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## Characterization of Major Allergens of *Parietaria officinalis*

### Abstract

The major allergens of *Parietaria officinalis* were characterized with a panel of nine monoclonal antibodies (mAbs). The binding of mAbs and patients' IgE in Western blots revealed two proteins with similar molecular weights in the range of 8–10 kD. Analysis of the mAb-binding patterns in Western blots of *P. officinalis* extract under reducing and nonreducing conditions allows the mAbs to be divided into three different groups. mAbs of group I recognize the higher-molecular-weight component (9.4 kD), mAbs of group II recognize the lower component (8.8 kD) and mAbs of group III recognize both proteins. A comparable mAb-binding pattern was observed with Western blots of *Parietaria judaica*. The mAbs were used for affinity purification of the corresponding proteins from a *P. officinalis* extract. The purified proteins obtained with mAbs of group I–III inhibit the binding of patients' IgE (serum pool) to a high degree, indicating that they possess the major IgE-reactive epitopes. The affinity-purified proteins were subjected to SDS-PAGE, blotted and immunologically stained by mAb binding. The results confirmed those obtained with the complete extracts. The N-terminal amino acid sequences of the blotted proteins were analyzed. The sequences of all the proteins contained highly conserved regions: GGTV (positions 4–7) and MPPLL (positions 11–15), alternating with highly variable regions (positions 1–3 for group II and 8–10 for group I). A specific group I sequence appears to be at position 1–3 with the amino acids APA and a specific group II sequence appears to be at position 8–10 with the amino acids GAL. It is possible that the two similar proteins are isoforms of Par o 1.

### Key Words

*Parietaria*  
*Parietaria officinalis*  
Major allergen  
Monoclonal antibodies  
Allergen characterization

### Introduction

Plants of the genus *Parietaria* are a major cause of allergic disease, particularly in the Mediterranean area and south-east Europe but also in southern parts of Great Britain and in Austria [1, 2]. The long pollen season of the *Parietaria* species underlines the importance of effective and safe immunotherapy. An important criterion for the standardization of allergen extracts used for diagnosis and immunotherapy is the quantification of major allergens [3, 4]. This requires their identification and analysis in the allergen ex-

tract preparation and, additionally, the characterization of components of test systems established for quantification purposes.

The major allergens of *Parietaria officinalis* and *Parietaria judaica*, Par o 1 and Par j 1, respectively, have been described. Par o 1 was identified as a glycoprotein of about 15 kD [5] or 14.5 kD with a protein:carbohydrate ratio of 100:21 [6]. The sequence of 12 N-terminal amino acids for a purified Par o 1 protein has been described, but it is still an open question whether there are other closely related major allergens in the *P. officinalis* extracts sharing dominant epi-

topes with this protein [6]. Several publications have dealt with the major allergen Par j 1 from the closely related species *P. judaica*. Estimates of the molecular weight of Par j 1 by SDS-PAGE varied between 10 and 15 kD [7–11]. Recently, Par j 1 was described as consisting of two isoforms with different molecular weights of 13 and 10.5 kD and similar, but not identical, N-terminal amino acid sequences [12]. To a certain extent, these sequences coincide with the N-terminal sequence described for Par o 1 [6]. A cDNA clone encoding Par j 1 has been isolated and its DNA sequence determined. The cDNA encoded an allergenic protein of 14,866 D and a deduced amino acid sequence containing 143 residues [13]. This sequence shows a certain homology to the N-terminal sequence described by Ayuso et al. [12], but is not identical.

A strong cross-reactivity between extracts of *P. officinalis* and *P. judaica* and for the group I allergens of three *Parietaria* species, *P. officinalis*, *P. judaica* and *Parietaria mauritanica* has been reported [6, 10, 14].

In this study, monoclonal antibodies (mAbs) against the major allergenic fraction of a *P. officinalis* extract were selected on the basis of their ability to contribute to the molecular characterization of the major allergen. This was performed in Western blots of *P. officinalis*. The binding patterns of the mAbs obtained with *P. officinalis* were compared with those obtained with *P. judaica* extract. Several mAbs were chosen for the affinity purification of the allergens from *P. officinalis* extracts. The purified allergens were analyzed by SDS-PAGE, by the determination of the N-terminal amino acid sequence and for their IgE reactivity by immunoblotting and an inhibition ELISA.

## Materials and Methods

### *Parietaria* Pollen Extract

As starting material, we used a commercially produced extract of *P. officinalis* pollen (Allergopharma, Reinbek, Germany). The raw material was purchased from Allergon (Engelholm, Sweden). Briefly, the pollen was extracted in Coca's solution (5 g NaCl and 2.5 g NaHCO<sub>3</sub>/l) at 4°C for 16 h, centrifuged, diafiltered (molecular weight cut-off of 5 kD), lyophilized and stored at –20°C.

### Patients' Sera

Seventeen serum samples from *Parietaria*-allergic patients were obtained from Greece and Spain. Their RAST classes were in the range 2.2–4. All the sera were included in a *Parietaria* serum pool in equal volumes.

### mAbs

mAbs were obtained by fusing spleen cells from BALB/c mice immunized with a Par o 1-enriched fraction of the supernatant of a 60% ammonium sulfate precipitation of *P. officinalis* extract and the myelo-

ma cell line P3X63Ag8U.1 (ATCC No. CRL 1591) according to the methods described by Köhler and Milstein [15] and Goding [16]. Screening of the hybridoma cells was performed in a direct ELISA in microtiter plates coated with 10 µg *P. officinalis* extract/ml 0.1 M carbonate buffer pH 9.6. The hybridoma supernatant was diluted 1:1 with PBS/Tween/BSA buffer (6.845 mM NaCl, 0.135 mM KCl, 0.507 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.073 mM KH<sub>2</sub>PO<sub>4</sub>, 0.05% Tween 20, 1% BSA) pH 7.4. Antibody binding was detected using an alkaline phosphatase (AP)-labeled goat anti-mouse IgG (H and L specific; Jackson ImmunoResearch, West Grove, Pa., USA), diluted 1:5,000 in the Tris/Tween buffer pH 7.4 and the phosphatase substrate 4-nitrophenylphosphate, 1 mg/ml DEA solution (0.1 M diethanolamine, 0.5 mM MgCl<sub>2</sub>, pH 9.8). The extinction was measured at 405 nm with an automatic ELISA reader photometer. In a second step, the ELISA-positive hybridomas were tested on Western blots of *P. officinalis* extract and those which showed a reactivity in a range of 8–14 kD were selected. These hybridomas were cloned twice by limiting dilution.

