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Characterization of grass group I allergens in timothy grass pollen

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Using Phl p V-depleted timothy grass pollen extract (Phleum pratense) as immunogen, we obtained a monoclonal antibody, QG 4, which recognized proteins of 33, 35, and 37 kd as determined by Western blotting. The antibody cross-reacted with pollen proteins of other grass species in the molecular weight range of 30 to 37 kd. By means of two-dimensional polyacrylamide gel electrophoresis blot of timothy grass pollen extract, we demonstrated at least seven protein spots: two of 37 kd with isoelectric points of 6.4 and 6.6; four of 35 kd with isoelectric points of 6.5, 6.8, 7.1, and 7.3; and one of 33 kd with an isoelectric point of 8.5. These protein spots were also detected by patients' pooled serum. Microsequencing of the 20 N-terminal amino acid residues revealed structures with sequence identities up to 90% to the well-established allergen, Lol p I of ryegrass (Lolium perenne). Therefore we assume that the monoclonal antibody QG 4 recognized the corresponding allergen Phl p I in timothy grass pollen. (J ALLERGY CLIN IMMUNOL 1993;92:789-96.)

Key words: Grass pollen allergens, immunoblotting, microsequencing, two-dimensional gel electrophoresis

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Abbreviations used

PAGE: Polyacrylamide gel electrophoresis
pI: Isoelectric point
SDS: Sodium dodecylsulfate

To improve hyposensitization therapy, components of the administered extracts should be clearly defined. It could be a promising step forward to use only the IgE-reactive components of an extract for immunization. Søndergaard and Weeke¹ proposed the preparation of individual, "tailored" extracts for each patient. The isolation of such components should be performed by affinity chromatography of crude extracts with IgE antibodies of the patient as "catcher." In our opinion, a better way to improve immunotherapy would be to isolate relevant extract components as a result of applying monoclonal antibodies in affinity chromatography. A suitable extract tailored for the individual patient would then be prepared by mixing.

As far as grass pollen allergy is concerned, timothy grass pollen (*Phleum pratense*) has been identified as one of the dominant allergen sources in Northern and Central Europe. The major allergen *Phl p V* constitutes about 6% of the entire timothy grass pollen extract.² More than 80% of our monoclonal antibodies raised against crude timothy grass pollen resulted in antibodies directed against *Phl p V* (unpublished data). Therefore this protein seems to be highly immunogenic in mice. For raising monoclonal antibodies against other extract components, a depletion of *Phl p V* is necessary.

We obtained a monoclonal antibody directed against grass group I. This antibody was used to identify the isoallergenic components of the pollen extract and to characterize them by NH₂-terminal microsequencing.

METHODS**Preparation of pollen extract**

One gram of grass pollen (ARTU Biologicals, Lelystad, The Netherlands) was suspended in 55 ml of 0.1 mol/L NH₄HCO₃ buffer (pH 8.0) and incubated under agitation at room temperature for 30 minutes. After centrifugation for 30 minutes at 17,000 g (4° C), the supernatant was dialyzed against double-distilled water. The freeze-dried extract was stored at 4° C until use.

Western blot

Proteins were separated by discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis

(SDS-PAGE) by means of the buffer system of Neville and Glossmann³ with a stacking gel of 5% T and 0.75% C and a running polyacrylamide gradient gel of 7.5% to 20% T and 0.75% C. The proteins were subsequently transferred to nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany) by semidry blotting⁴ for 30 minutes at 0.8 mA/cm².

Chromatographic separation of *Phl p V* from timothy grass pollen extract

Phl p V was isolated from timothy grass pollen extract by affinity chromatography. The monoclonal antibody Bo 1⁵ was covalently bound to activated CNBr-Sepharose 4B according to the description of the manufacturer (Pharmacia, Uppsala, Sweden). Crude timothy grass pollen extract was dissolved in 0.1 mol/L phosphate-buffered saline (pH 8.0) and loaded onto the column. The unbound fraction was collected and used for immunization. The Bo 1-reactive components were isolated by an elution buffer (0.1 mol/L glycine, 0.15 mol/L NaCl; pH 11.0).

Immunization and production of monoclonal antibodies

BALB/c mice were immunized intraperitoneally with 50 µg timothy grass pollen extract (unbound components after affinity chromatography) on days 1 and 8 with Freund's complete and incomplete adjuvant, respectively. Three days before splenectomy (day 28) the mice received boosters of 100 µg extract without adjuvant. Spleen cells were fused with myeloma cells P3X63Ag8U.1. The production of monoclonal antibodies was accomplished according to the method of Goding.⁶ Screening was performed by a dot test⁷ with timothy grass pollen extract as antigen.

