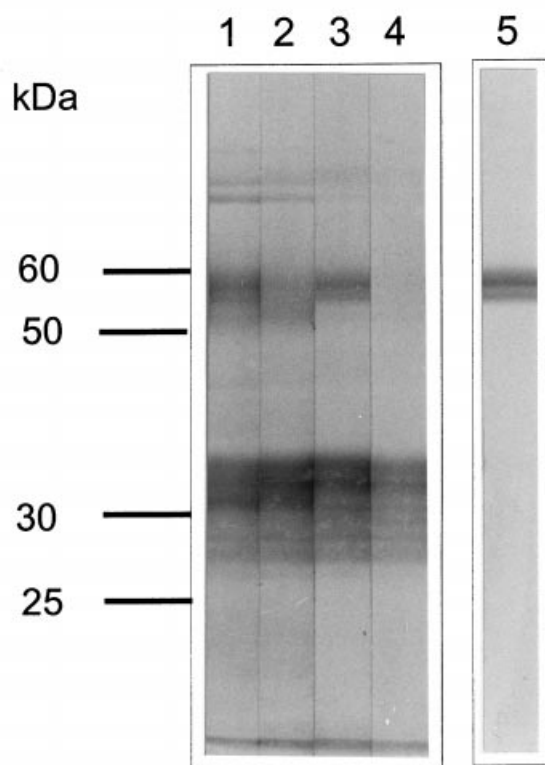


Fig. 4. Inhibition of IgE-binding to nitrocellulose-blotted timothy grass pollen extract with purified Phl p 4 and Phl p 13. Strips carrying separated grass extract were incubated with serum no. 47 already characterized in Fig. 2 (lane 1). Lane 2 contains IgE-binding pattern of the serum pre-adsorbed with Phl p 4, lane 3 with Phl p 13 and lane 4 with both Phl p 4/Phl p 13. Detection of Phl p 4 was performed with group 4-specific mab 3C4 (lane 5).

mass allergen designated as Phl p 4. Since both allergens exhibit similar molecular sizes, it has been suggested that former studies evaluating the allergenic importance of Phl p 4 using one dimensional SDS-PAGE indeed detected both allergens (Suck *et al.* submitted). Therefore, investigations were necessary to discriminate between Phl p 4 and Phl p 13 on the polyclonal IgE-level. Since the isoelectric points of the two allergens differ by approximately two units with 7.5 for Phl p 13 and 9.3 for Phl p 4 [8], two-dimensional immunoblotting would represent a reasonable method [22]. However despite possibilities for multiple reprobing of blots [23] only a limited number of sera could have been screened. Consequently, a novel allergen strip method similar to a dot blot was used for this purpose allowing reproducible high throughput IgE-screening with biochemically purified allergens. This analysis revealed that Phl p 4 and Phl p 13 displayed IgE-binding frequencies greater than 50% with 306 sera derived from patients allergic to grass pollens. Hence, both high molecular components have to be judged as major allergens. It seems likely that previous

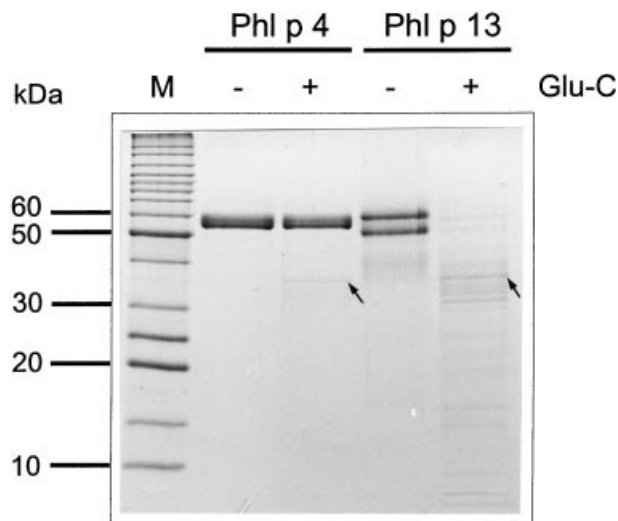


Fig. 5. Proteolytic digestion of Phl p 4 and Phl p 13 with Glu-C. Arrows indicate the position of Glu-C [29].

investigations detected not only Phl p 4, but in addition or even exclusively Phl p 13.