The detection of the immunoglobulin subclass was performed with a Hybridoma Sub-Isotyping Kit, mouse (Calbiochem, La Jolla, Calif., USA).

### SDS-PAGE and Western Blot

SDS-PAGE was performed according to Laemmli [17] in vertical slab gels with an acrylamide concentration of 13 and 14% T and 2.6% C. Disulfide bonds were reduced with dithiothreitol. 10–20 µg of *Parietaria* extract were applied/cm gel. Western blotting was performed by semi-dry electroblotting according to Kyhse-Andersen [18] with a Semi-Dry-Blotting System (Multiphor II, Novablot; LKB, Bromma, Sweden) with a current of 0.8 mA/cm<sup>2</sup> for 60 min. Nitrocellulose blotting membranes (pore size 0.45 nm) were purchased from Schleicher & Schuell (Dassel, Germany). The marker proteins used in these SDS-PAGE systems were rainbow markers 2.35–46 kD and 14.3–200 kD (Amersham, Braunschweig, Germany) or the LMW Calibration Kit: 14.4–94 kD from Pharmacia (Freiburg, Germany).

Affinity-purified *Parietaria* proteins were analyzed by the SDS-PAGE system according to Schägger and von Jagow [19] using acrylamide concentrations of 14.5, 16.5 or gradients from 8–20% T and 3% C in the separation gel. The stacking gel had an acrylamide concentration of 4% T, 3% C and the spacer gel one of 10% T and 3% C. The calibration kit for polypeptide-molecular-weight determination with a range of 2.5–17 kD was purchased from Pharmacia. When gel staining was performed, a solution of 0.15 (wt/vol) Coomassie brilliant blue G 250 (Serva, Heidelberg, Germany), 7% (vol/vol) acetic acid and 15% (vol/vol) methanol was used. Destaining of the background was performed with a solution of acetic acid and methanol in the same concentrations as above.

### Immunoblotting Analysis

For the detection of mAb binding, 3-mm wide strips of blotting membrane were incubated overnight with 2 ml culture supernatant and 2 ml TBS-Tween (0.1 M Tris, 0.1 M NaCl, 2.5 mM MgCl<sub>2</sub>, 0.05% Tween 20, pH 7.4). After three washes in TBS-Tween pH 7.4, the strips were incubated with AP-conjugated goat anti-mouse IgG (specific for H and L chains) diluted 1:20,000 with TBS-Tween for 3 h. mAb binding was visualized by the enzymatic reaction of AP with a substrate solution of nitro blue tetrazolium (NBT) 0.033% and 5-bromo-4-chloro-3-indolyl phosphate disodium (BCIP) 0.017% in TBS without Tween, pH 9.5.

The binding of IgE from patients' sera was performed by incubating the strips with human serum diluted 1:5 with TBS-Tween pH 7.4

overnight. After three washes with TBS-Tween, IgE binding was visualized either by incubation of an AP-conjugated monoclonal anti-human IgE (Allergopharma) for 3 h and subsequently with the substrate NBT/BCIP or by autoradiography with a  $^{125}$ I-labeled anti-human IgE (Allergopharma).

Protein staining of the membranes was performed with a solution of 0.1% India ink (Pelikan Fount India, Pelikan, Hannover, Germany) in TBS-Tween.

#### Affinity Chromatography

The procedure was performed according to Harlow and Lane [20]. For the preparation of an affinity column, the mAbs obtained from 300 ml hybridoma supernatant by precipitation with 50%-saturated ammonium sulfate were used. The precipitate was resuspended in 10 ml 0.2 M phosphate buffer pH 7.2 and incubated with 2 ml Protein-G Sepharose 4 Fast Flow (Pharmacia) overnight by slight agitation. The Sepharose was subsequently pelleted by centrifugation (3,000 g for 5 min), resuspended in 0.2 M borate buffer pH 9 and washed twice with the borate buffer. Finally, the gel was resuspended in 10 ml borate buffer, 52 mg dimethyl pimelimidate (Sigma, Deisenhofen, Germany) were added and the gel incubated for 30 min at room temperature under slight agitation. After pelleting, the reaction was stopped by incubation in 0.2 M ethanolamine buffer pH 8 for 2 h at room temperature under slight agitation, followed by another centrifugation step. Finally, the gel was resuspended in 0.2 M phosphate buffer pH 7.2, transferred to a column and equilibrated with the phosphate buffer. 0.8 mg *P. officinalis* extract were applied and the bound protein was eluted with 0.1 M glycine buffer pH 2.5. The affinity-purified proteins were analyzed by SDS-PAGE and their purity checked by Coomassie staining the gel. The protein concentration was determined according to Lowry et al. [21] and Wahl et al. [22].