Ouchterlony diffusion test

The isotypes of the monoclonal antibodies were determined by the Ouchterlony double diffusion test⁸ in agarose gel with goat-anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, and IgM (Nordic Immunological Laboratories, Tilburg, The Netherlands) as precipitating antibodies.

Two-dimensional PAGE blot

The two-dimensional PAGE blot was done according to the method of Görg et al.⁹ with modifications as described in detail.¹⁰ In the first dimension, isoelectric focusing was performed in immobilized pH gradients. The second dimension was run in SDS gels. For subsequent immunologic investigations thermoblotting (capillary blotting on a 50° C heating plate¹¹) was performed, whereas semidry blotting was used for biochemical studies.

Protein staining and immunologic detections

After blotting, the nitrocellulose membrane was stained with 0.1% (vol/vol) India ink (Pelikan AG, Hannover, Germany).¹²

For detection of allergens the nitrocellulose membrane was incubated in patients' pooled serum or in supernatants of monoclonal antibodies. IgE reactivity was identified by mouse-anti-human IgE (Immunotech, Hamburg, Germany). Subsequently, alkaline phosphatase-conjugated goat-anti-mouse IgG \pm IgM (Dianova, Hamburg, Germany) was added. The binding patterns were visualized by a substrate solution consisting of 0.033% (wt/vol) nitro blue tetrazolium chloride and 0.017% (wt/vol) 5-bromo-4-chloro-3-indolyl phosphate potassium salt (Serva, Heidelberg, Germany) in 0.1 mol/L Tris-buffered saline (pH 9.5).¹³

N-terminal sequence and amino acid composition

The ProBlott membrane (Applied Biosystems, Weiterstadt, Germany) was washed in double-distilled water after blotting for 30 minutes. Afterwards, the membrane was stained with 0.1% (wt/vol) Coomassie Brilliant Blue 250 (Serva, Heidelberg, Germany) in 50% (vol/vol) methanol for 10 minutes, destained in 50% (vol/vol) methanol, and air-dried.

Protein spots containing the presumed allergen were cut out. Microsequencing was performed by Edman degradation with a 473 A protein sequencer with online PTH amino acid analyzer (Applied Biosystems). The amino acid composition was determined by the 420 A amino acid analyzer (Applied Biosystems).

The estimation of relationship between different proteins was done by the index of Marchalonis and Weltman, ΔQ ¹⁴:

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

The factor ΔQ indicates the relatedness of proteins. The variable n_i is the content of amino acid i in the protein, N is the number of amino acids in the protein, A refers to the protein consisting of fewer amino acids, and B refers to the protein consisting of more amino acids.

RESULTS

Production of monoclonal antibodies against timothy grass pollen extract after immunoabsorption of *Phl p V*

The isolation of the 38 and 32 kd proteins from timothy grass pollen extract (*Phl p V*) was done by affinity chromatography with a CNBr-Sepharose column, which contained the monoclonal antibody, Bo 1, covalently bound to the matrix. The absorption of immunoreactive material was monitored by the chromatogram. The unbound fraction was tested by Western blotting for components still binding to the monoclonal antibody Bo 1 (Fig. 1).

As demonstrated in Fig. 1, the unbound fraction (II) shows only very faint bands of 32 and 38 kd detected by Bo 1, indicating that these aller-

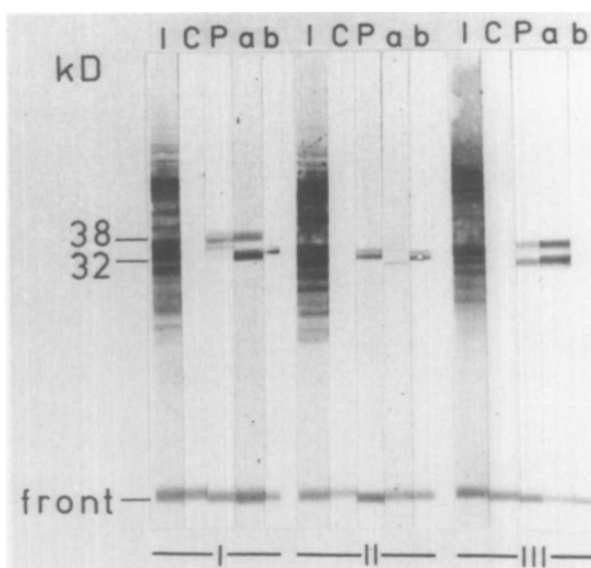


FIG. 1. Reactivity of the monoclonal antibody Bo 1 in crude timothy grass pollen extract (I), in the unbound pollen fraction (II), and in the bound fraction (III) after affinity chromatography. I, Protein staining by India ink; C, negative control; P, patients' pooled serum; a, monoclonal antibody Bo 1; b, monoclonal antibody QG 4.