Quantification of IgE-binding revealed comparable IgE-binding capacities of Phl p 4 and Phl p 13 and in addition another interesting aspect. Although the IgE-reactivity of some sera was more or less negative for Phl p 13 by allergen strip detection, large amounts of specific IgE directed against this allergen were measured with the ELISA. A possible explanation for this phenomenon might be that different allergen binding mechanisms influence the allergenicity of this molecule. Group 4 ELISA measurements showed that the allergen molecules possess at least two different reactive IgG and IgE epitopes [24,25], whilst IgE-binding was lost after two-dimensional immunoblotting [22]. It has been suggested that Dac g 4 was denatured in such a way that conformational changes occurred [22]. Since Phl p 13 was not harshly denatured for blotting onto nitrocellulose membrane, it seems more likely that it is the manner of the matrix that influences its conformation. Protein binding to nitrocellulose involves hydrophobic interaction [26], which probably effects a unidirectional orientation of some allergens. If this results in masking of epitopes which are recognized by dominant oligoclonal or even monoclonal IgE-pools of some sera, detection will be nearly impossible. In contrast, cyanogen bromide-activated paper, which was used for ELISA measurements, covalently binds proteins in a more random manner. This binding mechanism ensures for the availability of the whole epitope spectrum. Consequently, the number of sera possessing specific IgE against Phl p 13 should be even higher than 50.3% if screening were to be conducted throughout using the ELISA system.

Inhibition experiments were performed to investigate whether Phl p 4 and Phl p 13 represent the whole high molecular mass allergen fraction. Since pre-incubation of serum with both allergens completely eliminated IgE-reactivity in the range of 50–60 kDa, it is likely that this fraction is composed of only two allergens. Differences in the immunostaining intensity between Phl p 4 and Phl p 13 might be due to different proteolytic sensitivity. Group 4 allergens are known to be resistant against proteolytic attack [7], similar to Phl p 1 [26,27]. Thus, intact molecules that were focused sharply after SDS-PAGE were available for IgE-binding. In contrast, Phl p 13 was degraded rapidly with Glu-C and Lys-C (not shown). Assuming proteolytic activity in timothy grass pollen extract, for example group 1 allergens are thought to possess a cysteine proteinase function [28], Phl p 13 is a potential target for degradation. If this is the case it would explain why pre-incubation of sera with Phl p 13 resulted not only in the inhibition of the double band, but also in a decrease of background staining, assuming the smaller bands generating this background reaction are derived from Phl p 13. If this hypothesis is correct then precautions are necessary to protect Phl p 13 during and after extraction of pollens against proteolysis. Preincubation of sera with Phl p 13 also inhibited IgE-binding to very high molecular mass bands. Since Phl p 13 seems to be highly glycosylated (Suck *et al.* submitted), this effect might be due to IgE cross-reactivity between carbohydrate determinants [30,31], assuming glycosylation of these bands.

Another aspect to consider is the possible different extents of glycosylation of Phl p 4 and Phl p 13. Since many glycoproteins behave anomalously when SDS and thiol reagent are in excess [26], it is probable that the highly glycosylated Phl p 13 was not as sharply focused as Phl p 4 after SDS-PAGE. In this case, broadening of the bands would result in less intensive staining compared with Phl p 4, though equal amounts of allergen specific IgE and IgE-binding capacity were determined.

In summary, the data presented in this study clearly demonstrate that both Phl p 4 and Phl p 13 belong to the major allergens of *Phleum pratense*. Since separation of Phl p 4 and Phl p 13 by one dimensional SDS-PAGE is difficult to achieve and Phl p 13 is weakly immunostained, it becomes clear why the important new allergen Phl p 13 has not been recognized until now.

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