#### Amino Acid Sequence Analysis

Determination of the N-terminal amino acid sequences was performed on an Applied Biosystems (Weiterstadt, Germany) ABI 476A pulse liquid sequencer. About 70 µg of the affinity-purified protein separated by SDS-PAGE and blotted on ProBlott membrane (Applied Biosystems) using CAPS blotting buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% (vol/vol) methanol, pH 11) were applied according to Matsudeira [23]. The protein bands were stained with 0.1% (wt/vol) Coomassie blue R250 in 40% (vol/vol) methanol and 1% acetic acid by destaining the background with 50% (vol/vol) methanol. Separated bands were cut out of the membrane and arranged in the cartridge block of the sequencer. At least 15 amino acid residues starting from the N-terminus were analyzed per protein.

#### Inhibition of Patients' IgE

Microtiter plates were coated with *P. officinalis* extract at a protein concentration of 1 µg/ml in carbonate buffer (0.1 mol/l) pH 9.6 by incubation overnight at 4°C. After blocking free binding sites with PBS/Tween/BSA for 15 min, patient's serum diluted 1:2 was incubated with equal volumes of different concentrations (10, 1, 0.1, 0.01 and 0.001 µg/ml) of affinity-purified *Parietaria* allergens. The mixtures were preincubated for 30 min in vials and then applied to the microtiter plate and incubated for a further 2.5 h. After washing 5 times, a horseradish-peroxidase-conjugated anti-IgE mAb (Allergopharma) was applied and incubated for 3 h at room temperature followed by another washing step. Antibody binding was visualized by incubation with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma).

## Results

### Definition of Major Allergens by Patients' Sera

The seventeen serum samples of *Parietaria*-allergic patients were used to confirm the major allergens in *Parietaria* extracts (fig. 1). In both *P. officinalis* and *P. judaica* extracts, two narrow bands with molecular weights of 8.8 and 9.4 kD (9.8 kD for *P. judaica*) were detectable in Western blots of the Laemmli SDS-PAGE (13% T) system with the majority of patients' sera. The 9.4-kD band in the *P. officinalis* extract was recognized by fifteen of the seventeen sera, and six of these also reacted with the 8.8-kD band. All sera recognized the 9.8-kD component of *P. judaica* and ten of the seventeen patients the 8.8-kD band. In some cases, the reactivity pattern with the two *Parietaria* species did not coincide, e.g. patients No. 9, 10 and 17. These patients showed a strong reactivity with only the 9.4-kD band from *P. officinalis*, but in the *P. judaica* extract both bands of the doublet were recognized with intense staining. All seventeen sera were taken to prepare a serum pool.

### Characterization of mAbs by Western Blotting

From a panel of mAbs with reactivities spanning the whole molecular-weight range in Western blots of *P. officinalis* extract, nine mAbs with reactivities in the range of about 8–12 kD were selected. All mAbs belonged to the IgG1 isotype.

mAb characterization was performed with Western blots of *P. officinalis* and *P. judaica* extracts under reducing and nonreducing conditions (fig. 2, 3, table 1). The reactivity of patients' IgE from the serum pool was tested simultaneously on the same blots (documented for *P. judaica* only, fig. 3). It was possible to divide the mAbs into three groups (summarized in table 1) on the basis of similarities in their binding patterns in Western blots of *P. officinalis* extract. To demonstrate the small differences in the apparent molecular weights of the proteins, these are expressed at the level of 0.1-kD accuracy. With *P. officinalis* extract, group I mAbs exhibited a main reactivity at 9.4 kD, mAb 2F9 only very weakly and mAb 4C2 showing further reactivities with bands of higher molecular weight: 15, 23 and 43 kD. Under nonreducing conditions, the three mAbs possessed a main reactivity at 11 kD with similar intensity. Group II mAbs showed a reactivity at a band of slightly lower molecular weight, 8.8 kD, and a characteristic binding pattern (12, 16, 19, 22 kD) under nonreducing conditions. Group III mAbs possessed a reactivity at 9.4 kD and under nonreducing conditions another characteristic binding pattern (11, 19.4 kD) (fig. 2, table 1). The mAb reactivity patterns on Western blots of *P. judaica* (fig. 3) resembled those obtained with

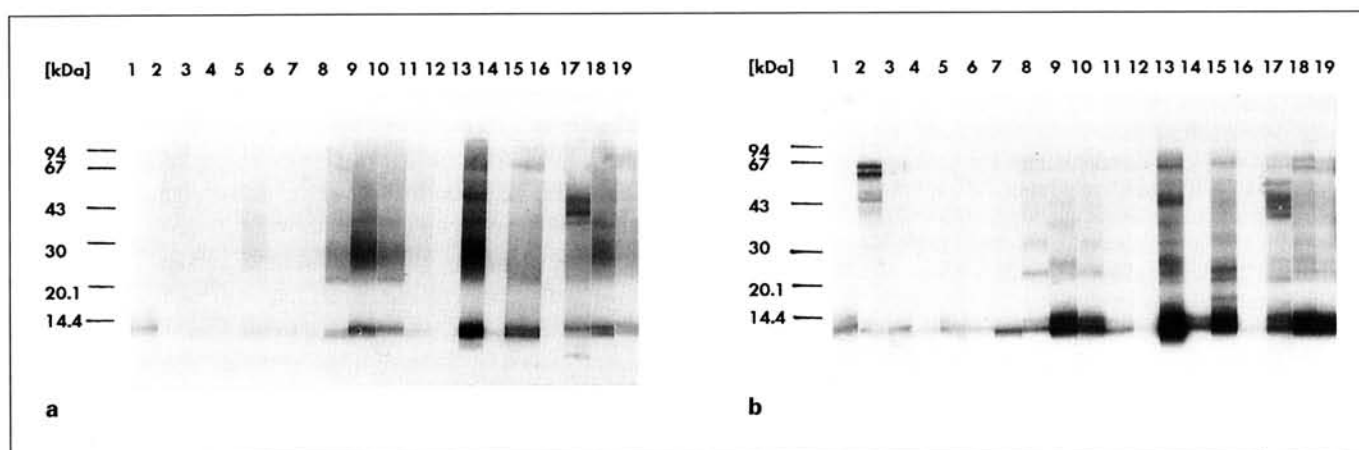


**Fig. 1.** Binding of IgE antibodies from the sera of seventeen *Parietaria*-allergic patients on Western blots (SDS-PAGE: 13% T) of *P. officinalis* (a) and *P. judaica* (b). Lane 1–17 = patients' sera; 18, 19 = pool of the seventeen sera at two different dilutions.

