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Identification and Characterization of the Major Allergens of Velvet Grass (*Holcus lanatus*), Hol I 1 and Hol I 5

Key Words

Velvet grass pollen
Major allergens
Immunoblotting
PAGE, two-dimensional
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Abstract

Although extract of velvet grass pollen is an important ingredient of commercial allergen extracts for immunotherapy, it is not defined concerning its major allergenic components. We have investigated the extract of velvet grass pollen by Western blotting using the sera of 97 patients and identified two major allergens of 34 and 30 kD. By two-dimensional immunoblotting with monoclonal antibodies and lectins, and by biochemical analysis the 34-kD protein was identified as a group I grass pollen allergen and the 30-kD protein as a group V grass pollen allergen. According to the nomenclature for grass pollen allergens we designated these allergens Hol I 1 and Hol I 5, respectively. Fragmentation of the velvet grass pollen extract by CNBr provided the first information on the localization of IgE binding epitopes on these two allergens. With Hol I 1 and Hol I 5 we identified two important components of commercial allergen extracts.

Introduction

Velvet grass (*Holcus lanatus*) is a common perennial grass species in Europe and North America. It flowers in the early summer and is wind-pollinated. In contrast to cultivated grass species like timothy grass or rye grass, velvet grass is often found at the roadside and in waste areas, and is considered as a weed. Unlike the cultivated grasses, which are regularly harvested, velvet grass reaches more often pollination and is therefore an important source for pollen causing type I allergy. Taking this into account, the extract of velvet grass pollen is an ingredient of commercial allergen preparations used for immunotherapy of type I allergy, although nothing is known about the relevant allergens [1, 2].

Hyposensitization therapy of allergy to grass pollen, house dust mite or bee venom is done with preparations based on crude allergen extracts. Best results are obtained in bee sting allergy where extracts contain few, well-defined allergens, as for example PLA₂ [3]. On the other hand, ex-

tracts of grass pollen contain up to 70 different components, mostly proteins, in varying amounts as shown by two-dimensional Page (2D-PAGE) [4, 5]. To improve immunotherapy for grass pollen allergy it seems desirable to use clearly defined and characterized allergens. Since it is known that patients' sera show an individual reactivity with the different pollen allergens [6], it might be useful to apply tailor-made allergen preparations for individual immunotherapy of each patient [7]. Purification of the relevant allergens is expensive and time-consuming. Since the application of molecular cloning techniques it seems possible to produce high amounts of purified recombinant allergens. To get detailed information on the relevant allergens, it is necessary to identify and characterize the major allergenic components of grass pollen extracts.

In some grass species, for example timothy grass, rye grass and Kentucky bluegrass, the major allergens were identified and classified into different allergen groups, according to their physico- and immunochemical properties

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[8]. Although this allergen classification suggests uniformity, single components of an allergen group reveal a variability in their primary structure, which might have an influence on the IgE reactivity. The most important and best-characterized allergens are the group I and group V allergens. They were found in nearly all investigated grass species [8]. Group I allergens have molecular weights of 31 to 36 kD and a carbohydrate content of approximately 5%. Group V allergens have molecular weights of 30–38 kD and consist of two isoforms, which were classified into the two subgroups a and b, for example the 38- and 32-kD group V allergens of timothy grass, Phl p 5a and Phl p 5b [9, 10].

In contrast to the pollen allergens of other grass species the allergens of velvet grass have been poorly characterized so far. We examined extract of velvet grass pollen by immunoblotting using the sera of 97 patients and monoclonal antibodies to identify the allergens and to analyze their structural variability in comparison to allergens from other grass species.

Materials and Methods

Patients' Sera and Monoclonal Antibodies

For identification of IgE-reactive proteins we used patients' sera collected in the Medical Clinic Borstel. The atopic phenotype was confirmed by clinical history and diagnosis, positive skin prick test to timothy grass pollen allergens and CAP score $\geq$ IV (Pharmacia CAP system, radioallergosorbent test for quantitation of specific IgE, scores according to manufacturer's manual, Pharmacia Diagnostics, Uppsala, Sweden). Control sera were collected from healthy donors. The monoclonal antibodies were raised against crude timothy grass pollen extract. Bo 1 is directed against Phl p 5a and b [11], BG6 against Phl p 5a and Bo9 against Phl p 5b [12]. IG 12 recognizes group I allergens [13].

Preparation of Velvet Grass Pollen Extract

One gram of velvet grass pollen (ARTU Biologicals, Lelystad, The Netherlands) was suspended in 55 ml of 0.1 M NH_4HCO_3 buffer (pH 8.0) and incubated under agitation in parallel for 30 min at room temperature, overnight at 4°C and overnight at 4°C in the presence of protease inhibitor (Complete, Boehringer, Mannheim, Germany) according to the manufacturer's instructions. After centrifugation for 30 min at 17,000 g (4°C), the supernatant was dialyzed against double-distilled water. After lyophilization the extract was stored at 4°C until use.

Western Blot

Proteins (5 $\mu\text{g}/\text{cm}$ gel) were separated by SDS-PAGE (12% T, 4% C; size 120 $\times$ 80 $\times$ 0.5 mm³) according to Laemmli [14]. The proteins were subsequently transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by semidry blotting for 30 min at 0.8 mA/cm² [15].

2D-PAGE Blot

The 2D-PAGE was performed according to Görg et al. [16]. For the first dimension we used gels (4% T, 4% C, size 240 $\times$ 80 $\times$ 0.5 mm³) on Gelbond film (Pharmacia/LKB, Bromma, Sweden) with an immobi-

lized pH gradient ranging from 3.6 to 9.3 and 4.0 to 7.0, respectively. The velvet grass pollen extract was diluted to a concentration of 100 μg per lane. Isoelectric focusing was performed for 7 h at 3,000 V and 2 mA. SDS gels (size 240 $\times$ 80 $\times$ 0.5 mm³) were prepared by casting the stacking gel (5% T, 4% C) first and the separating gel (8–12% T, 4% C) immediately afterwards. After the IEF gel stripes were incubated for 30 min in equilibration solution containing 6 M urea, 65 mM dithiothreitol and 260 mM iodacetamide, the separation by SDS-PAGE was carried out at 200 V, 30 mA and 30 W in the stacking gel and at 800 V in the separating gel. As pI marker the protein test mix 9 (Serva, Heidelberg, Germany) and as molecular weight standard the low range SDS-PAGE standard from BioRad (Richmond, Calif., USA) were used. For immunological studies blotting was performed onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by capillary force with a pressure of 0.2 Pa on a heating plate set to 50°C [17]. For amino acid analysis electroblotting was performed onto ProBlott membrane (Applied Biosystems, Weiterstadt, Germany) for 30 min at 200 mA, using 10 mM CAPS, pH 11.0 as a blotting buffer [18].

Protein Staining and Immunological Detection

After blotting the nitrocellulose membrane was incubated in 0.1 M Tris-buffered saline (TBS; pH 7.4) containing 0.05% (v/v) Tween 20 for blocking free binding sites. For protein staining the blotting membrane was incubated with 0.1% (v/v) India ink (Pelikan, Hannover, Germany) [19] and afterwards in AuroDye (Janssen Biochemica, Beerse, Belgium). For immunological detections of allergens the membrane was incubated with supernatants of hybridomas or patients' sera. As secondary antibodies we used goat anti-mouse IgG/M conjugated with alkaline phosphatase (Dianova, Hamburg, Germany) diluted 1:10,000 or alkaline phosphatase-conjugated monoclonal mouse anti-human IgE (Dianova, Hamburg, Germany) diluted 1:10,000. The binding patterns were visualized by a substrate/chromogen mixture of 0.033% (w/v) nitro blue tetrazolium (NBT) and 0.017% (w/v) 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Serva, Heidelberg, Germany) in 0.1 M TBS (pH 9.5) [20]. To study the carbohydrate moieties, blotted samples were incubated with different digoxigenin-labeled lectins (glycan differentiation kit, Boehringer, Mannheim, Germany). The following ten lectins were used AAA (*Aleuria aurantia*), ConA (*Conavalina ensiformis*), DSA (*Datura stramonium*), GNA (*Galanthus nivalis*), MAA (*Maackia amurensis*), PNA (*Arachis hypogaea*), RCA (*Ricinus communis*), SNA (*Sambucus nigra*), WGA (*Triticum vulgare*) and PHA (*Phaseolus vulgaris*). Lectin binding was visualized by alkaline phosphatase-labeled anti-digoxigenin and an NBT/BCIP substrate/chromogen mixture.

Glycoprotein Detection

Carbohydrate detection was performed by the glycan detection kit (Boehringer, Mannheim, Germany) according to the manufacturer's description. In brief, vicinal hydroxyl groups of carbohydrates were oxidized to aldehydes by 10 mM NaIO_4 in 100 mM sodium acetate buffer (pH 5.5) for 20 min. Then the steroid hapten digoxigenin was covalently bound to the aldehyde groups via a hydrazide group. Glycoproteins were visualized by alkaline phosphatase-labeled anti-digoxigenin followed by incubation in NBT/BCIP substrate.

Amino Acid Sequence Analysis

The ProBlott membrane was washed in double-distilled water after blotting for 30 min. Afterwards the membrane was stained by 0.1% (w/v) Coomassie R-250 (Serva, Heidelberg, Germany) in 50% methanol for 10 min, destained in 50% methanol and air-dried. Protein spots

containing the presumed allergen were cut out. Microsequencing was performed by Edman degradation using a 473 A protein sequencer with on-line PTH amino acid analyzer (Applied Biosystems, Weiterstadt, Germany).

CNBr Fragmentation [21]

One milligram of pollen extract was dissolved in 100 µl 70% (v/v) formic acid. After adding 4 mg CNBr the solution was kept in the dark under agitation for 24 h. To eliminate excess CNBr the extract was diluted 10-fold with double-distilled water and lyophilized.

Results

Preparation of Velvet Grass Pollen Extract

To determine the most effective method for preparation of velvet grass pollen extract, several methods described in the literature were compared. Extracts were prepared by incubation for 30 min at room temperature, overnight at 4°C and overnight at 4°C in the presence of a protease inhibitor mix. By extraction for 30 min at room temperature the same number and intensity of IgE-reactive bands were detected as compared to the other extraction conditions. No proteolytic degradation was observed [data not shown]. Therefore, this method was chosen for all further experiments.