gens are nearly depleted from this fraction. The incubation with patients' pooled serum (II-P) does not identify any Bo 1-reactive proteins, but allergens of 37 and 35 kd are detectable. On the other hand, intensive protein bands of 32 and 38 kd are demonstrated in the eluted fraction (III) and in untreated extract (I) by the monoclonal antibody Bo 1, as well as by patients' pooled serum. By immunization with the preabsorbed fraction (II) we obtained a monoclonal antibody called QG 4. It detects proteins of 37 and 35 kd in both the crude timothy grass pollen extract (I) and the unbound fraction (II). This indicates that QG 4 has bound to IgE-reactive components.

Ouchterlony diffusion test demonstrated that QG 4 belonged to the IgM class.

Characterization of the monoclonal antibody QG 4

For further characterization of the antigens detected by QG 4 we performed two-dimensional PAGE blot.

Fig. 2, A gives an impression of the timothy grass pollen proteins stained by India ink. The size of the proteins range from about 14 to 60 kd. The intensive protein spots of 35 and 37 kd are IgE-reactive as shown after incubation with patients' pooled serum (Fig. 2, B). Additional allergenic molecules of 55, 38, and 32 kd were identified. These proteins are not detectable by protein

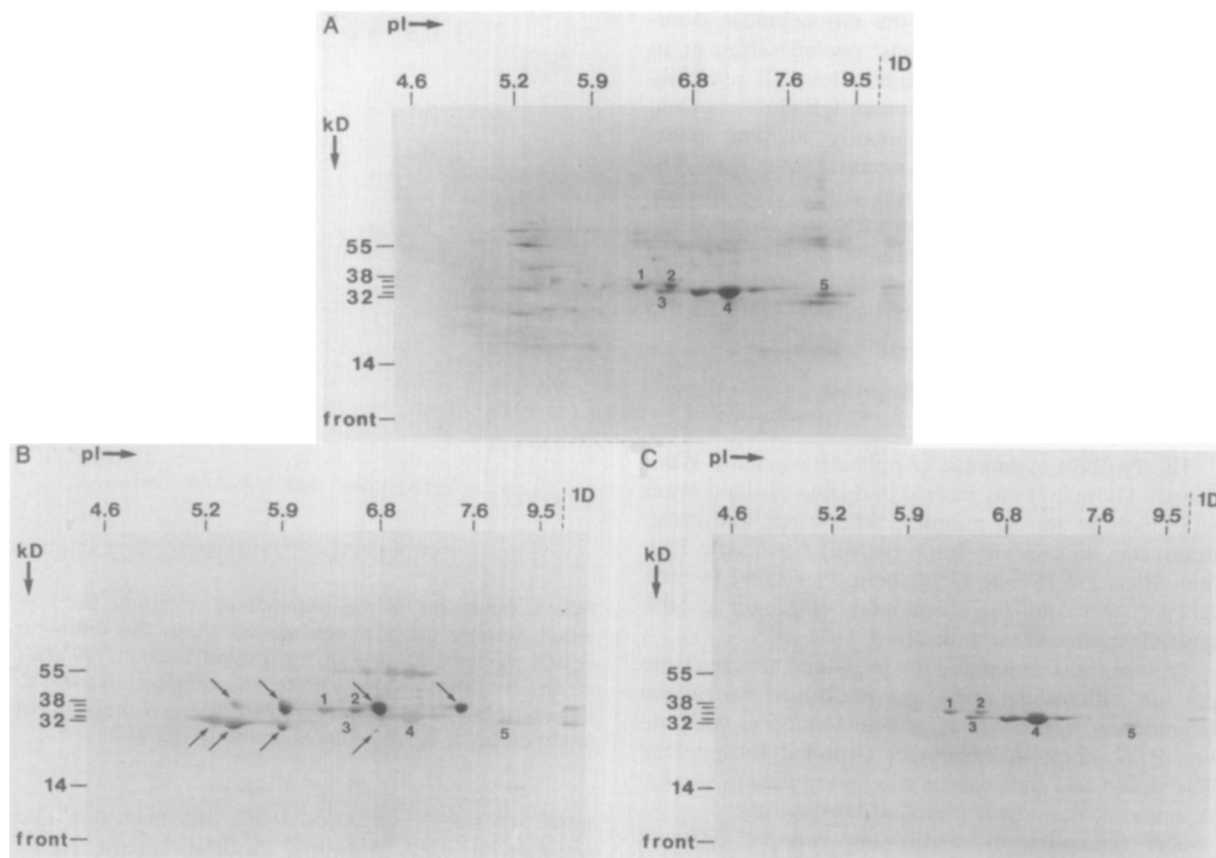


FIG. 2. Two-dimensional and one-dimensional PAGE blot of timothy grass pollen extract. Protein staining by India ink (**A**), detection of IgE binding proteins by patients' pooled serum (**B**), antigen detection by the monoclonal antibody QG 4 (**C**). The protein spots 1 to 5 were isolated for amino acid investigations. In **Panel B** the arrows indicate the Bo 1-reactive proteins.

staining. The four components of 38 kD and four of 32 kD (indicated by arrows) are Bo 1-reactive. The monoclonal antibody QG 4 (Fig. 2, C) bound to components of 37 kD with isoelectric points (pIs) of 6.4 and 6.6; 35 kD with pIs of 6.5, 6.8, 7.1, and 7.3; and 33 kD with a pI of 8.5. These components were congruent to those identified by patients' pooled serum. To determine the antibody specificity, we investigated whether the antibody QG 4 cross-reacted with pollen extract components of other grass species.