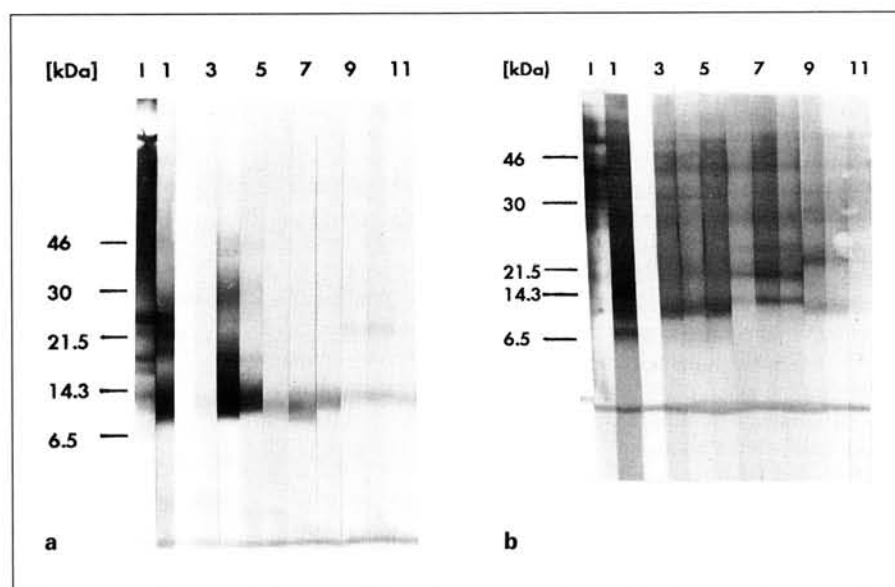


**Fig. 2.** Binding of mAbs on Western blots (SDS-PAGE: 14% T) of *P. officinalis* extract under reducing (a) and nonreducing (b) conditions. Lane 1 = protein staining with India ink; 2 = negative control; 3 = mAb 2F9; 4 = mAb 4C2; 5 = mAb 7D9; 6 = mAb 1C5; 7 = mAb 8B6; 8 = mAb 3F8; 9 = mAb 8C7; 10 = mAb 3E3; 11 = mAb 6C11.

*P. officinalis*, with some variations in the molecular weights of the allergen, summarized in table 1 (only the main reactivities are included for the nonreducing conditions).

The reactivities of patients' IgE antibodies from the serum pool were tested on blots of *P. officinalis* and *P. judaica* (only shown for the latter in fig. 3). The two main bands at 8.8 and 9.4 (9.8) kD showed a strong IgE reactivity (fig. 1, 3).

#### Affinity Purification of Major Allergens

The major allergens from *P. officinalis* extract were purified by affinity chromatography using mAbs 2F9, 7D9, 3F8, 8B6 and 8C7. The molecular weights of the different affinity-purified proteins were estimated by peptide SDS-PAGE according to Schagger and von Jagow [19]. The molecular weights of the proteins from *P. officinalis* extract recognized by mAbs of group I, II, and III were not clearly distinguishable (fig. 4), but precise measurements lead to an apparent

**Table 1.** Binding pattern of mAbs and IgE from *Parietaria*-allergic patients in Western blots of *P. judaica* and *P. officinalis* extract under nonreducing and reducing conditions

| mAb           | <i>P. officinalis</i><br>reduced | <i>P. judaica</i><br>reduced | <i>P. officinalis</i><br>nonreduced | <i>P. judaica</i><br>nonreduced | mAb<br>group |
|---------------|----------------------------------|------------------------------|-------------------------------------|---------------------------------|--------------|
| 2F9           | 9.4                              | 9.8                          | 11                                  | 9.4                             | I            |
| 4C2           | 9.4, 15, 23, 43                  | 9.8, 23, 43                  | 11                                  | 9.4                             |              |
| 7D9           | 9.4                              | 9.8                          | 11                                  | 9.4                             |              |
| 1C5           | 8.8                              | 8.8                          | 12, 16, 19, 22                      | 6.5, 9.4, 14.6, 20              | II           |
| 8B6           | 8.8                              | 8.8                          | 12, 16, 19, 22                      | 6.5, 9.4, 14.6, 20              |              |
| 3F8           | 8.8                              | 8.8                          | 12, 16, 19, 22                      | 6.5, 9.4, 14.6, 20              |              |
| 8C7           | 9.4                              | 8.8, 9.8                     | 11, 19                              | 9.4, 17.6                       | III          |
| 3E3           | 9.4                              | 8.8, 9.8                     | 11, 19                              | 9.4, 17.6                       |              |
| 6C11          | 9.4                              | 8.8, 9.8                     | 11, 19                              | 9.4, 17.6                       |              |
| Patients' IgE | 8.8, 9.4                         | 8.8, 9.8                     | 11, 12                              | 6.5, 9.4, 12, 14.6, 17.6, 20    |              |

