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Characterization of the Allergen Group VI in Timothy Grass Pollen (Phl p 6)

I. Immunological and Biochemical Studies

Key Words

Epitopes
Grass pollen allergen
IgE binding
Immunoblotting
Microsequencing
Two-dimensional electrophoresis

Abstract

For the identification and characterization of allergen grass group VI we performed two-dimensional immunoblotting of timothy grass pollen (*Phleum pratense*). Two intense 13-kD protein spots of pI 5.2 and 5.5 were found to be IgE reactive. By N-terminal microsequencing and amino acid analysis we identified them as Phl p 6 isoallergens. An antiserum was raised against Phl p 6 by immunizing BALB/c mice with allergen bearing nitrocellulose particles of one isoform. The antiserum revealed an intense reactivity to Phl p 6 isoforms, but also showed a weak cross-reactivity with Phl p 5 allergens. After immunoabsorption of patients' serum to Phl p 6 spots on the blotting membrane, we were able to demonstrate that the eluted human IgE antibodies cross-react with the grass group V allergens as well. Therefore, Phl p 5 and Phl p 6 possess one or more common IgE binding epitopes.

Introduction

Grass pollens are one of the main causes of IgE-mediated allergy. Of all the temperate grasses the major allergens belong to groups I and V. Timothy grass (*Phleum pratense*) possesses an additional major allergen which was not detected in other grasses [1]. This was classified as group VI (Phl p 6), formerly termed Ag 19 [2]. The skin test and CRIE (cross radioimmuno-electrophoresis) showed an IgE prevalence of 75% [3]. Monitoring of IgE antibodies to Ag 19 before and during hyposensitization therapy followed typical courses: before immunotherapy starts the titer of IgE antibodies against Phl p 6 is usually low, during initial hyposensitization it reaches a high titer or remains unchanged.

This high IgE response was thought to be a suitable marker for prospective evaluation of clinical effect [4].

Löwenstein [5] studied Phl p 6 components in depth and determined a molecular mass of 11 kD by gel filtration and a pI of 4.9. Due to the difficulty in separating the allergen from Phl p 5 by gel filtration, ion exchange chromatography and copper chelate affinity chromatography [5, 6], it was suspected that it would be similar to grass group V components. Furthermore, Matthiesen et al. [1] observed a high degree of similarity between the N-terminus of group VI and an internal part of the Phl p 5. They did not detect any cross-reactivity between the two grass groups after raising an antiserum against Phl p 6 by immunizing rabbits.

Received:
June 22, 1994
Accepted after revision:
April 4, 1995

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1018-2438/95/1081-0049
\$8.00/0

Our aim was to characterize Phl p 6 in detail at the protein level and to clarify its relationship to allergen grass group V.

Materials and Methods

Preparation of Pollen Extract. 1 g of timothy pollen (ARTU Biologicals, Lelystad, The Netherlands) was suspended in 55 ml of 0.1 M NH_4HCO_3 buffer (pH 8.0) and was agitated at room temperature for 30 min. 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.

Patients' Pooled Serum, Monoclonal Antibody and Polyclonal Antisera. For the identification of IgE-reactive proteins we used a mixture of sera from 3 grass pollen sensitive patients (Pharmacia CAP System, CAP score VI). The grass group V directed monoclonal antibody Bo 1 was raised against crude timothy pollen extract [7]. The hyperimmune serum against grass group VI was raised in BALB/c mice after immunization with allergen bearing nitrocellulose particles from two-dimensional (2D) blotting [8, 9]. Rabbit antisera to Lol p 2 and profilin were kindly provided by Dr. H. Löwenstein (ALK, Hørsholm, Denmark) and Dr. P. Deviller (Immuno-Virologie Moleculaire et Cellulaire, Lyon, France).

Western Blotting. Proteins were separated by discontinuous SDS-PAGE using the buffer system (T=15%, C=4%) according to Laemmli [10]. The proteins were subsequently transferred onto nitrocellulose membrane [Schleicher and Schüll, Dassel, Germany] by semi-dry blotting [11].

2D PAGE Blot. The 2D electrophoresis was performed according to Görg et al. [12] by isoelectric focusing in immobilized pH gradients (T=4%, C=4%; pH 4–9.5) in the first dimension and gradient SDS-PAGE (T=8–12%, C=4%) in the second. Afterwards the gels were blotted onto nitrocellulose membrane for immunological analysis [13] or onto PVDF membrane (Immobilon-P, Millipore, Eschborn, Germany) for biochemical studies.

