

Group V allergens in grass pollens: IV. Similarities in amino acid compositions and NH₂-terminal sequences of the Group V allergens from *Lolium perenne*, *Poa pratensis* and *Dactylis glomerata*

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Summary

Monoclonal antibodies (PpV4) raised against *Phleum pratense* group V allergen were used for immuno-affinity chromatography of cross-reacting group V allergens from related grass species. Fractions enriched in group V allergen were obtained from *Lolium perenne*, *Poa pratense* and *Dactylis glomerata* extracts. The major components in these fractions were found in the M_w, range 25–28 kD. IgE binding to these components was shown using a pool of grass allergic sera, by SDS-PAGE immunoblotting. These fractions were electroblotted from tricine SDS-PAGE gels onto a polyvinylidene-difluoride membrane and selected group V bands were directly cut out and used for amino acid analysis and NH₂-terminal sequencing.

Both the amino acid compositions and the NH₂-terminal sequences obtained for each group V allergen were almost similar to each other and to the sequence and composition of the previously described allergen *Phl p V* from *Phleum pratense*. A common trait of the investigated allergens, is the very high contents of alanine (25–32%) and the presence of the modified amino acid, hydroxyproline.

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Introduction

Grass pollen has been shown to be important triggers of allergic diseases during the pollination season [1]. The grass pollen allergens can generally be divided into six major groups, of which group I and V are the most dominating [2–7]. In previous studies the group V allergens have been shown to be present in 8 out of 10 investigated grasses [8]. *Phl p V* has been isolated [4] and shown to consist of at least 2–3 isoallergenic components. The *Phl p V* isoallergenic mixture was used to raise monoclonal antibodies (MoAbs), named *Pp V1–8*. These MoAbs were shown to have specificity for group V allergens from a number of related species as shown by immunoblotting, ELISA and crossed immunoelectrophoresis (CIE) overlay techniques, and they did not bind to any other identified grass allergens, including group I [9].

The study reported here makes use of this group specific cross-reactivity by applying MoAbs raised against *Phl p V* for immuno-affinity purification of group V allergens from *Lolp*, *Poap* and *Dacg*. The aim was to obtain information about these allergens at the basic structural level (amino acid composition and NH₂-terminal sequence) for comparative purposes and to determine whether the immunological similarities are reflected in the basic protein structure.

Materials and methods

Pollen extracts

Grass pollens were obtained from Greer Laboratories (North Carolina, U.S.A.) as dried, non-defatted material. The species applied were: *Dactylis glomerata* (*Dacg*), orchard grass; *Lolium perenne* (*Lolp*), rye grass; and *Poa pratensis* (*Poap*), Kentucky blue grass. According to the manufacturer's certificates, the collected pollen had less

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than 1% (w/w) contamination with foreign material. The pollens were stored and extracted as described elsewhere [4].

Antibodies

Polyspecific and monospecific rabbit anti-grass pollen antibodies were raised, isolated and purified as described previously [4,8]. The murine MoAbs were raised and purified from ascites on protein G columns as described elsewhere [9]. The clone PpV4 was selected for MoAb affinity purification of *Dac g* V, *Lol p* V and *Poa p* V because of the group V cross binding seen in ELISA, crossed radio immunoelectrophoresis (CRIE) overlay and SDS-PAGE immunoblotting [9]. The antibodies were stored at 5°C in a 0.015 M NaN₃, 1% (v/v) aprotinin (Novo Nordisk A/S, Denmark) solution.

Sera

A positive serum pool was made with equal volumes of sera from 30 grass sensitized individuals [8]. A pool of 1000 Danish individuals was used as control serum.

The MoAb affinity column

Cyanogen bromide (CNBr) Sepharose 4B (Pharmacia A/S, Sweden) was activated with 0.001 M HCl, according to the manufacturer's recommendation. The coupling procedure was optimized for approximately 85% coupling of the MoAbs (as estimated from $E_{280}^{1\%} = 13.1$), which has been shown to be optimal [10]. One millilitre activated gel material was mixed with 2–3 mg protein G purified MoAb [9] in 5 ml phosphate-buffered saline (PBS, 0.075 M Na₂HPO₄, 0.025 M NaH₂HPO₄, 0.5 M NaCl, pH 7.2). The suspension was stirred gently for 45 min at 5°C. The gel was washed with 5 ml 0.1 M NaHCO₃, 0.5 M NaCl (pH 8.0) on a Büchner funnel, followed by blocking of the active groups by washing the gel with 3 × 2.5 ml 0.1 M NaHCO₃ + 0.5 M NaCl, 0.5 M ethanolamine (pH 8.0); and allowed to stand overnight in the solution at 5°C.

The gel material was transferred to 1.0 × 10 cm Econo-columns (Bio-Rad, Richmond, California, U.S.A.) and washed with 6 ml 0.1 M NaAc/HAc-buffer, 0.5 M NaCl (pH 4) followed by 6 ml 0.1 M NaHCO₃ + 0.5 M NaCl (pH 8.0). Washing was repeated three times and 10 ml PBS was used for equilibration (flow 45 ml/min). To avoid cross-contamination of group V allergens from the different species, each column was used for one species only.

Purification of the allergens

Extracts (20 mg/ml in PBS) of *Dacg*, *Lolp* and *Poap* were filtered (Millipore, 0.45 µm) and 0.5 ml was applied to 1

ml columns (PBS, flow 45 ml/min, 20°C) and unbound material eluted until A_{280nm} reached baseline level. Bound material was eluted with 0.1 M glycine, 0.15 M NaCl (pH 11, 20°C). The material was collected in 1 ml fractions and immediately neutralized with 2 M HCl.

Immuno-electrophoresis

Fused rocket immuno-electrophoresis (FRIE) was performed at pH 8.6 using 1% (w/v) agarose gel (type HSA, Litex, Copenhagen, Denmark), 2 mA/cm for 15–20 hr [11]. Group V allergens were identified with monospecific polyclonal anti *Phl p* V antibodies in the intermediate gel.

Dialysis

Fractions containing the purified group V allergen were pooled and dialysed (Servapor) at 5°C (20 hr, 0.01 M NH₄CO₃, 0.01% (w/v) NaN₃ and 20 hr, 0.001 M NH₄CO₃, pH 8.3). The resulting material was freeze dried and stored at –20°C.

SDS-PAGE, electroblotting and immunoblotting

Electrophoresis in polyacrylamide gels (SDS-PAGE), electroblotting, goldstaining and IgE immuno-blotting were performed as previously described [4]. Electroblots for IgG immunoblot were incubated overnight with 50 µl/lane rabbit antiserum diluted 1:1000 v/v in the incubation buffer (0.05% [v/v] Tween 20 in 0.05 M Tris and 0.15 M NaCl, adjusted to pH 10.3), followed by three washes in incubation buffer, and 3 hr of incubation in alkaline phosphatase conjugated swine anti-rabbit IgG antibodies (D306, Dakopatts A/S) diluted 1:1000 v/v in incubation buffer. The blots were developed using freshly prepared alkaline phosphatase substrate (0.00011 M nitroblue tetrazolium, 0.00014 M 5-bromo-4-chloro-3-indolylphosphate, 0.0036 M MgCl₂ in 0.1 M ethanolamine buffer, adjusted to pH 9.6). The molecular weight markers used were the same as in tricine SDS-page.

Tricine SDS-PAGE and electroblotting

Affinity purified material was further separated in a tricine SDS-PAGE system [12], modified as described by M. Ploug *et al.* [13]. The marker used was a Bio-Rad low M_w range mixture of rabbit phosphorylase b (97.4 kD), bovine serum albumin (66.2 kD), hen egg white ovalbumin (42.7 kD), bovine carbonic anhydrase (31.0 kD), soybean trypsin inhibitor (21.5 kD) and hen egg white lysozyme (14.4 kD). The proteins were blotted onto a polyvinylidene difluoride (PVDF, Millipore) membrane using a 10 mM 3-[cyclohexylamino]-1-propane sulphonic acid (CAPS, Sigma, St Louis, Missouri, U.S.A.), 20%

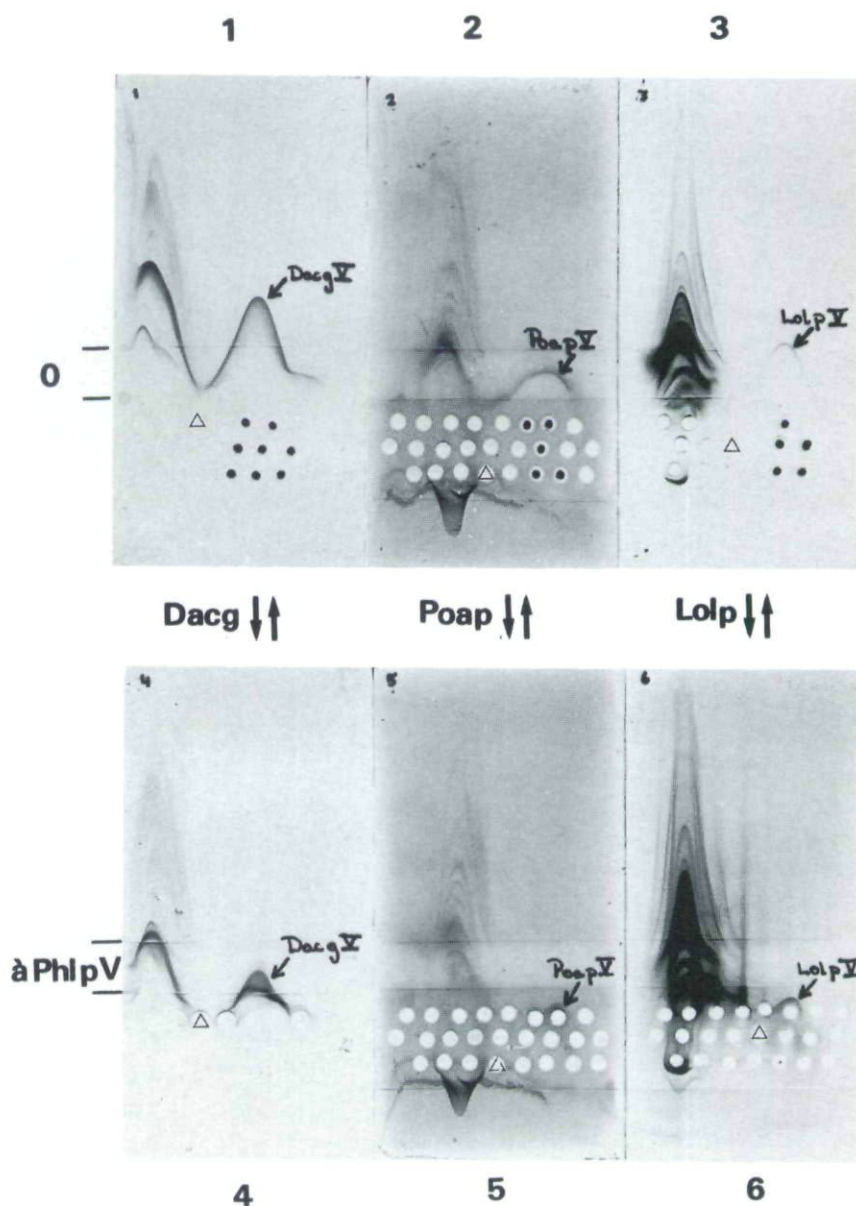


Fig. 1. FRIEs (Coomassie stained) of the fractions obtained from the MoAb affinity chromatography of *Dacg* (1 and 4) *Poap* (2 and 5) and *Lolp* (3 and 6). Triangles indicate where the elution of bound material starts. Lanes 1–3, using only polyspecific rabbit anti whole pollen extract antibodies. Lanes 4–6, identifying the group V allergen using monospecific rabbit anti *Phl p V* in the intermediary gel.

(v/v) methanol, pH 11 transfer buffer, 8 mA/cm² for 1.5 hr in a semi-dry blotting apparatus.

After 2 × 5 min washing in H₂O, the membranes were stained with Coomassie Brilliant Blue R-250 (0.1% w/v in 50% v/v methanol for 1 min) and destained (40% v/v MeOH, 10% (v/v) HAc) until the bands were visually distinct from the background (5–10 min). Finally, the membranes were washed thoroughly (2 × 15 min) in Milli-Q H₂O.

Selecting and cutting bands

Bands identified by immunoblotting as group V allergens, were directly cut out from the wet membrane and dried in N₂. The membranes were stored under N₂ at –20°C.

Amino acid analysis and NH₂-terminal sequencing

The selected bands were used directly for amino acid analysis [14] and NH₂-terminal sequencing, performed on an Applied Biosystems (Foster City, California, U.S.A.) 477 Pulsed Liquid Phase Protein/Peptide Sequencer, with a 120A HPLC on-line PTH-amino acid analyser [15,16].

Results

Material eluted from the PpV4 coupled columns gave single precipitation arcs in the FRIEs of all three species, thus indicating the presence of only one immunochemically dominant component. The precipitates were identi-

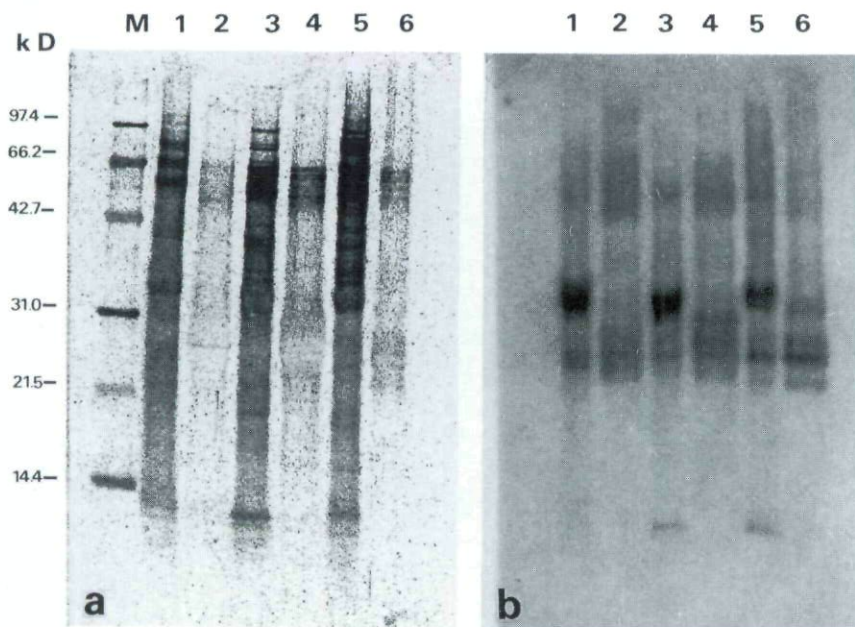


Fig. 2. (a) Goldstaining and (b) radiostaining, showing the IgE binding bands in the full extracts (1 = *Dacg*, 3 = *Poap* and 5 = *Lolp*) and the of the MoAb affinity chromatography products (2 = *Dacg*, 4 = *Poap* and 5 = *Lolp*). M is the molecular weight marker.

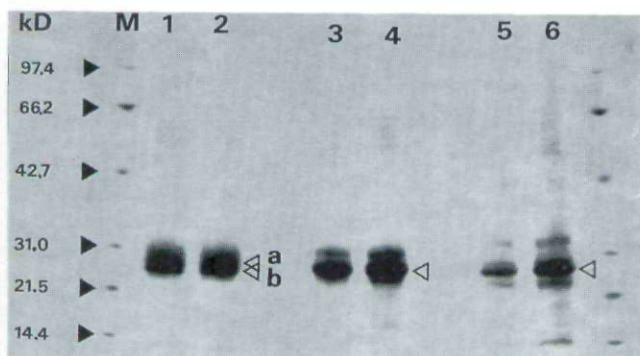


Fig. 3. The tricine SDS-PAGE blots of the purification products (Lanes 1 and 2 = *Dacg*, lanes 3 and 4 = *Poap*, lanes 5 and 6 = *Lolp* and 'M' is the molecular weight marker). The bands that were cut for further analyses are marked with arrowheads.

fied as group V components using mono-specific anti-*Phl p* V antibodies in the intermediate gel. The FRIEs are shown in Fig. 1. The eluted fractions containing group V components were pooled, dialysed and freeze-dried.

The gold stained SDS-PAGE blots of the group V pools revealed two–three dominating bands in the 25–30 kD area (Fig. 2a). These were shown to be IgE binding by SDS-PAGE immunoblotting and radiostaining, using a serum pool of grass sensitized patients (Fig. 2b). Some weaker contaminating bands were also seen, both above and under this area in all three grasses. The gold stained blots also contained the usual artefactual bands, produced by the oxidized dithiothreitol (DTT) at 58, 63, and 68 kD [17]. This material was separated in a tricine SDS-PAGE system combined with electroblotting onto

PVDF, followed by cutting of selected bands for amino acid analysis and NH_2 -terminal sequencing. An attempt to apply this system to obtain the bands directly from the crude extract was not successful, probably due to contamination from overlapping bands and traces of bands with lower Mw_r (not shown).

In tricine SDS-PAGE, the lanes with the MoAb-purified material were deliberately overloaded, aiming both at obtaining a maximum of protein blotted on the PVDF membrane for the best quality of the latter analyses and to reveal the contaminants. Figure 3 shows a PVDF blot of purified material from all three grasses. The most dominating bands in the blot were: *Dacg* 25/28 kD, *Poap* 28 kD and *Lolp* 28 kD. As in the goldstaining (Fig. 2a), weaker, contaminating bands were also seen. The dominating bands were cut out and used for amino acid analysis and NH_2 -terminal sequence analysing. The actual cutting of the bands was performed from the rear of the membrane, where only the cores of the selected bands were seen. The amino acid compositions of these bands and *Phl p* V [8], are shown in Table 1. All the analysed bands had very high contents of alanine (26–32%), as previously seen for *Phl p* V allergens. Traces of hydroxyproline (Hyp) were present in the amino acid analysis.

The SAQ values indicating protein relationships were determined for the following pairs ($\Delta\text{N} < 18$): *Dac g* Va/*Poap* V = 25.9, *Dac g* Va/*Lolp* V = 31.6 and *Poap* V/*Lolp* V = 23.1. The 'strong Cornish-Bowden test for relatedness of proteins' gives $\text{SAQ} = 34.8$ as the limit value (confidence level 92.5%, $n = 243$) [18].

Table 1. Amino acid composition of the group V components from the three grasses, including *Phl p V*. The ΔQ values estimated for the analysed components of the same size (28 kD) are shown below the table. ΔQ values were estimated (Table 1) for *Dac g Va*, *Poa p V* and *Lol p V*, using the formula:

$$\Delta Q = 10^4 \sum \left(\frac{n_{iA}}{N_A} - \frac{n_{iB}}{N_B} \right)^2$$

where n_{iA} (n_{iB}) is the content of amino acid 'i' in the protein A (B) and N_A (N_B) is the number of amino acids in protein A (B). The 'strong Cornish-Bowden test for relatedness of proteins' [18] gives a threshold limit at $\Delta Q = 34.8$ (confidence level 92.5%, $n = 243$) for these components.

Amino acid	Mole %				
	<i>Dac g Va</i>	<i>Dac g Vb</i>	<i>Poa p V</i>	<i>Lol p V</i>	<i>Phl p V</i>
Asx	5.7	6.0	6.1	6.3	5.4
Thr	8.6	8.4	6.5	8.6	7.6
Ser	1.0	2.0	3.5	2.0	5.1
Glx	9.2	9.3	9.0	9.8	10.2
Pro	4.7	4.4	4.7	4.9	5.9*
Gly	2.7	4.1	5.2	4.0	6.4
Ala	32.2	29.7	29.4	29.0	25.7
Cys	0.8	0.7	0.3	1.0	0.0
Val	7.3	7.0	8.7	6.4	6.6
Met	0.9	1.0	0.6	0.3	0.7
Ile	4.0	3.9	4.5	3.4	3.6
Leu	3.6	3.6	3.4	5.9	4.7
Tyr	3.4	3.3	2.3	3.0	3.5
Phe	5.8	5.8	5.2	5.0	4.1
His	0.2	0.3	0.6	0.3	0.8
Lys	10.0	10.2	9.5	9.8	8.8
Arg	0.0	0.3	0.6	0.4	1.0

ΔQ values:	25.9 23.1 31.6
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* 1.4% of the 5.9% proline is found as hydroxyproline.

The NH₂-terminal sequences are aligned with the previous published sequence of *Phl p V* [2] in Table 2. All analyses were performed twice with material from independent, identical purifications. The aligned sequences are 60–100% identical, including the least similar combination of the variable sequences.

All sequences contained a modified amino acid which eluted at a position identical to that of hydroxyproline (P*). This, together with the observed traces of Hyp in the amino acid analyses, strongly indicated that the modified amino acid found in these group V allergens was Hyp.

Discussion

The cross-reactivity observed for the investigated group V allergens is reflected in the primary protein structure. The amino acid composition of the analysed *Poa p V*, *Lol p V* and *Dac g V* (Table 1) are very similar and the NH₂-terminal sequences are homologous (Table 2). A common trait for all the investigated proteins are the presence of hydroxyproline and a high content of alanine (25–30%). These observations are in agreement with the results from previously published analyses on *Phl p V* [2]. The evolutionary relationships derived from the amino acid compositions (the Cornish-Bowden test), and the similarities in the NH₂-terminal sequences, indicate that the group V allergens represent homologous protein in grass pollens. This is in agreement with the commonly found cross-species sensitization that is seen in grass pollen allergic individuals [19–21].

The similarities of the group V allergens are evident when comparing the amino acid compositions of the investigated allergens (Table 1), including the previously purified *Phl p V* [4]. According to the Cornish-Bowden 'strong test' for relatedness of proteins [18] the ΔQ values (Table 2) calculated for *Poa p V*, *Lol p V* and *Dac g Va* indicate that the proteins are related. The fact that the composition of the impure preparation of *Dac g Va* still holds in this test, further confirms the idea of a mixture of homologous proteins. Even though *Dac g Vb* and *Phl p V* do not fit the prerequisites of the test (regarding size and identical analysis conditions), it is notable that their amino acid composition profiles are very similar to the other group V allergens (Table 1). Comparing *Lol p V* with *Lol p I* [13] reveals no such similarity. The main contributor to this significant degree of integrity of the group V allergens is the extremely high alanine content. This alone could be seen as a marker of the group V allergens, as it has not been found in the composition of any previously reported grass pollen allergens [22,23].

From the sequence data presented (Table 2) it is seen that the analysed bands from *Lol p* and *Poa p* were sufficiently pure to determine unambiguous NH₂-terminal sequences. The two bands from *Dac g V* (a & b, Table 1) resulted in double sequences, probably due to insufficient separation of the bands in the tricine SDS-PAGE system. The results indicate that the sample contains a mixture of two proteins which have the characteristic group V amino acid composition. The result may therefore be taken as a tentative indication of an isoallergenic variation of *Dac g V* (comparable to the results for *Phl p V* [4]).

The high identity (60–100%) of the aligned group V allergen NH₂-terminal sequences (Table 2), including *Phl p V*, is in agreement with the results of the amino acid

Table 2. The one letter code of the NH₂-terminal sequences of the group V allergens, including the previously analysed *Phl p V* [4]. The sequences are aligned with that of *Phl p V*, according to the highest similarity, obtained by aligning the conserved six amino acid 'block', position 7–12. The frame indicates the possible identical positions in this fit.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
<i>Phl p V</i>		A	S		A													
		G	D	L	G	Y	G	P*	A	T	P*	A	A	P*	G	A	E	Y
<i>Poa p V</i>		A	D	A	G	Y	A	P*	A	T	P*	A	A	A	G	A	A	A
<i>Lol p V</i>				A	A	E	T	P*	A	T	P*	A	A	T	P*	A	A	
<i>Dac g Va</i>						A							A					
				A	Y	T	P*	Y	T	P*	A	G	A					
<i>Dac g Vb</i>				A	Y	T	P*	A	A	P*	A	G	A					

analysis. A notable feature of the NH₂- terminals are six amino acid residues: -Hyp-Ala-Thr-Hyp-Ala-Ala- (Table 2), found in both *Lol p V* and *Poa p V* (the first four residues are also found in *Dac g Vb*). The same six residue sequence has previously been reported as a repeated sequence in the NH₂-terminal of *Phl p V* [4]. This could indicate a role in the function of the group V allergens. A database search [24] for homologies to the total sequences and to this motif alone, scored no other sequence.

The previously reported cross-reactivity of MoAbs raised against *Phl p V* and the group V allergens from related species [9] could itself be seen as an indication of similarities at the protein structure level that leads to the presence of homologous B-cell epitopes. The phenomenon of cross-reactive MoAbs has also been reported for grass pollen group I allergens by several investigators [25–27], who have demonstrated that grass pollen group I allergens possesses both unique and group specific B-cell epitopes. This also seems very likely for the group V allergens and would explain the different specificities of the previously investigated MoAbs [9], from which PpV4 was selected.

Another important feature of the grass pollen allergens is the isoallergenic variation, mentioned above. This variation is further underlined in IEF, where the products from the affinity purifications reveal a number of bands, that greatly exceed the number seen in SDS-PAGE (not shown). To which degree this variation is due to sub-species variation, differences in the post-translational modifications, proteolytic degradation, etc. is still subject

for future studies. As this variation is still not characterized, it can not be excluded that the selection of a single MoAb for an affinity purification step, might exclude some of the isoallergenic variants. A considerable isoallergenic variation has been shown in other pollen allergen systems, e.g. *Amb a* [28]. The presence of multiple highly homologous, non-identical clones coding for the major allergen, *Amb a I*, suggests the possibility of variation at the cDNA level. The probable existence of the grass major allergen groups as families of isoallergens rather than single proteins do not conflict with the classical division in major allergen groups. However, it greatly accentuates the complexity of these allergen systems.

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