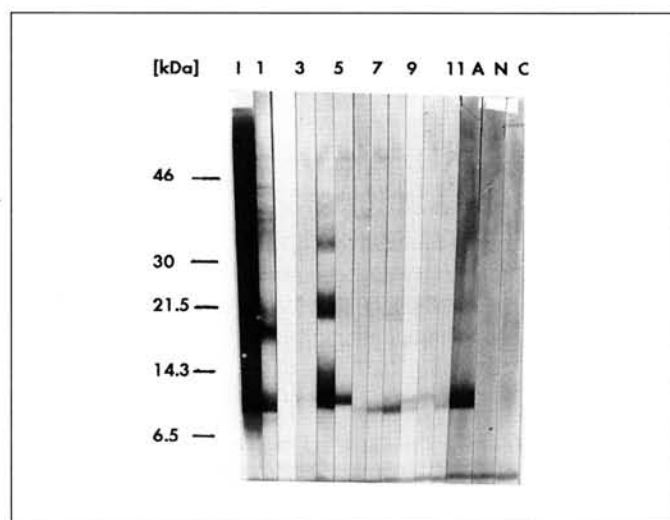


**Fig. 3.** Binding of mAbs on Western blots, (SDS-PAGE: 14% T) of *P. judaica* extract (reduced). Detection of human IgE binding: Lane A = serum pool of *Parietaria*-allergic patients; N = serum pool of non-allergic persons; C = control, buffer only. Lane 1 = protein staining with India ink. Detection of mouse IgG binding: Lane 1 = hyperimmune serum; 2 = buffer control; 3 = mAb 2F9; 4 = mAb 4C2; 5 = mAb 7D9; 6 = mAb 1C5; 7 = mAb 8B6; 8 = mAb 3F8; 9 = mAb 8C7; 10 = mAb 3E3; 11 = mAb 6C11.

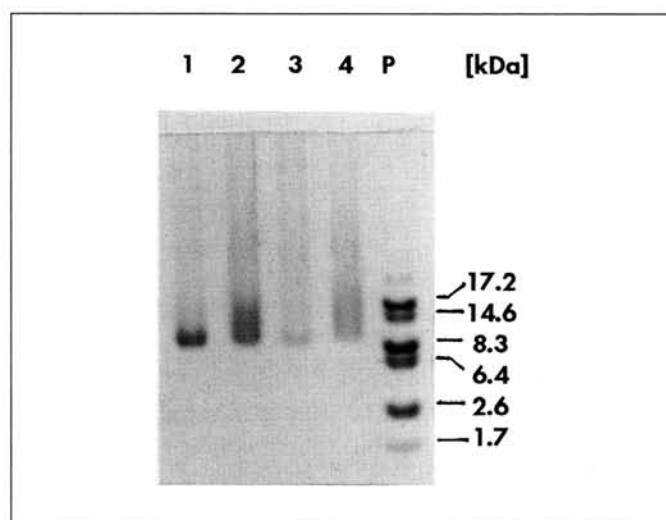


**Fig. 4.** SDS-PAGE (Schägger and von Jagow system) of affinity-purified *P. officinalis* proteins obtained with several mAbs. Coomassie staining. P = Peptide-molecular-weight marker; lane 1–4 = affinity-purified proteins obtained with mAb 3F8 (1); 8C7 (2), 8B6 (3) and 2F9 (4).

molecular weight of 8.8 kD for the group II protein and 9.4 kD for the group I and III proteins. The protein eluting from the column amounted to approximately 5–15% of the material applied.

The reactivities of the nine mAbs were tested on Western blots of the affinity-purified proteins (fig. 5). On blots of proteins purified with a group I mAb (2F9), the mAbs of group I showed the strongest reactivity with the 9.4-kD

band, as expected. Group III mAbs were also reactive with this protein, whereas the group II mAbs showed no reactivity. Conversely, whilst blotted 8B6- and 3F8-purified proteins showed very strong binding with the group II mAbs, group III mAbs also showed some degree of binding. Concerning group I mAbs, mAb 4C2 showed weak reactivity, whereas the others did not. On blots of a protein obtained with a group III mAb (8C7), all mAbs showed strong