Amino Acid Analysis. NH_2 microsequencing was performed on the blotted samples using a 473 A protein sequencer (Applied Biosystems, Weiterstadt, Germany). The determination of amino acid composition was performed by Dr. H.G. Meyer (Institut für Physiologische Chemie, Ruhr-Universität Bochum, Germany) using an Applied Biosystems 420 A amino acid analyzer.

Identification of Shared Allergens. The identification of similar immunological reactivity between allergens was performed according to the method of Donoval and Baldo [14] with modifications. Phl p 6 protein spots were excised from Coomassie-stained 2D blotting membranes. 10 spots (each containing about 10 µg protein) were blocked with TBS containing 0.1% Tween 20 and afterwards incubated overnight in patients' pooled serum. After washing thoroughly three times, the bound antibodies were eluted using 0.1 M glycine-HCl buffer pH 11.0 containing 0.5% BSA and immediately neutralized with 0.5 M hydrochloric acid to pH 7.5. The eluted fractions were pooled, concentrated twofold under vacuum (Speed vac), and analyzed by Western blotting.

Results

Detection and Investigation of Phl p 6 at the Protein Level

The separation of Phl p 6 components from crude timothy grass pollen extract was performed by 2D PAGE blotting. Since several IgE-reactivity proteins demonstrate similar characteristics, e.g. molecular mass, acidic pI, we tried to differentiate between them by using specific antisera directed against these allergens.

By comparing the 2D patterns of the protein staining (fig. 1a) and the immunostaining by patients' IgE (fig. 1b), two prominent 13-kD protein spots of pI 5.2 and 5.5 were visualized. These components were not detected by the antisera directed against certain allergens and by monoclonal antibody Bo 1. The antisera raised against group II (data not shown) and III allergens (fig. 1c) of ryegrass (*Lolium perenne*) identified timothy pollen components with an apparent molecular weight of about 15 kD in the acidic (pH 4.6) as well as in the basic region (8.7 and 9.5). Profilin antiserum (fig. 1d) raised against ragweed (*Ambrosia elatior*) showed a strong affinity to a 15-kD component of pI 4.6 and weak affinities to 64-, 55- and 48-kD proteins of similar pI. The monoclonal antibody Bo 1 (fig. 1e) directed against group V allergens recognized components of 38 and 32 kD corresponding to Phl p 5a and Phl p 5b, respectively. Since we did not detect any cross-reactivity with the two intense 13-kD spots, we concentrated on our efforts on their characterization. Using a glycan detection kit, we could not demonstrate any detectable carbohydrate content (data not shown).

The primary structures of the spots 1 (pI 5.2) and 2 (pI 5.5) were studied by N-terminal microsequencing of the blotted samples.

Protein sequencing of spot 1 (fig. 2) revealed two different isoforms, thus, demonstrating that it is a mixture of two Phl p 6 components. Although the two proteins have similar size and pI, one of them possesses two additional amino acid residues, glutamic acid and glycine (fig. 2, lower row of spot 1). This results in a shift of two residues in the primary structure. Except for this difference the N-terminal sequences of spots 1 and 2 are identical. The similarity of the two protein spots was further demonstrated by amino acid analysis.

Figure 3 depicts the amino acid compositions showing only slight variations between the two spots. Thus, we obtained at least three isoforms of Phl p 6: spots 1 and 2 differ in their pI, while the two isoforms of spot 1 were of the same molecular mass and pI but differed by their NH_2 -terminal sequence.

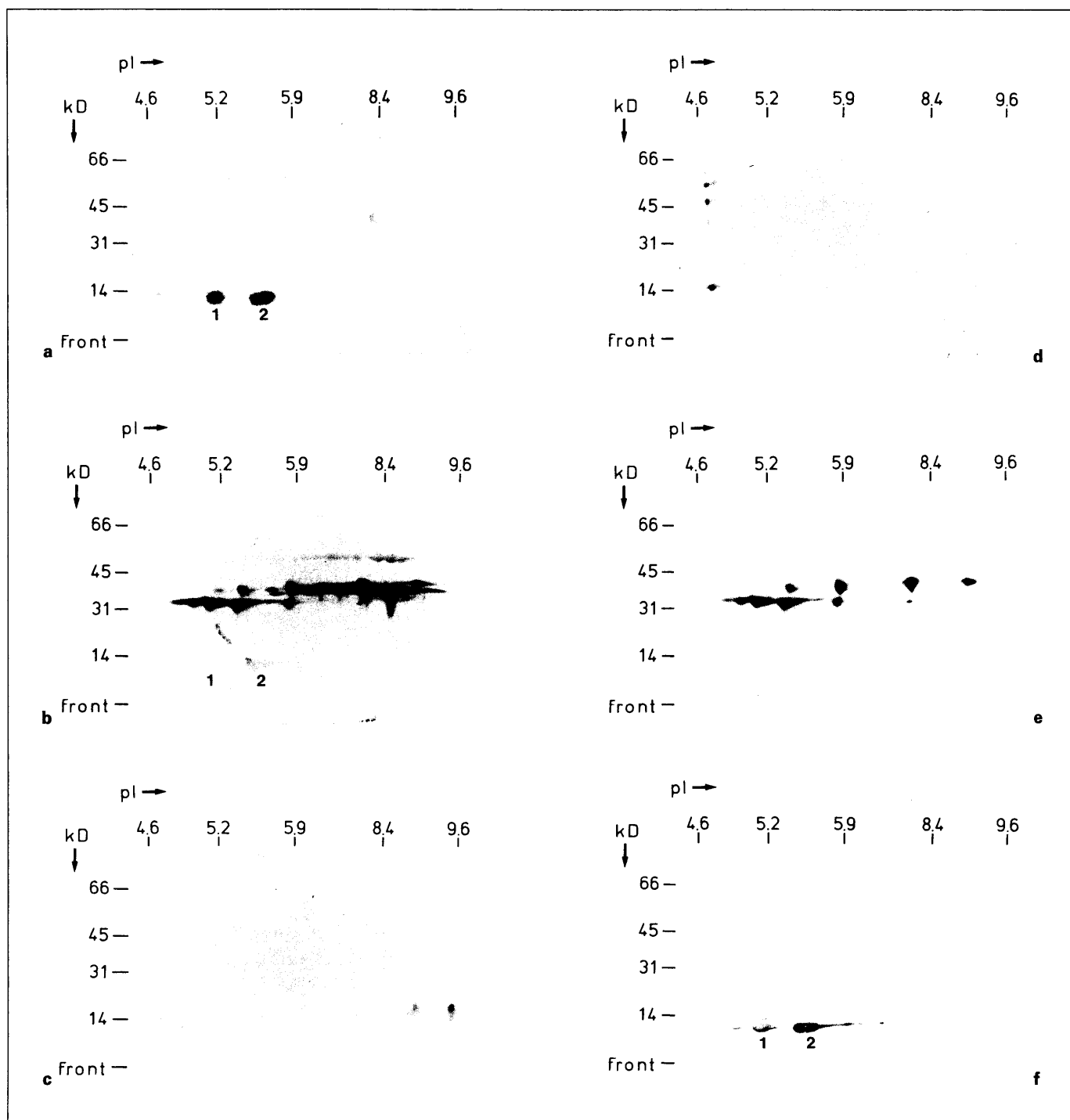


Fig. 1. 2D immunoblotting of timothy grass pollen extract for the detection of allergen grass group VI. Protein staining was done by Ponceau S (a). IgE-reactive proteins were detected by use of a patients' pooled serum (b). Identification of the following allergen grass groups: Phl p 3 (by rabbit antiserum; c), profilin (by rabbit antiserum; d), Phl p 5 (by monoclonal antibody Bo 1; e), Phl p 6 (by mouse antiserum; f). The two isoallergenic spots of Phl p 6 are indicated by 1 and 2.

Fig. 2. Microsequencing of the 20 N-terminal amino acid residues of the 13-kD proteins. Isoallergen I is a mixture of two components with equal amounts.

AA	1	5	10	15	20	25	30	35
Spot 1	G	K	A	T	T	E	E	Q
	K	L	I	E	D	V	N	A
	S	F	R	A				
	E	G	G	K	A	T	T	E
	E	Q	K	L	I	E	D	V
	N	A	S	F				
Spot 2	G	K	A	T	T	E	E	Q
	K	L	I	E	D	V	N	A
	S	F	R	A	A	M	T	T
	A	N	V	P	P	A	D	V
	Y	K	T	F				

	spot 1		spot 2	
	%	number	%	number
A	19.5	23	21.4	25
D	10.3	12	8.5	10
E	11.6	14	9.7	11
F	3.8	4	4.2	5
G	5.6	7	4.4	5
H	1.9	2	2.1	2
I	2.3	3	2.6	3
L	5.3	6	5.6	7
M	1.5	2	1.6	2
P	7.1	8	6.8	8
R	3.3	4	3.1	4
S	4.4	5	4.4	5
T	4.8	6	5.2	6
V	7.1	8	7.6	9
Y	4.1	5	4.4	5

Fig. 3. Amino acid analyses of the two 13-kD isoallergens (1 = pl 5.2, 2 = pl 5.5) indicating the percent distributions of the amino acid residues and number of residues in the 117 amino acid proteins.

Immunological Investigations on Shared Epitopes of Phl p 5 and Phl p 6

For the selective identification of grass group VI, we immunized BALB/c mice with a suspension of Phl p 6 bearing nitrocellulose particles. In order to avoid a mixture of isoallergens we prepared the antigens from spot number 2.

10 days after short-term immunizations (two boosters) blood was taken from 4 mice. The antisera were investigat-

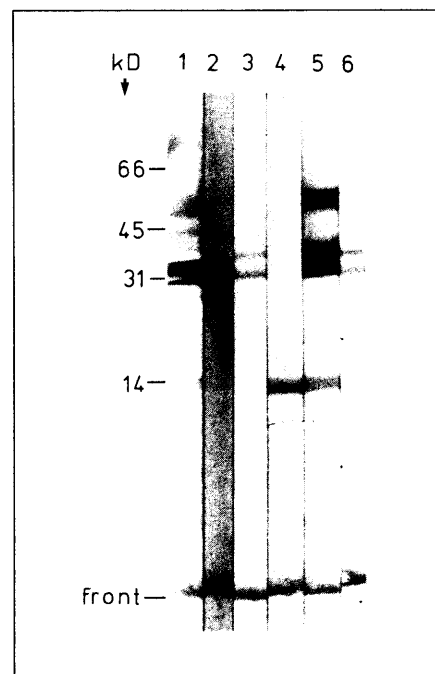


Fig. 4. Identification of common IgE binding epitopes on grass groups V and VI. 1 = Protein staining (Ponceau S); 2 = monoclonal antibody IG 12 (-> Phl p 1); 3 = monoclonal antibody Bo 1 (-> Phl p 5); 4 = Phl p 6 antiserum; 5 = IgE reactivity of patients' pooled serum without prior absorption; 6 = IgE reactivity of patients' pooled serum eluted from Phl p 6 protein spots.

ed for their reactivity by 2D immunoblotting. Each of the sera bound intensely to the two Phl p 6 isoallergenic components. While two of them were monospecific to group VI, the other two showed a weak cross-reactivity to components of 38 and 32 kD that correspond to Phl p 5a and Phl p 5b, respectively, as shown by the group V directed monoclonal antibody Bo 1 (fig. 1f). The binding pattern of a cross-reactive antiserum is demonstrated in figure 4. These results indicate that grass group V and VI share one or more common epitopes. In order to investigate whether human IgE recognizes common epitopes on Phl p 6 and Phl p 5, an immunoabsorption experiment was performed. After absorption

of patients' serum to excised Phl p 6 spots, the absorbed antibodies were eluted and tested by Western blotting.

The results of the immunoabsorption test are shown in figure 4. Lane 6 shows the binding pattern of the preabsorbed and eluted IgE antibodies. The identified protein bands of 13, 32 and 38 kD correspond to the allergens Phl p 6, Phl p 5b and Phl p 5a, respectively. The Phl p 6 preabsorbed antibodies also bind to the Phl p 5 components. For comparison lane 5 shows the binding pattern of patients' IgE without pretreatment; bands are present at 33–37 and 55 kD. This clearly demonstrates that Phl p 6 and 5 share one or more identical IgE binding epitopes.

Discussion

In this study we set out to characterize the major allergen Phl p 6 by immunological techniques and by protein chemistry, in order to investigate the relationship between allergen grass groups V and VI more precisely.

We performed 2D electrophoresis to obtain purified Phl p 6, and followed this with N-terminal microsequencing and immunological investigations. In this way the crude pollen extract was separated and purified for molecular mass and pI determination without recourse to the structural and immunological characteristics of the proteins. Separation by copper chelate affinity chromatography and ion exchange chromatography [6] was unsuitable since components of groups V and VI could not be separated completely.