Fig. 3 shows the binding pattern of the monoclonal antibody QG 4 to pollen extracts of the main allergen sources in Europe. In each of the grass pollen extracts proteins of about 35 kD were detected. Although identical concentrations of the extracts were loaded onto the gel, there were differences in the intensity of the protein bands. Although *Dactylis glomerata* (1) and *Festuca elatior* (2) reveal intensive bands, we only identified faint bands of 35 kD in *Lolium perenne* (3) and

Secale cereale pollen (6). *Poa pratensis* (5) and *Holcus lanatus* pollen (7) showed two bands with molecular weights of 33 kD, 37 kD and 30 kD, 38 kD, respectively. *Plantago lanceolata* (weed pollen) and *Betula verrucosa* pollen (tree pollen) were used as controls and did not show any reactivity to the antibody.

Amino acid analyses of the QG4-detected allergens

For amino acid analysis we separated timothy grass pollen extract by two-dimensional PAGE blotting and cut out the five spots indicated in Fig. 2, C for microsequencing and determination of the amino acid composition.

Table I demonstrates the sequences of the 20 N-terminal amino acid residues of all components detected by QG 4. The five proteins reveal similar sequences with the exception of some variations in the basic protein of 33 kD. The components of 37 kD (1 and 2), as well as the 35 kD proteins (3

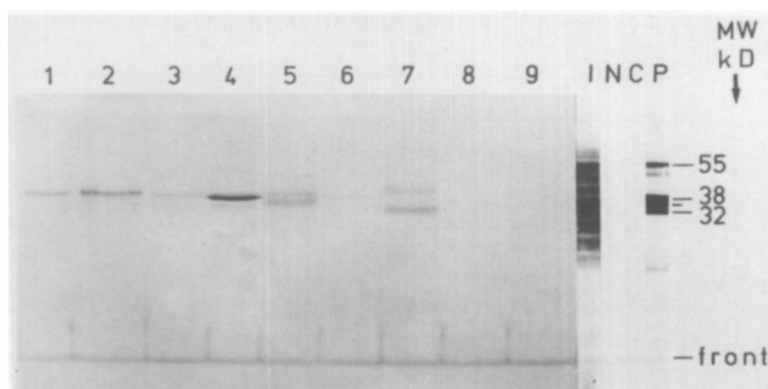


FIG. 3. Cross-reactivity of the monoclonal antibody QG 4 after Western blotting. *I*, Protein staining by India ink; *C*, negative control; *N*, normal serum; *P*, patients' pooled serum; 1, *Dactylis glomerata*; 2, *Festuca elatior*; 3, *Lolium perenne*; 4, *Phleum pratense*; 5, *Poa pratensis*; 6, *Secale cereale*; 7, *Holcus lanatus*; 8, *Plantago lanceolata*; 9, *Betula verrucosa*.

and 4), consist of identical primary structures up to position 20. When the 35 kd antigens are compared with the 37 kd proteins, sequences are identical, with the exception of position 18. The 33 kd protein (spot 5) shows differences in four positions (2, 12, 13, and 15) compared with the other proteins. In position 9 none of the five antigens under investigation reveal detectable amino acid residues. Presumably, this is due to carbohydrate structures attached to the amino acid. Besides proline, we identified posttranslationally modified hydroxyproline in two positions (5 and 8). For further comparison of the five proteins we performed amino acid analysis of the blotted samples.

Table II indicates the percent of amino acid contents in the five proteins. Tryptophan and cysteine residues were not determined, because these amino acids are usually destroyed in the amino acid analysis. At first sight, the amino acid compositions are very similar: only small amounts of the amino acids methionine and histidine were detected. The 33 kd protein (spot 5) differs from the other proteins especially in the content of glutamic acid and proline.

Using the index of Marchalonis and Weltman, SAQ, we determined the relationships among the five proteins from the amino acid analyses data.

All values indicate that the five proteins are related (Table III). The degree of relationship between proteins of identical molecular weights matches well, as shown by small SAQ indices. Although the connection between spots 1 to 5 and spots 2 to 5 is only within the limits of the "weak" test, the other values reveal strong evidence of interrelation.

DISCUSSION

We describe a method for raising monoclonal antibodies against timothy grass pollen allergens other than the immunodominant 32 and 38 kd proteins. Because these allergens have already been exhaustively studied and characterized with regard to timothy grass pollen, termed grass group V (*Phl p V*),^{2, 15} we wanted to circumvent the production of antibodies against components by use of the "cascade technique" described by Milstein and Lennox,¹⁶ according to which clearly identified antigens are removed in different steps from a mixture of antigens by affinity chromatography. The monoclonal antibody Bo 1⁵ was suitable for the affinity purification, because it binds to the 38 and 32 kd allergens at pH 8.0 and releases these proteins under elution conditions at pH 11.0 without changes in the immunologic reactivity.

The monoclonal antibody QG 4 that was obtained showed high similarities in its binding patterns to patients' pooled serum. It recognized proteins of 37 kd (spots 1 and 2), 35 kd (spots 3 and 4), and 33 kd (spot 5), with pIs varying from 6.3 to 8.5. Because of the different pIs of the protein spots, we were able to distinguish between proteins that only differ by 1 kd. These proteins, which ranged between 38 and 37 kd and between 33 and 32 kd, were not detectable after the one-dimensional separation by Western blotting (Fig. 3). The less intensive binding to the 33 kd was also only identified after two-dimensional immunoblotting.

The existence of isoallergens exhibiting similar immunologic and molecular characteristics but different pIs was first described by Johnson and

TABLE I. N-terminal sequences of the 37, 35 and 33 kd proteins identified by the monoclonal antibody QG 4.

Position		1			5			10		
37 kd	Spots 1 + 2	Ile	Pro	Lys	Val	Hyp	Pro	Gly	Hyp	? Ile
35 kd	Spots 3 + 4	Ile	Pro	Lys	Val	Hyp	Pro	Gly	Hyp	? Ile
33 kd	Spot 5	Ile	Thr*	Lys	Val	Hyp	Pro	Gly	Hyp	? Ile

Hyp, Hydroxyproline.

*Variations in the 33 kd proteins.

†Differences in the amino acid residues between the 37 and 35 kd allergens.

TABLE II. Amino acid analyses of the 37, 35, and 33 kd components detected by the monoclonal antibody QG 4

Amino acids	37 kd		35 kd		33 kd
	1	2	3	4	5
Ala	8	7	8	9	8
Arg	3	3	4	3	4
Asx	11	11	9	9	9
Glx	12	13	11	12	9
Gly	15	14	15	14	15
His	1	1	1	1	0
Ile	4	4	4	5	5
Leu	4	5	4	4	5
Lys	11	11	13	12	13
Met	1	1	1	1	1
Phe	3	3	3	3	2
Pro	7	7	6	7	9
Ser	5	5	5	5	5
Thr	6	7	5	6	5
Tyr	4	4	4	4	4
Val	6	5	6	6	5

The table indicates the percentual distributions of the amino acid residues.

Marsh¹⁷ for the major allergen of ryegrass, *Lol p I*. One of the reasons for such microheterogeneity might be posttranslational modifications (e.g., differences in the carbohydrate content¹⁸). By use of the glycan differentiation kit (Boehringer, Mannheim, Germany) we demonstrated that the 33, 35, and 37 kd timothy grass pollen proteins contained carbohydrate residues.¹⁹

The QG 4-binding antigens were not only present in timothy grass pollen but also in other grass pollen species. Cross-reactivity among grass pollen allergens was demonstrated by many research groups, including Weber²⁰ and Matthiesen and Løwenstein.²¹ Because of these similarities, identical IgE-binding epitopes were assumed to be found on allergens of different grass pollens, and this led to the classification of IgE-reactive

TABLE III. Determination of the relatedness between the protein spots 1 to 5 by the index of Marchalonis and Weltman, SΔQ

Protein spots compared	SΔQ	Critical values of SΔQ	
		"Strong" test	"Weak" test
1 - 2	7.76	25.2	55.9
1 - 3	17.64	25.2	55.9
1 - 4	10.83	25.2	55.9
1 - 5	27.01	25.2	55.9
2 - 3	20.84	25.2	55.9
2 - 4	16.53	25.2	55.9
2 - 5	36.60	25.2	55.9
3 - 4	7.26	26.7	59.0
3 - 5	17.67	26.7	59.0
4 - 5	22.29	26.7	59.0

proteins into allergen grass groups.²² The N-terminal sequences of the isoantigens detected by QG 4 are identical among allergens of the same size (spots 1 to 2 and 3 to 4). Even the comparison of 35 and 37 kd proteins revealed 95% sequence identity concerning the 20 N-terminal amino acid residues. But protein spot 5 showed only a sequence identity of about 77% with regard to the 17 amino acids identified. This corresponds to the results of the amino acid compositions and the SΔQ values,¹⁴ whereas the timothy grass pollen proteins of 35 kd and those of 37 kd are very similar to each other. Although there is a relatedness among all five proteins, the relationship between the different-sized molecules is much weaker.

The demonstrated sequences are quite similar to those of the ryegrass allergen *Lol p I*, published by Cottam et al.²³ and Singh et al.²⁴ Except for protein spot 5 there is a sequence identity of 90% in the N-termini: in positions 2 and 13 we found proline and threonine, whereas Singh et al.²⁴ de-

11			15			20			
Thr	Ala	Thr	Tyr	Gly	Asp	Lys	Trp†	Leu	Asp
Thr	Ala	Thr	Tyr	Gly	Asp	Lys	Lys†	Leu	Asp
Thr	Thr*	Lys* Thr	Tyr	Asp* Tyr*	Asp	Lys			

tected alanine and glutamic acid, respectively. Furthermore, the 35 kd proteins have leucine instead of tryptophan in position 18. Although Singh et al.²⁴ discovered proline residues in positions 5 and 8, we identified hydroxyproline residues in both positions. Therefore we assume that the proteins recognized by QG 4 have identical or similar epitopes and belong to group I allergens in timothy grass pollen (*Phl p* I). Recently, Matthiesen et al.²⁵ compared the N-terminal sequences of grass group I allergens of four grass species (including timothy grass). In contrast to our results, they only isolated one form of *Phl p* I. Their findings were identical to ours with the exception of position 2. Although the N-termini of the group I allergens among different grass species seem to be highly conservative, position 2 is quite variable. Matthiesen et al.²⁵ identified an alanine residue in *Poa p* I and a proline residue in *Sec c* Ia, and we determined a threonine amino acid residue in the case of *Phl p* I isoallergen spot 5.

The reasons for the microheterogeneity leading to allergens of different pIs are still unclear. Perez et al.²⁶ deduced the complete amino acid sequences of two isoallergens of *Lol p* I from complementary DNA clones and identified four changes at the amino acid level in the 240 amino acid proteins. Presumably the variations in pIs of protein spots 1 to 2 and 3 to 4 in timothy grass pollen are also caused by single exchanges of amino acid residues.

To understand allergenic mechanisms, it is necessary to determine the IgE-binding epitopes. Therefore inhibition tests with monoclonal antibodies and patients' pooled serum must be performed. By fragmentation of the allergenic proteins, we intend to encircle the IgE-binding epitopes in order to determine their primary structures. For improving hypersensitization therapy it is necessary to know whether the IgE-binding structures are identical within one grass group so that one extract is sufficient or whether the IgE-binding epitopes reveal species-dependent variations requiring extracts of all potential allergen sources.

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