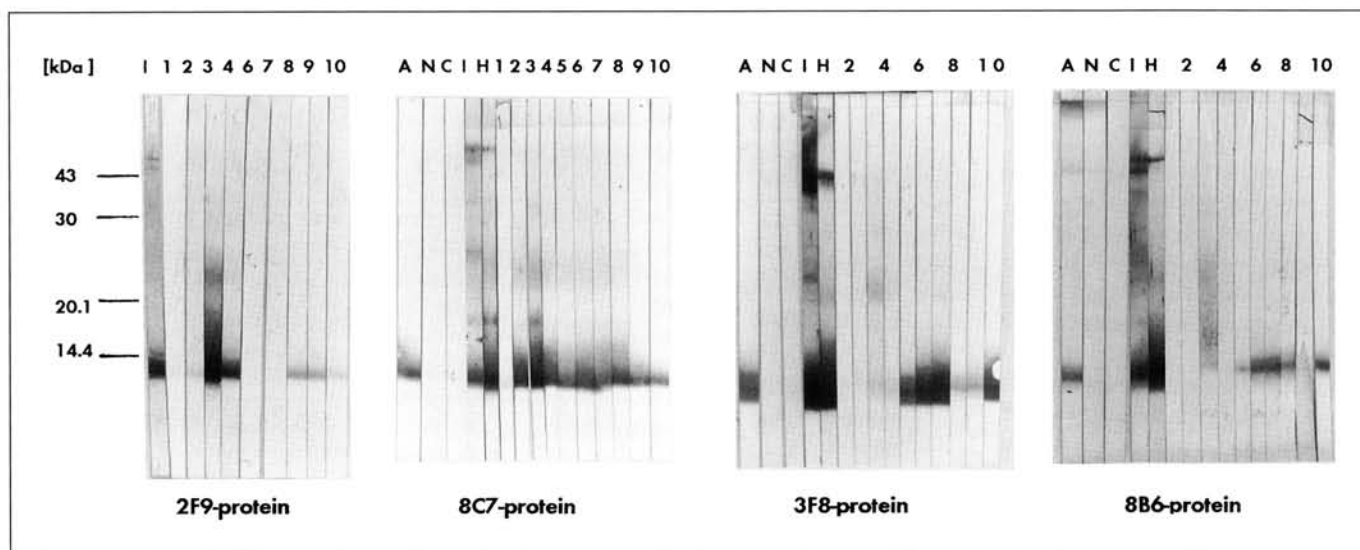


**Fig. 5.** Binding of mAbs on Western blots of the *P. officinalis* allergen obtained by affinity purification with mAbs 2F9, 8C7, 3F8 and 8B6. Lane A = IgE from patients' serum (pool from sera 1–17); N = IgE reactivity from a serum pool from nonallergic people; C = negative control for human IgE; I = protein staining with India ink; H = hyperimmune serum; 1 = negative control for mouse IgG; 2 = mAb 2F9; 3 = mAb 4C2; 4 = mAb 7D9; 5 = mAb 1C5; 6 = mAb 8B6; 7 = mAb 3F8; 8 = mAb 8C7; 9 = mAb 3E3; 10 = mAb 6C11.

binding, the reactivity of the group II mAbs lying at a slightly lower molecular weight than those of the group I and III mAbs. The incubation of these blots with serum pool of *Parietaria*-allergic patients revealed a strong IgE reactivity at the relevant band with all the affinity-purified proteins tested (obtained with mAbs 3F8, 8B6 and 8C7).

#### Mouse mAb-Purified Proteins Represent the Major IgE-Binding Allergens

In an ELISA with *P. officinalis*-extract-coated microtiter plates, almost complete inhibition (88%) of binding of patients' IgE was achieved (fig. 6). To establish whether the affinity-purified proteins represent the major IgE-reactive components in the extract, their inhibitory capacity was tested in this inhibition ELISA. As shown in figure 6, the IgE-binding could be inhibited to a high degree, with maximal inhibition values ranging between 79.6 and 93%. 50% inhibition was achieved with 65 and 75 ng/ml of the purified group III and I proteins respectively, with 105 ng/ml of the purified group II proteins and 110 ng/ml of the extract.

#### N-Terminal Sequence Analysis of Affinity-Purified Proteins

The affinity-purified proteins of *P. officinalis* extract obtained with mAbs from each group (mAbs 2F9, 7D9, 3F8, 8B6, 8C7 and 7D9) were used to perform an N-terminal

amino acid sequence analysis after SDS-PAGE under reducing conditions and blotting. With mAb 8C7, the two bands of 11 and 19 kD obtained under nonreducing conditions were taken. The sequences are shown in table 2.

The N-terminal sequences of the six proteins (3F8: 8.8 kD; 7D9: 9.4 kD; 2F9: 9.4 kD; 8B6: 8.8 kD; 8C7: 11 and 19 kD; table 2) are identical at positions 4, 5, 6 and 7 (GGVV) and at position 11 (M), 13 (P) and 15 (L). In positions 11–15, the sequence is nearly identical between all the proteins investigated, consisting always of three identical amino acid residues (M, P, L) but in different compositions (MPPLL, MPPPL, MMPLL), demonstrating that amino acid exchanges appeared at positions 12 and 14. For the 11-kD component of the 8C7 proteins, there appeared to be an exchange or addition at position 14 (L/P), and for the 19-kD component an exchange at position 12 (P/M).

The comparison of the N-terminal sequence of the major allergens recognized by mAbs of group I, II and III revealed homologous regions in the sequence alternating with more or less variable regions (table 2).

Variability in the N-terminal sequence is evident at positions 1–3 and 8–10. The first three amino acids are considered to be identical for the affinity proteins of mAbs 7D9, 2F9 and 8C7 with 9.4 kD; similarities are also evident for the 19-kD component of mAb 8C7, but the amino acids 1–3 differ completely in the 3F8 and 8B6 protein. In positions 8,

**Fig. 6.** Inhibition of the binding of IgE from *Parietaria*-allergic patients by *P. officinalis* extract (○) and four affinity-purified *Parietaria* allergens obtained with mAbs 3F8 (●), 2F9 (□), 8B6 (■) and 8C7 (△) at different concentrations. Values are as a percent of the uninhibited binding of IgE.

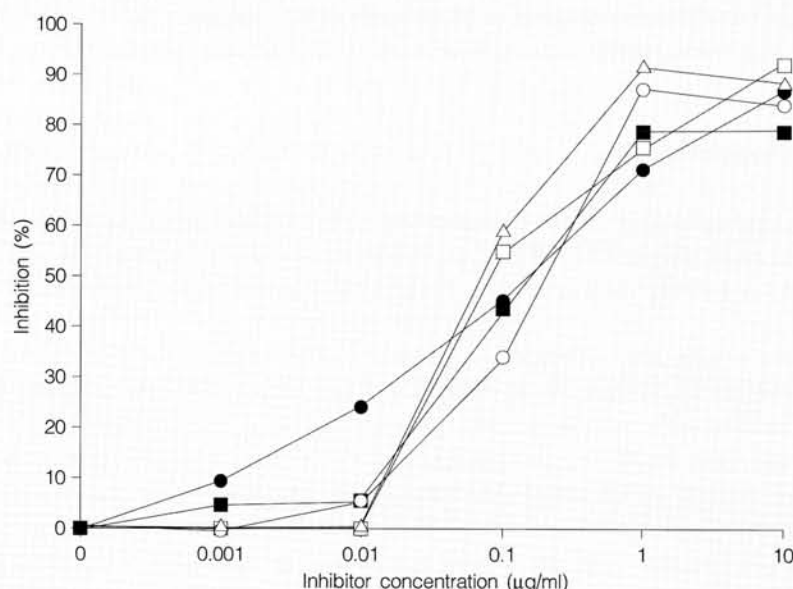


**Table 2.** N-terminal sequences of six affinity-purified proteins from *P. officinalis* extract obtained with mAbs 2F9, 8C7, 7D9, 3F8 and 8B6