Identification of the Major Allergens of Velvet Grass Pollen

For identification of the major allergens, crude velvet grass pollen was separated by SDS-PAGE and analyzed by immunoblotting for IgE reactivity of patients' sera ($n = 97$) and for reactivity of the following monoclonal antibodies: the group I-specific monoclonal antibody IG12 and the group V-specific monoclonal antibodies Bo1 (directed against 5a and b), BG6 (directed against 5a) and Bo9 (directed against 5b) (fig. 1, b–e). Patients' sera reacted mainly with a 34- and a 30-kD protein of crude velvet grass pollen extract in an individual manner. The 34-kD protein was recognized by 67% of the sera and the 30-kD protein by 64% of the sera. Fifty-three percent of the patients' sera showed IgE reactivity to both allergens, 14% only to the 34-kD allergen, 11% only to the 30-kD allergen. Few patients' sera reacted to a protein of 55 kD (17%) and a protein of 14 kD (5%). The 34-kD allergen was recognized by the group I-specific monoclonal antibody IG12, the 30-kD allergen by the group V-specific monoclonal antibody Bo1 and the group Vb-specific antibody Bo9. No reactivity was found with the group Va-specific antibody BG6. According to the nomenclature for grass pollen allergens [8] the 34-kD allergen was named Hol 11 and the 30-kD allergen Hol 15.

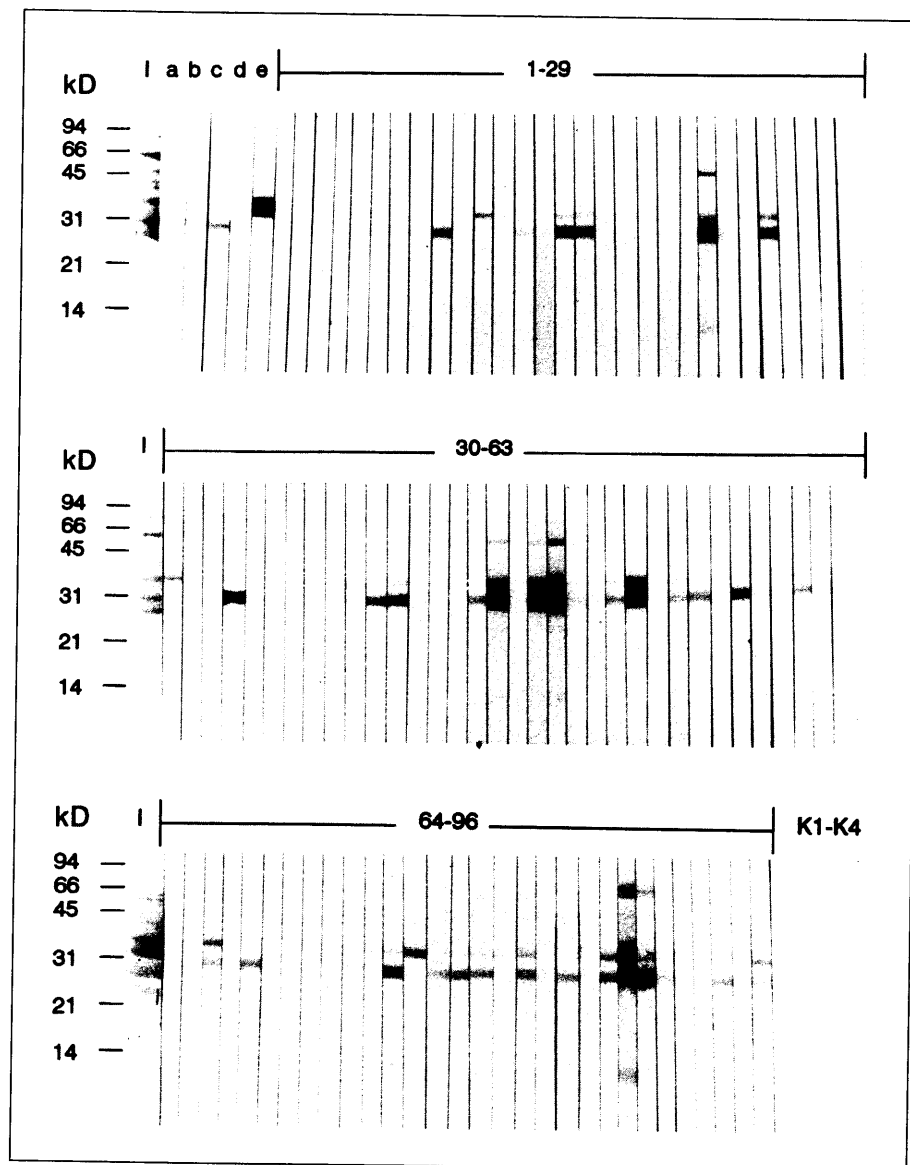
Characterization of the Major Allergens of Velvet Grass Pollen, Hol 11 and Hol 15, by Two-Dimensional Immunoblotting

For further analysis the crude extract of velvet grass pollen was separated by 2D-PAGE with a pH gradient ranging from 3.6 to 9.3 followed by sequential thermoblotting onto four sheets of nitrocellulose resulting in four identical protein blots. Proteins were detected by staining of the membrane with india ink and afterwards AuroDye (fig. 2; I.I.). The IgE-reactive allergens were detected by incubation of the membrane with patient's serum W (PS-W), which covers most of the IgE reactivities, and group I and group V allergens were detected by the monoclonal antibodies IG12 and Bo1. Only proteins of 34 and 30 kD from the crude mixture of extracted pollen proteins were detected by patients' IgE. The 34-kD allergen (Hol 11, see arrow, fig. 2) was identified by the group I-specific monoclonal antibody IG12, the 30-kD allergen (Hol 15, see arrow, fig. 2) was recognized by the group V-specific monoclonal antibody Bo1. It consists of 6 isoallergens of pI 4.7–6.2 (indicated spots 1–6, fig. 2). Under these conditions the separation of Hol 11 isoallergens was insufficient. To improve resolution 2D-PAGE was done in a pH range of 4.0–7.0 and again analyzed with india ink and AuroDye, patients' IgE and monoclonal antibodies. As indicated by numbers (fig. 3, 1–5) Hol 11 consists of at least 5 isoallergens with pI ranging from 4.6 to 5.2.

Analysis of the N-Terminal Amino Acid Sequences of Hol 11 and Hol 15

The indicated isoallergen spots 1–6 of Hol 15 (fig. 2) and 1–5 of Hol 11 (fig. 3) were cut out from ProBlott membranes and analyzed by microsequencing. As demonstrated in table 1 spots 1–6 of Hol 15 have identical N-terminal amino acid sequences as well as spots 1–5 of Hol 11. Both allergens show a high content of the amino acid alanine and the post-translational modified amino acid hydroxyproline. In position 9 of the sequence of Hol 11 no amino acid residue could be detected. Table 2 shows an N-terminal amino acid sequence alignment of some known group I and group V allergens of different grass species with Hol 11 and Hol 15, respectively. Within the group I allergens a very high similarity of about 90% is found. Sequences of group I allergens derived from recombinant allergens (rLol p1 and rPhl p1, table 2) reveal at position 9 the amino acid N within the context NXT, a possible motif for N-glycosylation. Group V allergens show a similarity of about 70%, which is a higher variability than the group I allergens. This also reflects the presence of the isoforms a and b which differ in molecular weights and sequences. Poa p 9 (belongs to the group V allergens) even possesses some additional amino acids at the

Fig. 1. IgE reactivity of patients' sera and reactivity of monoclonal antibodies (b–e) with velvet grass pollen extract analyzed by immunoblotting. I = Protein staining with india Ink., a = PS-W; b = Bo1 (group V-specific); c = Bo9 (group Vb-specific); d = BG6 (group Va-specific); e = IG12 (group I-specific). 1–96 represent individual patients' sera, K1–K4 sera of nonatopic individuals.



N terminus. To achieve the highest possible similarity of the sequences we aligned the amino acids 4–24.

Detection of Glycoproteins in Extract of Velvet Grass Pollen

A 2D-PAGE blot of velvet grass pollen extract was investigated for the presence of glycoproteins with the glycan detection kit (Boehringer, Mannheim, Germany; fig. 4). In parallel we developed an identical 2D-PAGE blot with the monoclonal antibody IG12 (specific for group I allergens). On both nitrocellulose sheets the corresponding spot was recognized by glycan detection and the monoclonal antibody IG12, respectively. For further characterization of the glycans an immunoblot of velvet grass pollen extract was

incubated with digoxigenin-labeled lectins and in parallel with the monoclonal antibodies IG12 and Bo1 (fig. 5). The 34-kD band of Hol 11 showed an intense reactivity to the lectin AAA (*A. aurantia* agglutinin), which mainly binds to α -fucose, and weak reactivity to Con A. The investigated lectins did not show any binding to the 30-kD band of Hol 15. Further components of velvet grass pollen extract bound to the lectins AAA, Con A, MAA, SNA.

Fragmentation of Velvet Grass Pollen Extract by CNBr

Extract of velvet grass pollen was treated with CNBr and investigated by immunoblotting with patients' serum (PS-W) and monoclonal antibodies BO1 and IG12 before (fig. 6A) and after (fig. 6B) fragmentation. After CNBr

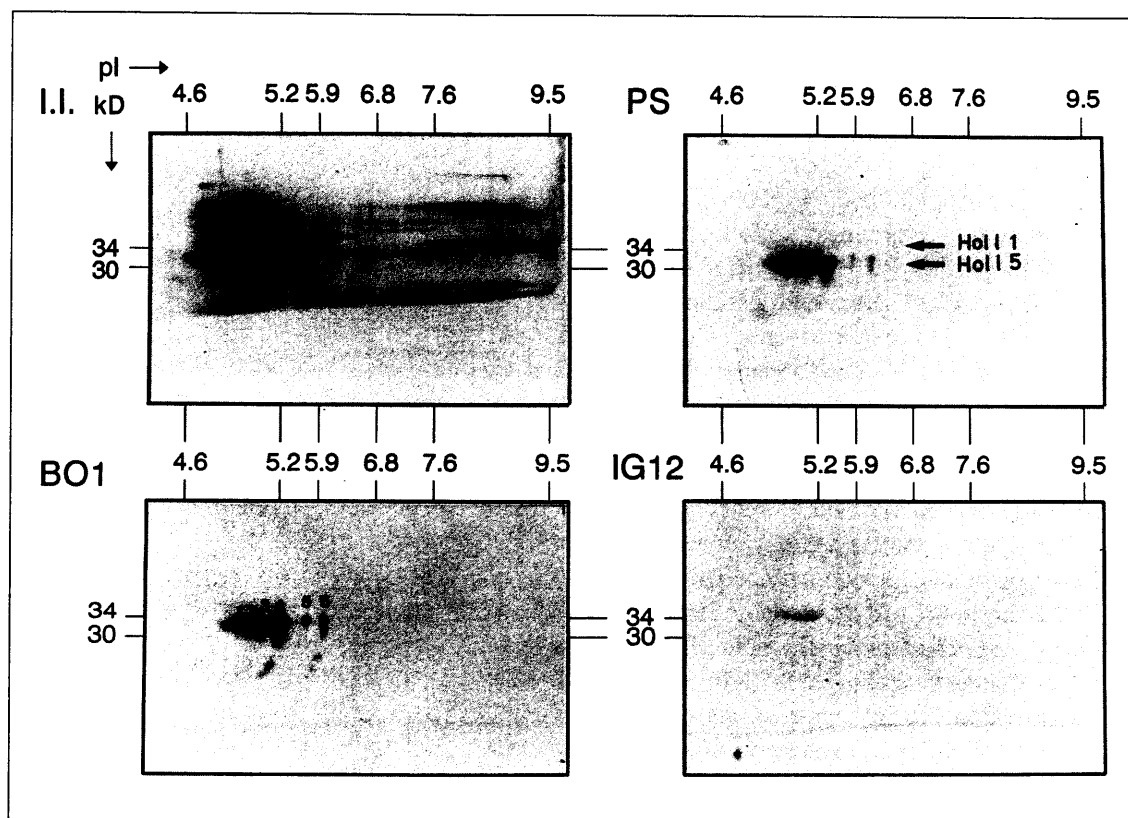


Fig. 2. Two-dimensional immunoblotting of velvet grass pollen extract with a pH gradient ranging between 3.6 and 9.3 incubated with PS-W and monoclonal antibodies BO1 (group V-specific) and IG12 (group I-specific). I.I. = Protein staining with india ink and afterwards with AuroDye. The two identified major allergens of velvet grass are indicated with arrows. The indicated protein spots (1–6) show the six isoallergens of Hol 1 5 and were isolated for amino acid sequence analysis.

treatment no reactivity of patient's IgE and of the group I-specific monoclonal antibody IG12 to fragments of Hol 1 1 was detectable. In the case of Hol 1 5 two bands of 29 and 30 kD were immunoreactive to patient's IgE as well as to the group V-specific monoclonal antibody BO1.

Discussion

In this study we identified the major allergens of velvet grass pollen by one- and two-dimensional immunoblotting of velvet grass pollen extract. For extract preparation various methods have been described. Several studies investigated the rate of extraction of the allergens from pollen and observed that most of the allergens were extracted after 5–15 min at room temperature as well as at 4°C [22–24]. Marsh et al. [22] showed that the extraction rate of ragweed pollen allergens increased 10-fold by increasing the pH of the extraction buffer from 6.0 to about 8.0. Also in the in

vivo situation in the nose the allergens are released from the pollen within minutes and in rhinitis an increased nasal pH of 8.0 compared to the normal pH 6.0 was determined [22]. From other groups different methods for preparation were used. For example an extraction method for 1 h at room temperature [25], overnight at 4°C [9] or overnight at 4°C in the presence of protease inhibitor [26]. Therefore, we tested three different methods for the preparation of velvet grass pollen extract with a buffer of pH 8.0 but no visible differences regarding the number and amount of extracted allergens were found. Altogether this prompted us to choose an extraction procedure of 30 min at room temperature in a buffer of pH 8.0, mimicking more closely the in vivo situation.

For immunoblot analysis we used 97 individual patients' sera and monoclonal antibodies specific for group I and group V grass pollen allergens. Two proteins of 34 and 30 kD, respectively, were recognized by more than 50% of patients' sera and, thus, defined as major allergens [27]. Pa-

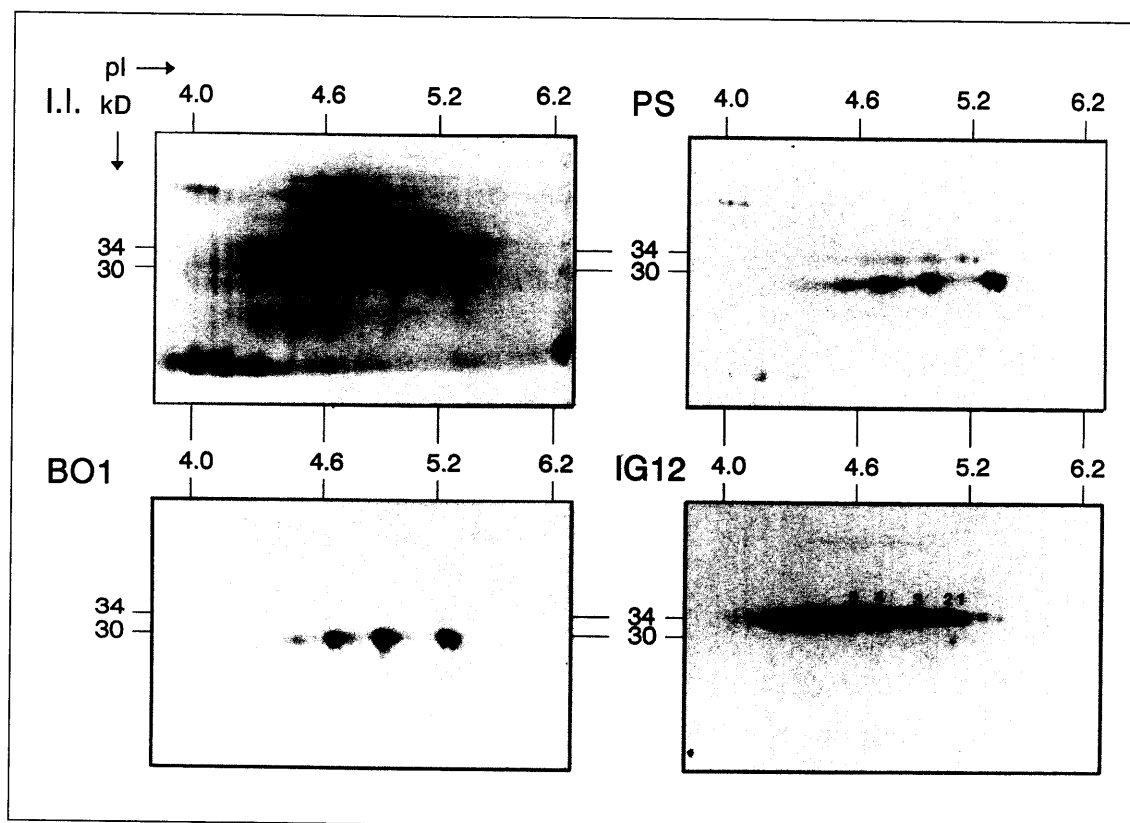


Fig. 3. Two-dimensional immunoblotting of velvet grass pollen extract with a pH gradient ranging between 3.6 and 7.0 for improved separation of Hol 11 isoallergens. I.I. = Protein staining with india ink and afterwards AuroDye. PS = PS-W (IgE); Bo1 = group V-specific monoclonal antibody Bo1; IG12 = group I-specific monoclonal antibody IG12. The indicated protein spots (1–5) show the isoallergens of Hol 11 and were isolated for amino acid sequence analysis.

tients' sera showed individual reactivity to the major allergens of velvet grass. Most reacted to both allergens, some to only one of them at individual intensities. An individual reactivity of patients' sera to a mix of 6 grass pollen and single grass pollen extracts by immunoblotting after isoelectric focusing was also shown by Haas et al. [6]. Based on the reactivity to group specific monoclonal antibodies, posttranslational modifications and similarities of N-terminal amino acid sequences, we designated the 34-kD allergen Hol 11 and the 30 kD allergen Hol 15 according to the nomenclature for grass pollen allergens [8].

Hol 11 was recognized by the group I-specific monoclonal antibody IG12, Hol 15 by the group V-specific monoclonal antibody Bo1. Interestingly, reactivity was only found to the group Vb-specific antibody Bo9, not to the group Va specific antibody BG6, indicating a higher similarity of Hol 15 to the Vb grass pollen allergens.

2D-PAGE analysis of velvet grass pollen extract revealed that Hol 11 and Hol 15 consist of isoallergens, a phenom-

enon which is also known from other grass pollen allergens [28, 29]. This microheterogeneity might be due to differences in posttranslational modifications as glycosylations or phosphorylations [30] or due to differences on the gene level leading to amino acid exchanges in the primary structure. Amino acid variations were found within the complete sequences of two recombinant isoallergens of Lol p 1 [31], and are also probable for the isoallergens of Hol 11 and Hol 15, respectively. Hol 15 displays a higher microheterogeneity presenting 6 isoallergens in a pI range from 4.7 to 6.2 than Hol 11 with 5 isoallergens in a pI range from 4.7 to 5.2. The reason for the presence of isoallergens in pollen has not been known so far. There are first reports regarding the birch pollen allergen Bet v1 that the heterogeneity is contributed by gene polymorphism among individuals of the same species [32].

Microsequencing of the N-terminal amino acid sequences of Hol 11 and Hol 15 [see also 12] revealed the presence of the posttranslational modified amino acid hydroxypro-

Table 1. N-terminal amino acid sequences of the 34-kD allergen Hol 11 (spots 1–6) and the 30-kD allergen Hol 15 (spots 1–6)

	Position																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
34kD	I	P	K	V	p	P	G	p	?	I	T	A	T	Y	G	D	E	W	L	D
30 kD	A	D	A	G	Y	T	p	A	A	p	A	A	p	G	A	G	G	D	A	T

p = Hydroxyproline, ? = blocked amino acid in microsequencing.

Table 2. Alignment of the N-terminal amino acid sequences of group I and group V grass pollen allergens of different grass species

Allergen Group I	Amino acid 1–20																				Reference No.
Cyn d 1	A	M	G	D	K	P	G	P	?	I	T	A	T	Y	G	D	K	W	L	D	40
rLol p 1	I	A	K	V	P	P	G	P	N	I	T	A	G	Y	G	D	K	W	L	D	31
	I	A	K	V	P	P	G	P	N	I	T	A	E	Y	G	D	K	W	L	D	41
Phl p 1	I	P	K	V	p	P	G	p	?	I	T	A	T	T	G	D	K	W	L	D	33
rPHl p 1	I	P	K	V	P	P	G	P	N	I	T	A	T	Y	G	D	K	W	L	D	13,42
Poa p 1	I	A	K	V	p	P	G	p	?	I	T	A	T	Y	G	D	K	?	L	D	43
Hol 11	I	P	K	V	p	P	G	p	?	I	T	A	T	Y	G	D	E	W	L	D	this work ¹
Group V																					
Dac g 5a				A	Y	T	p	Y	T	p	A	G	A								34
Dac g 5b				A	Y	T	p	A	A	p	A	G	A								34
rLol p 1b	A	D	A	G	Y	T	P	A	A	A	A	T	P	A	T	P	A	A	T	P	44
rLol p 5b	A	D	A	G	Y	A	P	A	T	P	A	T	P	A	A	P	A	T	A	A	45
Phl p 5a	A	D	L	G	Y	G	p	A	T	p	A	A	p	A	A	G	Y	T	p	A	10
Phl p 5b	A	D	A	G	Y	A	p	A	T	p	A	A	A	G	A	E	A	G	K	A	10
rPhl p 5	A	D	L	G	Y	G	P	A	T	P	A	A	P	A	A	G	Y	T	P	A	46
rPhl p 5a	A	D	L	G	Y	G	P	A	T	P	A	A	P	A	A	G	Y	T	P	A	47
rphl p 5b	A	D	A	G	Y	A	P	A	T	P	A	A	A	G	A	A	A	G	K	A	48
Hol 15	A	D	A	G	Y	T	p	A	A	p	A	A	T	G	A	G	G	D	A	T	12, this work ¹
rPoa 31	P	P	A	S	K	F	P	A	K	P	A	P	K	V	A	A	Y	T	P	A	49
p 41	P	A	T	L	A	T	P	A	T	P	A	A	P	A	A	G	Y	T	P	A	49
9 60	D	L	S	Y	G	A	P	A	T	P	A	A	P	A	A	G	Y	T	P	A	49

Grey shading indicates that amino acids are identical in all sequences. r = Sequences obtained from recombinant allergens; p = posttranslationally by modified amino acid hydroxyproline.

¹ Sequences obtained in this work.

line. This amino acid was also found in grass pollen allergens of other species [33–35]. It is part of crosslinks between amino acid chains stabilizing proteins as for example collagen [36]. Sequencing of Hol 11 revealed at position 9 a not detectable amino acid residue. Presumably this might be due to carbohydrate structures attached to the amino acid. This phenomenon was also reported for the same position of the amino acid sequence of the timothy grass pollen aller-

gen Phl p 1 [33]. Comparison of the amino acid sequence of Hol 11 with other known recombinant group I allergens (see table 2), revealed at position 9 the residue asparagine in the motive NXT, which is typical for N-glycosylation [37]. The presence of glycocomponents of Hol 11 was confirmed by analysis of the velvet grass pollen extract with the glycan detection kit (Boehringer) and by immunoblot analysis with digoxigenin-labeled lectins. Hol 11 bound to AAA, a lectin

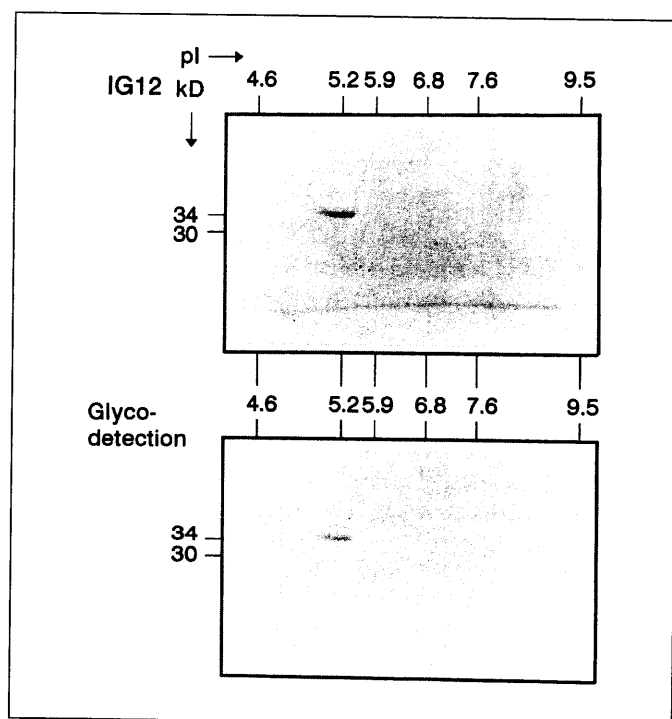


Fig. 4. Detection of glycoproteins on the 2D-PAGE blot of velvet grass pollen extract. For comparison one of the immunoblots was incubated with the group I-specific monoclonal antibody IG12.

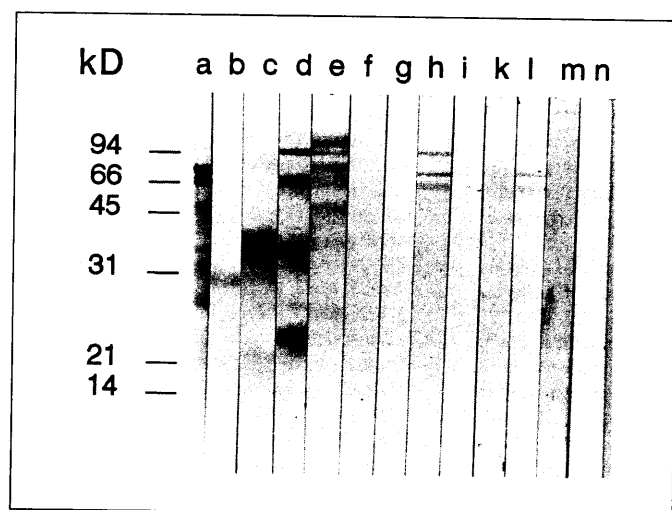


Fig. 5. Immunoblot of velvet grass pollen extract for detection of different glycoproteins incubated with india ink for protein staining (a), group V-specific monoclonal antibody Bo1 (b), group I-specific monoclonal antibody IG12 (c) and different lectins: d = AAA, e = Con A, f = DSA, g = GNA, h = MAA, i = PNA, k = RCA, l = SNA, m = WGA, n = PHA-L.

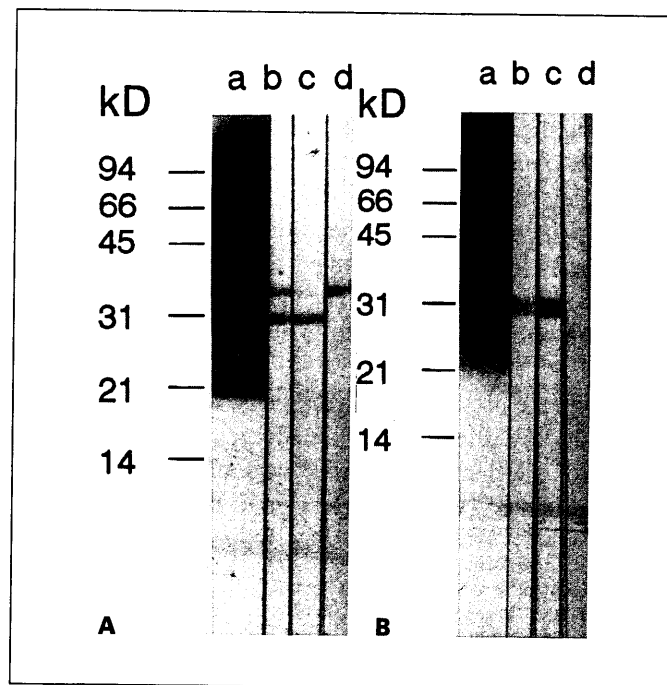


Fig. 6. Immunoblot of velvet grass pollen extract before **A** and after **B** fragmentation with CNBr incubated with india ink for protein staining (a), PS-W (IgE; b), group V-specific monoclonal antibody Bo1 (c) and group I-specific monoclonal antibody IG12 (d).

recognizing α -fucose residues. Recently Petersen et al. [38] reported strong binding of AAA to Phl p 1 and IgE reactivity of one patient's serum to the carbohydrate moiety of Phl p 1.

Comparison of the N-terminal amino acid sequence of Hol 15 with the corresponding sequences of other known group V allergens showed lower similarity (about 70%) than the alignment of Hol 11 with other group I allergens (similarity about 90%). There is also a higher number of group V allergens divided into the subgroups with a different molecular weight and sequence differences as shown for Phl p 5 [10, 39] and Dac g 5 [35]. Furthermore, group V allergens show higher microheterogeneity concerning the pI range of their isoallergens. In summary one might say that group V allergens are much more heterogeneous than group I allergens. These findings might play a role for further concepts of immunotherapy, because the individual IgE reactivity might not only apply to different allergens but also to the different isoallergenic forms of one allergen.

To get first informations on the localization of IgE binding epitopes of the major allergens of velvet grass pollen we performed a fragmentation of crude velvet grass extract with CNBr. Analysis by immunoblotting revealed the complete loss of immunoreactivity to Hol 11, indicating the presence of the amino acid methionine at positions important for

epitope structure. Hol 15 was split into two bands of 30 and 29 kD with identical N-terminus, indicating partial digestion of Hol 15 at the C-terminus of the molecule without destroying the allergenic and antigenic epitopes. Similar results were obtained by cleavage of timothy grass pollen extract by Petersen et al. [4]. The authors show the loss of IgE reactivity to Phl p 1 after fragmentation and the partial cleavage of the 38-kD isoform of Phl p 5 to 32 kD. The investigation was complicated by the existence of the 32-kD isoform of Phl p 5, which was cleaved to fragments of 30, 21 and 15 kD.

Despite the presence of isoallergens, velvet grass pollen extract shows a relatively simple allergen pattern with only two major allergens not divided into subgroups and isoforms. Pollen of other important grass species revealed a

much more complicated allergen pattern with allergen belonging to further allergen groups and subgroups. The presence of these additional isoforms of one allergen group may negatively affect characterization of a given allergen and analysis of its epitope structure.

Since velvet grass pollen contains only two major allergen groups, namely I and V, which show no further subgroups, it provides a good subject for further analysis of the allergenic epitopes of group I and group V allergens.

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