We carried out immunodetection with polyclonal antisera in order to prevent confusion with proteins of molecular mass and pI such as grass group II (Lol p 2: 11 kD, pI 5.0–5.3 [14]), grass group III (Lol p 3: 11 kD, pI 9.0–9.4 [15]), and profilin (maize profilin: 14 kD, pI 5.1–5.8 [16]). The use of polyclonal antisera was assumed to be more suitable than monoclonal antibodies because they reveal a broad spectrum of antibody specificities to a certain allergen and are more likely to identify cross-reactions. We used antisera raised against the corresponding allergens in ryegrass in order to detect grass groups II and III. In contrast to ryegrass the binding patterns of both antisera to timothy grass pollen extract were similar. This agrees with the findings of Dolecek et al. [17], who did not differentiate between the cDNA sequences of Phl p 2 and 3, while Ansari et al. [18] determined a sequence homology of only 59% between Lol p 2 and Lol p 3.

We also tested for profilin, a protein which is termed a 'panallergen' [19] because it belongs to a ubiquitous family of IgE-reactive proteins. The profilins of different organisms reveal a high sequence homology which is responsi-

ble for cross-reactivities [16]. 2D immunoblotting (separation and immunodetection) clearly distinguished these allergens from Phl p 6.

Two intense spots were identified as Phl p 6 isoallergens by microsequencing. Our sequence data correspond well with the previously published data for Phl p 6 as performed by Matthiesen et al. [1]. It differs in that we have identified two additional amino acid residues in spot 1.

We were able to define three isoforms: two components reveal different pIs, but show identical NH₂-termini, while two isoforms have the same pI but different sequences. From Coomassie-stained 2D PAGE gels we estimated that the protein of Phl p 6 in the crude timothy pollen extract was about 15% (w/w). This concentration is higher than expected if levels of group II, III and profilin antigens are taken into consideration. This finding may be explained by the fact that our extraction procedure is shorter (60 min) than that used by other groups [6]. From our results, we presume that Phl p 6 is liberated quickly from the pollen grains. Despite the high concentration of Phl p 6, we could not detect monoclonal antibodies directed against the group VI antigen after immunization of BALB/c mice with crude timothy pollen extract [unpubl. data]. It is interesting to note that more than 50% of the monoclonal antibodies obtained were directed against group V antigens. This leads us to suppose that antigens from grass group VI are less immunogenic. This is supported by our observations of blotting membranes. Although Phl p 6 is present at a higher concentration than Phl p 5, it stains less intensely. Perhaps there are fewer antigenic binding sites on Phl p 6 molecules than are present on Phl p 5.

Two investigative techniques enabled us to identify that grass group VI and V share one or more common epitopes.

In order to obtain an antiserum specifically directed against group VI, we used 2D separated Phl p 6 spots for immunization. Because of the separation technique we were able to avoid contamination. The method has already been successfully applied to the separation and isolation of single components from complex mixtures [8, 9]. Even though the cross-reactivity to group V was weak, we were able to identify it in 2 of 4 mouse sera tested. Matthiesen et al. [1] could not detect this when they investigated antisera raised in rabbits. To investigate cross-reacting human IgE, we used the technique of immunoabsorption in conjunction with high concentrations of Phl p 6 antigen. It is apparent from the results that binding also occurred to Phl p 5a and Phl p 5b, thus supporting the partial structural similarity of group V and VI demonstrated by Matthiesen et al. [1].

The described relationship between Phl p 6 and Phl p 5 may be similar to that of Lol p 1, Lol p 2 and Lol p 3.

Although no IgE antibody cross-reactivity has been determined, the three allergens possess similar IgG binding epitopes, as demonstrated using human and animal sera [20], and similar sequences [18]. Grass group VI antigens are only detectable in timothy pollen [1], but they provide an important source of IgE-reactive proteins as this grass species is very common throughout north and central Europe. Therefore, Phl p 6 proteins should be considered for use in immunotherapy. Phl p 6 and 5 are major allergens [3] and

share only one or a few IgE binding sites; both allergens are therefore necessary extract components for an efficient hyposensitization therapy.

Acknowledgment

The authors would like to thank Mrs. Maren Blümke for her excellent technical assistance.

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