|          | 1   | 2   | 3   | 4   | 5   | 6 | 7 | 8   | 9   | 10  | 11  | 12 | 13 | 14  | 15  | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|----------|-----|-----|-----|-----|-----|---|---|-----|-----|-----|-----|----|----|-----|-----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 2F9      | A   | P   | A   | G   | G   | V | V | P   | I   | I   | M   | P  | P  | ?   | ?   | F  | F  |    |    |    |    |    |    |    |    |    |    |    |    |    |
|          |     |     |     |     |     |   |   | (A) |     |     |     |    |    |     |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 7D9      | A   | A   | A   | G   | G   | V | V | H   | H   | I   | M   | P  | P  | L   |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|          |     | (P) | (K) |     | (K) |   |   |     |     |     |     |    |    |     |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 8C7      | A   | P   | A   | G   | G   | V | V | V   | A   | A   | M   | P  | P  | P   | L   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| (1)      |     |     |     |     |     |   |   | (G) | (I) |     | (D) |    |    |     |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 8C7      | G   | P   | V   | G   | G   | V | V | H   | A   | H   | M   | M  | P  | L   | L   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| (2)      | (A) |     |     | (P) |     |   |   | (P) | (H) | (I) |     |    |    |     |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 3F8      | E   | E   | X   | G   | G   | V | V | G   | A   | L   | M   | P  | P  | L   | L   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|          |     | (T) | (T) |     | (T) |   |   | (G) | (A) | (L) | (M) |    |    |     | (P) |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 8B6      | G   | T   | T   | G   | T   | V | V | G   | A   | L   | M   | P  | P  | L   | F   | V  | Q  | G  | K  | E  | K  | E  | ?  | P  | P  | S  | ?  | K  | ?  | G  |
|          | (E) |     |     |     | (G) |   |   |     |     |     |     |    |    | (P) |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| P.o.1    | A   | T   |     | G   | K   | V | V | Q   | G   | A   | M   | P  | P  |     |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|          |     |     |     |     | (T) |   |   | (A) | (I) |     |     |    |    |     |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| P.j.1    | A   | D   |     | G   | K   | V | V | Q   | D   | I   | M   | P  | P  | L   | L   | F  | V  | K  |    |    |    |    |    |    |    |    |    |    |    |    |
|          |     | (T) |     |     |     |   |   |     |     |     |     |    |    |     |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| P.j.2    | G   | P   | X   | G   | K   | V | V | X   | D   | I   | M   | P  | P  | L   | L   | F  | V  | K  | G  |    |    |    |    |    |    |    |    |    |    |    |
| P.j.cDNA |     |     |     |     | M   | V | R | A   | L   | M   | P   | C  | L  | P   | F   | V  | Q  | G  | K  | E  | K  | E  | P  | S  | K  | G  | C  | C  |    |    |

The sequence analysis was performed from blotted proteins after SDS-PAGE under reducing conditions, except for mAb 8C7, where the two proteins under nonreducing conditions were taken: 1 = 11-kD protein; 2 = 19-kD protein. The lower part of the table lists the known sequences from the literature: P.o.1 = Par o 1 allergen, 14.4 kD [6]; P.j.1 = Par j 1 isoallergen, 10.5 kD [12]; P.j.2 = par j 1 isoallergen, 13 kD [12]; P.j. DNA = sequence of Par j 1 deduced from a cDNA [13]. The amino acids in parentheses could substitute at these positions.

9 and 10 the two 8.8-kD proteins obtained with mAbs 3F8 and 8B6 have identical sequences (GAL), whereas the other proteins of 9.8 kD differ from each other at these positions.

## Discussion

The mAbs distinguished between two proteins with very similar molecular weights in *P. officinalis* extract. Patients' IgE (pooled serum from seventeen allergic patients and the single sera) bound strongly to both proteins as demonstrated in Western blots of the extract (fig. 1, 3) and the affinity-purified proteins (fig. 5) and by the inhibition of IgE binding to the extract with the affinity-purified proteins (fig. 6).

Using SDS-PAGE and immunoblotting, we found molecular weights of 8.8 and 9.4 kD for the two proteins in *P. officinalis* extract (8.8 and 9.8 kD in *P. judaica* extract). These molecular weights are slightly lower than those described in the literature [5, 6] for the major allergen, but from the degree of homology between the N-terminal sequences of the affinity-purified proteins with those described in the literature, it is possible that the two proteins correspond to isoforms of Par o 1 and, furthermore, to the Par j 1 isoforms.

Several reasons may account for the variation in molecular weight obtained by us and other researchers: (a) variability of the raw material obtained from different locations, probably an inherent variability in the amino acid sequence of the proteins representing the major allergen of *P. officinalis* and *P. judaica*; (b) different methods employed for the detection of molecular weights, i.e. HPLC and different SDS-PAGE systems, and (c) different procedures employed for protein extraction from the pollen.

The detection of two similar proteins, distinguished by differences in molecular weight, mAb-binding, N-terminal amino acid sequence and IgE reactivity, is in accordance with the publications of Oreste et al. [6] and Ayuso et al. [12]. The proteins probably represent two isoforms of Par o 1.

The reactivities of the mAbs in Western blots of the affinity-purified proteins and the extract under reducing and nonreducing conditions suggest that one epitope appears on both isoforms of Par o 1 (recognized by mAbs of group III), whereas the other two epitopes (recognized by mAbs of group I and II) are associated with the different isoforms. Since we extended our mAb characterization to the other important *Parietaria* species, *P. judaica*, we could show that the mAbs had very similar binding characteristics and the difference between the two molecules/isoforms persists between these two species.

When testing the seventeen sera of *Parietaria*-allergic patients in Western blots of *P. officinalis* and *P. judaica* extracts, we could show that the two proteins of the doublet must exhibit additional IgE-reactive epitopes, since the intensity of IgE binding to the two bands differs from patient to patient and within the IgE of one patient between the two *Parietaria* species. Ayuso et al. [11] made similar observations for the two different forms of Par j 1 with a panel of twenty sera. On the other hand, all the affinity-purified proteins obtained with our mAbs possess the major IgE-reactive epitopes, since they strongly inhibit (80–93%) the binding of IgE to allergen extract on a solid-phase. This may indicate that the main IgE epitopes on both molecules/isoforms are identical. Although the Par o 1 isoforms account for only 15–30% of the total protein in the extract, they nevertheless inhibit binding of the majority of IgE antibodies. Residual IgE binding activity must be attributable to other IgE-reactive proteins. When comparing the amount of allergen necessary to achieve 50% inhibition, the group I and III proteins have the highest inhibitory activity, whereas the group II proteins and finally the extract give values which are slightly higher, indicating a smaller inhibitory activity.

Table 2 shows our sequences in comparison with those from other publications [6, 11, 13], for two isoforms of Par j 1 and for a Par o 1 protein. The sequences MPPLL and GKVV instead of GGVV were found for these proteins at nearly the same positions. The blotting results suggested that the 9.4-kD proteins obtained with mAbs 7D9, 2F9 and 8C7 were identical, but their N-terminal sequences differ at positions 8–10. From the results obtained by microsequencing, it seems that there are several very similar proteins in *Parietaria* extracts possessing conserved sequences beside sequences of high variability and that the two main isoforms of different size possess a certain microheterogeneity. Such microheterogeneity within isoforms of different size has been described for other allergens, like Phl p 5 [24] or Amb a 1 and 2 [25]. The biological significance of this variability is unknown. In Par o 1, a specific sequence for the 9.4-kD protein/isoform seems to be APA at positions 1–3 and for the 8.8-kD proteins, GAL at positions 8–10.

The N-terminal sequence of the 19-kD protein obtained with mAb 8C7 also shows an extensive degree of homology. The protein may be a dimer of the 11-kD protein, since its molecular weight is nearly double and it is only evident under nonreducing conditions. The difference in the amino acid sequence at positions 1–3 leads us to suggest that the 19-kD component may be a heterodimer.

Comparison of the sequence for the 8B6-purified protein, where more than 30 amino acids were analyzed, with that deduced from a cDNA of Par j 1 [13] shows extensive

homology (83%) spanning the sequence: \*V\*-ALMP\*L-FVQGKEKE-P-S-K-G (with \* indicating a non-identical amino acid residue), when inserting artificial gaps into the sequences. This homology is further evidence of similarities between the allergens of the two *Parietaria* species. Furthermore, the sequence found for the 8B6-purified protein from *P. officinalis* has a greater homology to this cDNA sequence (83%) than the N-terminal sequences described by Ayuso et al. [12] for Par j 1, where only 50% homology could be found for this part of the sequence. The differences in the sequence obtained by different researchers may be a further indication of the heterogeneity of the major allergens in *Parietaria* extracts. Investigation of individual plants from the same and from different locations, as described for Amb a 1 and 2 [25] on the genetic level, may resolve this phenomenon.

The heterogeneity of the major allergens in *Parietaria* extracts has to be considered when developing alternative or better-defined preparations for hyposensitization treatment, e.g. when using recombinant allergens or peptides. This will require further investigation of the epitopes at the B and T cell levels. This heterogeneity must also be considered when developing test systems for the quantification of allergens in *Parietaria* extracts.

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## References

- 1 D'Amato G, Ruffilli A, Sacerdoti G, Bonini S: *Parietaria* pollinosis: A review. *Allergy* 1992; 47:443–449.
- 2 D'Amato G, Liccardi G: Pollen related allergy in the European Mediterranean area. *Clin Exp Allergy* 1994;24:210–218.
- 3 Chapman MD: Allergen specific monoclonal antibodies: New tools for the management of allergic disease. *Allergy* 1988;42(suppl 5):7–14.
- 4 Diener C, Jäger L: Monoclonal antibodies in the standardization of allergens. *Allergy Clin Immunol News* 1989;1:180–183.
- 5 Giuliani A, Pini C, Bonini S, Mucci N, Ferroni L, Vicari G: Isolation and purification of a major allergen from *P. officinalis* pollen. *Allergy* 1987;42:434–440.
- 6 Oreste U, Coscia MR, Scotto d'Abusco A, Santonastaso V, Ruffilli A: Purification and characterization of Par o 1, major allergen of *Parietaria officinalis* pollen. *Int Arch Allergy Appl Immunol* 1991;96:19–27.
- 7 Ayuso R, Carreira J, Polo F: Cross-reactivity among the major allergens from three different *Parietaria* species (abstract 2223). *Allergy* 1993;48(suppl 16):129.
- 8 Cocchiara R, Locorotondo G, Parlato A, Guarnotta G, Ronchi S, Albegiani G, Amoroso S, Falagiani P, Geraci D: Purification of Par j 1, a major allergen from *P. judaica* pollen. *Int Arch Allergy Appl Immunol* 1989;90:84–90.
- 9 Polo F, Ayuso R, Carreira J: Studies on the relationship between structure and IgE binding ability of *P. judaica* allergen. *Mol Immunol* 1991;28:169–175.
- 10 Corbi AL, Ley V, Sanchez-Madrid F, Carreira J: Isolation of the major IgE-binding protein from *Parietaria judaica* pollen using monoclonal antibodies. *Mol Immunol* 1985;22:1081–1089.
- 11 Ayuso R, Polo F, Carreira J: Purification of Par j 1, the major allergen of *Parietaria judaica* pollen. *Mol Immunol* 1988;25:49–56.
- 12 Ayuso R, Carreira J, Lombardero M, