

Comparison of Four Grass Pollen Species Concerning Their Allergens of Grass Group V by 2D Immunoblotting and Microsequencing

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Summary: The identification and characterization of allergenic components is a vital step towards improving diagnosis and therapy. Members of the grass family (Poaceae) reveal a high cross-reactivity among each other caused by the close phylogenetical relationship. In order to investigate the variability between allergenic components, we studied the allergen grass group V, one of the major allergens. Pollen extracts of 4 different tribes (timothy grass (*Phleum pratense*) – Agrostidae, perennial rye grass (*Lolium perenne*) – Festuceae, meadow velvet (*Holcus lanatus*) – Aveneae, and rye (*Secale cereale*) – Triticeae) of the Festucoideae subfamily were sepa-

rated by 2D PAGE and investigated by immunoblotting using patients' poolserum and monoclonal antibodies (raised against group V allergens of timothy grass pollen). The antibodies identify different allergens in the four grass species. The components vary from 30–50 kDa and pI 4.8–7.0. The eight NH₂-terminal amino acids were determined and indicated high similarities between the different components. These results cast doubt on the suitability of classifying allergens into groups based only on their molecular mass, isoelectric point and N-terminal sequence analysis. It suggests to classify allergens according to their IgE-reactive epitopes.

Key terms: Grass group V, immunoblotting, microsequencing, pollen allergens, two-dimensional polyacrylamide gel electrophoresis (2D PAGE).

Grass pollen are the most common allergens causing immediate type reactions that are mediated by IgE-antibodies. Usually the allergenic symptoms are not provoked by only one grass species but by exposure to other grass pollen as well. The reason for their cross-reactivity to allergens of different grass pollen species is the close phylogenetical relationship^[1,2]. For immunotherapy, e.g. hyposensitization therapy, the treatment with well characterized extracts is considered that should only contain relevant IgE-reactive components^[3,4]. It is, therefore, necessary to investigate whether or not the allergenic components of different grasses are identical.

In Northern and Central Europe about 90% of patients suffering from pollinosis reveal IgE-reactivity against the major allergen of timothy grass pollen (*Phleum pratense*) termed Phl p V^[5]. By cross radio immuno electrophoresis (CRIE) Matthiesen and Løwenstein^[6] demonstrated that similar immunological reactivities are also detectable in many other grass pollen. The variability of cross-reactivity of IgE antibodies was investigated by means of RAST-inhibition tests in several groups^[7,8]. Although the degree of cross-reactivity was determined between different grasses, the structure of the allergenic molecules and the IgE-reactive epitopes remained obscure. Klysner

Abbreviations:

IEF: isoelectric focusing, IgE: immunoglobulin E, PAGE: polyacrylamide gel electrophoresis, P': hydroxyproline, pI: isoelectric point, SDS: sodium dodecyl sulfate, 2D: two-dimensional.

et al.^[9] and Roberts et al.^[10] isolated allergens of group V from grass pollen extract for amino acid sequencing by affinity chromatography using immobilized monoclonal antibodies.

We separated crude pollen extracts with high resolution by 2D electrophoresis in order to prevent loss of any component that might not be detected by the catching antibody and to circumvent time-consuming purification steps. Using this method we were able to investigate the extract components for their immunological reactivities as well as for their NH₂-terminal amino acids. The four tribes under investigation belong to the Festucoideae subfamily and are widespread in our areas. Based on the studies of timothy grass pollen^[11] we investigated allergens of group V in perennial rye grass (*Lolium perenne*), meadow velvet (*Holcus lanatus*) and rye (*Secale cereale*).

Materials and Methods

1) Monoclonal antibodies

The monoclonal antibodies Bo 1^[12], BG 6 and Bo 9 were raised against crude timothy pollen extract. In timothy grass pollen they are specific for Phl p V, Phl p Va and Phl p Vb, respectively.

2) Patients' poolserum

Detections of allergenic components were performed by a poolserum consisting of 3 patients' sera of RASTscore IV (radioallergo sorbent test (Pharmacia, Bromma, Sweden) for the quantification of specific IgE, scores according to manufacturer's manual from RAST I (low concentration) to RAST IV (high concentration)).

3) Preparation of the pollen extracts

1 g of grass pollen (ARTU Biologicals, Lelystad, The Netherlands) was suspended in 55 ml of 0.1M (NH₄)HCO₃ buffer (pH 8.0) and incubated under agitation at room temperature for 30 min. After centrifugation the supernatant was removed from the sediment, dialysed against double-distilled water overnight and lyophilized afterwards.

4) 2D PAGE blotting

The 2D PAGE was performed according to Görg et al.^[13]. In the first dimension (IEF) we used flat gels (T = 4%, C = 4%; size 240 × 80 × 0.5 mm³) with an immobilized pH gradient from 3.6–9.3. Pollen extract was diluted to a concentration of 100 µg per gel strip (8 cm). Isoelectric focusing was performed for 7 h limited to 3000 V, 2 mA, 5 W. SDS gradient gels (size 240 × 80 × 0.5 mm³) were prepared with stacking gel (T = 5%, C = 4%) and separating gel (T = 8–12%, C = 4%). After equilibration of the IEF strips with SDS-containing sample buffer for 30 min, the separation by SDS-PAGE was limited to 800 V, 30 mA and 30 W.

For immunological investigations blotting was performed onto nitrocellulose membrane by thermoblotting^[14]. Each gel was blotted 4 times for 15 min onto different sheets of nitrocellulose membrane. For NH₂-terminal sequencing of the allergens we employed elec-

troblotting onto ProBlott membrane (Applied Biosystems, Weiterstadt, BRD) using 10mM CAPS (3-cyclohexylamino-1-propyl-sulfonic acid) pH 11.0 as blotting buffer^[15].

5) Protein staining and immunological detections

After blotting the nitrocellulose membrane was incubated in 0.1M Tris-buffered saline (pH 7.4) containing 0.05% (v/v) Tween 20 for blocking free binding sites. (This buffer was also used for washing steps and for the dilutions of antibodies.)

The protein staining was performed by incubating the blotting membrane at first in 0.1% (v/v) India ink (Pelikan AG, Hannover, BRD)^[16] and afterwards in AuroDye solution (Janssen Biochimica, Beerse, Belgium).

For immunological detections of antigens and allergens the membrane was incubated with supernatants of hybridomas and patients' sera diluted 1:40 and 1:20, respectively. For secondary antibodies we used alkaline phosphatase (AP)-conjugated goat-anti-mouse IgG/M (Dianova, Hamburg, BRD) diluted 1:10000 or AP-conjugated goat-anti-human IgE (Medac, Hamburg, BRD) diluted 1:1000. The binding patterns were visualized by a substrate solution of 0.1M Tris-buffered saline (pH 9.5) containing 0.033% (w/v) nitroblue tetrazolium chloride (NBT) and 0.017% (w/v) 5-bromo-4-chloro-3-indolyl phosphate, potassium salt (BCIP) (Serva, Heidelberg, BRD)^[17].

6) Amino acid sequence analysis

After electroblotting the ProBlott membrane was stained with Coomassie and the spots of interest were cut out. Microsequencing was performed by Edman degradation using the 473 A protein sequencer with on-line PTH (phenylthiohydantoin) amino acid analyser of Applied Biosystems (Weiterstadt, BRD).

Results

1) Identification of allergens by patients' serum and timothy grass group V directed monoclonal antibodies

For the identification of timothy grass group V related structures in perennial rye grass, meadow velvet and rye pollen we performed 2D immunoblotting. The binding patterns of the IgE-reactive components were compared with the patterns identified by the monoclonal antibodies Bo 1, BG 6 and Bo 9.

As indicated in Fig. 1 and Table 1 all spots detected by the 3 monoclonal antibodies were also detected by patients' IgE, thus proving that these proteins are allergens.

Different monoclonal antibodies were used for identifying allergens of group V, since they showed different reactivities to group V allergens in timothy grass pollen. The molecular masses of the detected proteins are 30–40 kDa except for a 50 kDa reactive protein in perennial rye grass. In some cases there are protein spots of identical molecular mass but different pI detectable, e.g. perennial rye grass reveals at least five components of 30 kDa with pI of 4.9, 5.1, 5.3, 6.1 and 7.2. (For more detailed information please refer to Fig. 1 and Table 1.) The binding patterns of monoclonal antibody BG 6 is peculiar, since it does not bind

* Petersen, A., Becker, W.M. & Schlaak, M., unpublished results.

to any components in meadow velvet and rye. The extracts of both grass species reveal identical binding patterns by use of Bo 1 or Bo 9. In both cases we only identified components of 30 kDa.

Protein staining of the three grass pollen extracts after 2D PAGE blotting revealed the following results (Fig. 1): All components recognized by patients' pool-serum as well as by one of the monoclonal antibodies were not detectable on the nitrocellulose membrane by India ink staining. After subsequent staining of the membranes with AuroDye the allergens under inves-tigation could be visualized as red spots. Using this two-step staining procedure also smaller IgE-reactive proteins of about 14 kDa were identified.

2) *Comparison of the NH₂-terminal sequences of the allergens*

Those protein spots that have been detected by one or more monoclonal antibodies and by patients' IgE (presumably allergens of grass group V) were used for sequence analysis. We determined the sequences of the most prominent spots (indicated spots in Fig. 1).

Table 1. Reactivities of monoclonal antibodies directed against timothy grass group V in pollen of different grass species. The molecular mass of the allergens detected by the monoclonal antibodies is indicated (kDa). PS = patients' poolserum; Bo 1, BG 6, Bo 9 = monoclonal antibodies directed against allergens of timothy grass group V. Molecular masses of antigens with weak antibody reactivities are indicated by brackets.

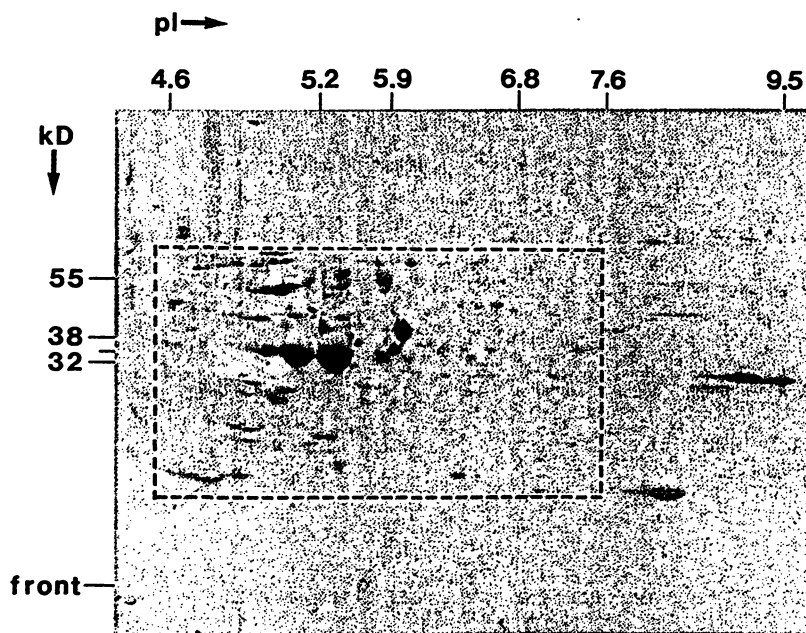
Grass spec. Antibodies	Perennial rye grass (<i>Lolium perenne</i>)	Meadow velvet (<i>Holcus lanatus</i>)	Rye (<i>Secale cereale</i>)	Timothy grass (<i>Phleum pratense</i>) ^[11]
PS	55 50 40 34 32 30 (25) (20) 14	36 (33) 30 14	38 30	55 38 37 35 32 14
Bo 1	32 30 (25) 20	(33) 30	30	38 32
Bo 9	(32) 30	30	30	32
BG 6	50 40 34			38

Table 2. N-Terminal microsequencing of the allergens detected by the timothy grass group V directed monoclonal antibodies (sequence identities are highlighted). The protein spots analysed are shown in Fig. 1.

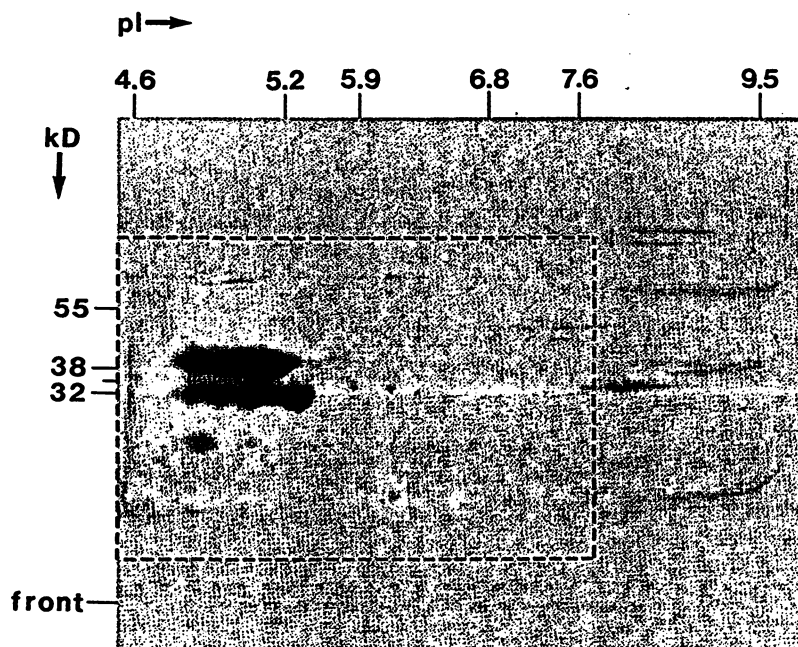
Grass spec.	Allergen	Spot No	N-Terminal sequence																			
			1	5	10	15	20															
Perennial rye grass	50kDa	1	A	D	A	G	Y	A	P'	A	T	P'	A	T	P'	A	A	G	A	T	P'	A
	34kDa	2	A	D	A	G	Y	A	P'	A	T	P'	A	T	P'	A	A	P'	A	T	A	
	30kDa	3a,3b	A	D	A	G	Y	T	P'	A	A	A	A	T	P'	A	T	P'	A	A	T	P'
Meadow velvet	30kDa	4a,4b	A	D	A	G	Y	T	P'	A	A	P'	A	A	T	G	A	G	G	D	A	T
Rye	30kDa	5a	G Y A P' A A P' G T Q P K A																			
		5b	G Y A P' A A P' G T Q P K A A D A G Y																			
Timothy grass ^[11]	38kDa		A	D	L	G	Y	G	P'	A	T	P'	A	A	P'	A	A	G	Y	T	P'	A
	32kDa		A	D	A	G	Y	A	P'	A	T	P'	A	A	A	G	A	E	A	G	K	A

Fig. 1. Detection of allergens of grass group V in perennial rye grass (I), meadow velvet (II) and rye (III) pollen extract. General view of the protein patterns stained first by India ink and subsequently by AuroDye (a). Identification of components by patients' IgE (b) and by monoclonal antibodies Bo 1 (c), BG 6 (d) and Bo 9 (e). (Protein spots that were investigated by micro-sequencing are indicated by numbers).

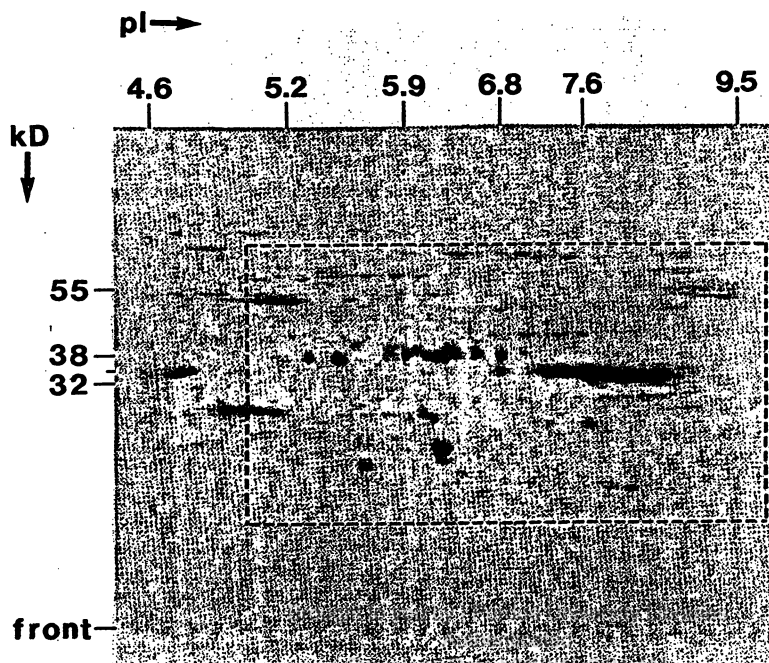
1a



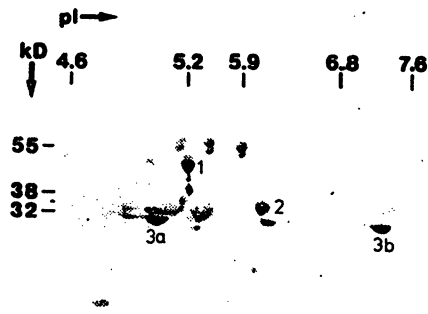
2a



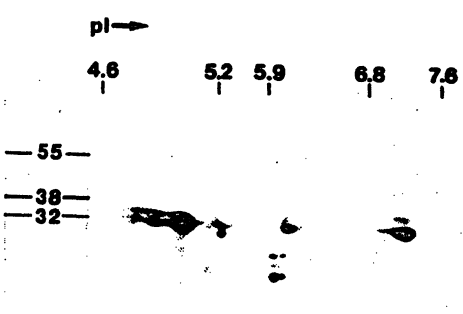
3a



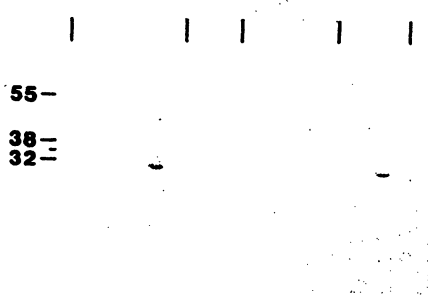
1b



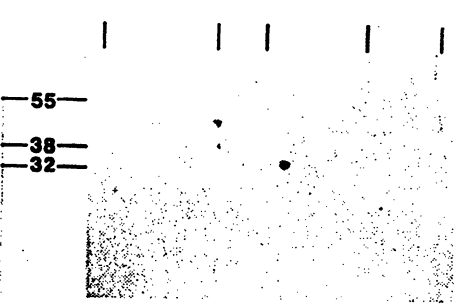
1c



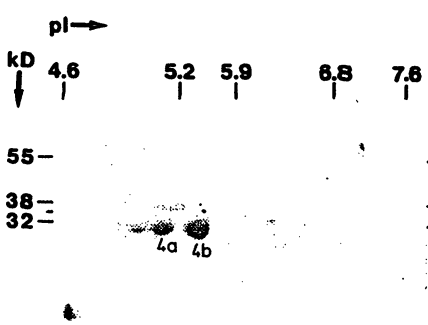
1d



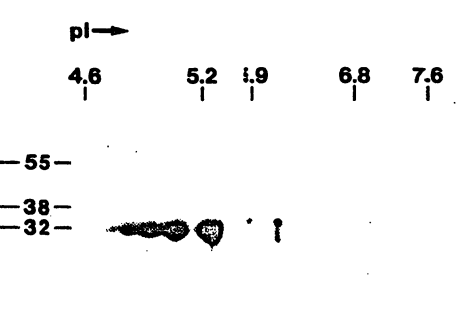
1e



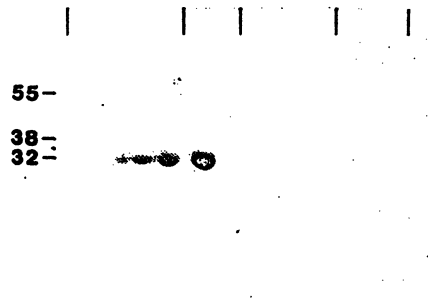
2b



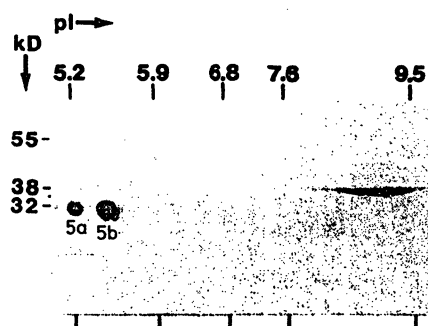
2c



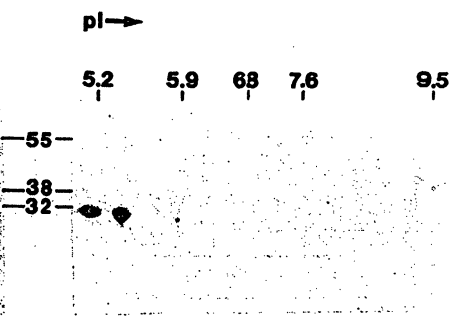
2d



3b



3c



3d

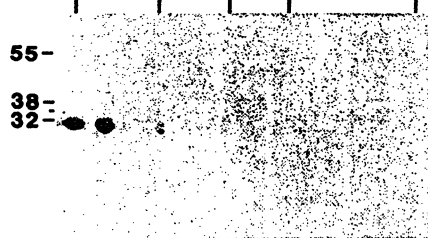


Table 2 demonstrates the 20 N-terminal amino acids of perennial rye grass, meadow velvet, rye and timothy grass pollen (as a control). The sequences are very similar indicating nearly identical proteins. Each of the grasses contains hydroxyproline residues. As regards the eight NH₂-terminal amino acids each of the four grasses reveals identical sequences, except in position 3 of the 38-kDa timothy grass allergen leucine has occupied the position of alanine. By sequencing the 30-kDa protein (5b) of rye we identified each two amino acids in the HPLC chromatogram. One signal was quite strong (main sequence), while the other showed an intensity of only about a quarter. The less concentrated component revealed the identical sequence compared to those of the other grasses, while the main sequence was lost of the first 3 NH₂-terminal amino acids. Above position 8 the variations in the amino acid residues increased.

Discussion

We studied the variability of major allergens in pollen extracts, termed group V, in four grass species provoking IgE-mediated allergy. Since the IgE-reactive epitopes were not determined till now, we had to use several criteria for the identification of such components.

The classification into allergen grass groups, e.g. group V, is based on the assumption that there is at least one kind of IgE-reactive epitopes on an allergenic molecule. Allergenic components with identical IgE-binding epitopes are assumed to show similar immunological reactivities, molecular sizes, isoelectric points and chemical criteria, e.g. N-terminal sequence^[18].

The isolation of certain allergens from extracts is often done by affinity chromatography using "grass group specific" monoclonal antibodies^[9,10]. We expect that this might cause a loss of IgE-reactive components, since the monoclonal antibody and patients' IgE bind to different epitopes. On the other hand, the antibody-binding fraction might be a mixture of different components, as in the case of Phl p V: Matthiesen and Løwenstein^[6] determined the NH₂-terminal amino acid sequences of the whole group of Phl p V isoallergens, a mixture of the two subgroups Va and Vb that show structural differences between each other.

In order to circumvent these problems, we used the 2D electrophoresis for the separation of the crude extract components with subsequent blotting. We were thus able to apply several classification criteria for fine-tuning of the identification process of allergens. The 2D PAGE blot has proved to be a suitable

technique for the high resolution separation of such complex protein extracts, since the separation criteria are independent from each other. This method is the prerequisite for immunological detections as well as for biochemical investigations (microsequencing) in our studies. Thus, in contrast to other studies in this field done by affinity chromatography^[9,10], we were able to differentiate molecules with similar immunological reactivity through molecular mass and pI. The risk that some allergens were not identified after 2D PAGE blot was minimized by use of three monoclonal antibodies binding to different sites of the group V allergens in timothy grass pollen.

Most of the group V allergens identified by the monoclonal antibodies reveal molecular masses of 30–40 kDa, but we also detected a 50-kDa allergen in perennial rye grass. We identified this protein several times and the microsequencing data clearly indicated the absence of impurities and aggregated complexes. Furthermore, the molecular mass seems to be no suitable criterion for classification, since many allergens are in the range of 30–40 kDa, e.g. the major allergen grass group I^[19].

A lot of the identified allergens consists of isoforms of similar molecular sizes but different isoelectric points. E.g. the 30-kDa allergens of perennial rye grass consist of at least five isoforms ranging from 4.9–7.2. Therefore, an allergen classification by molecular charge is inappropriate, since it would be the result of an isoallergenic mixture or a citation of pI values of each isoform, which might cover a wide pI range. The occurrence of such isoallergens indicates slight differences in the molecule structure. Perez et al.^[20] sequenced two cDNA clones of Lol p I that coded for proteins of 240 amino acids only differing in four amino acids from each other. It is imperative to investigate the reason for the differences in the isoforms and their possible influence on the IgE-binding sites.

Another criterion for defining allergens of grass group V was the protein staining on the blotting membrane. Former studies of timothy grass pollen demonstrated that India ink did not stain allergens of group V, whereas they were capable of being stained later with AuroDye^[11]. Although the staining mechanism of India ink has not been understood till now, the sensitivity depends largely on the protein nature^[16]. But the classification according to this staining behavior is not absolute: Apart from allergens of group V there are IgE-reactive components of about 14 kDa in each pollen extract. These proteins show similar staining behavior, but they do not react with the monoclonal antibodies. Microsequencing of the 14-kDa proteins of timothy grass pollen assigned

them as grass group VI (unpublished results). The N-terminal sequence of Phl p VI showed high similarities with an internal sequence part of grass group V^[21]. Although it is not clear whether Phl p VI is a cleavage product of grass group V or whether it stems from a partly duplicated gene (Matthiesen, pers. information), similarities between both allergen grass groups are obvious. Such facts demonstrate the difficulties in classification.

The immunologically identified allergen grass group V components of the four species revealed high similarities concerning their NH₂-terminal sequences. Especially the amino acid positions 1, 2, 3, 4, 5, 7 and 8 were identical, except of the main sequences of the 30-kDa allergens from rye that lost the first three amino acid residues. Roberts et al.^[10] determined the identical sequence for the Sec cV, confirming our data. Besides such structural changes in the N-terminal sequence, crossing-over may have taken place during phylogenesis of the grasses, resulting in the cleavage of the N-terminal sequence from the IgE-epitope. Therefore, we cannot be sure whether the IgE-binding epitopes and the NH₂-terminal sequence are parts of the same protein, although the N-terminal sequences are similar. Thus, the classification of allergens by their NH₂-terminal amino acid structure is possible but not compelling for molecules of similar IgE-binding epitopes. Partial gene duplications may have taken place as we identified allergens of grass group V of only one molecular size in meadow velvet and rye, while we detected two subforms in timothy grass (38 and 32 kDa) and six subforms in perennial rye grass (50, 40, 32, 30, 25 and 20 kDa).

The investigation of the allergens in four different grass species clearly demonstrates that the allergen grass group classification must be handled with care. There are a lot of exceptions for each of the classification criteria. Since we used several criteria for the identification of allergens, we probably detected allergenic molecules of similar IgE-reactive epitopes. We must aim at identifying the IgE-binding epitopes of the allergenic molecules to make the classification dependent on the structure of the IgE-reactive epitopes.

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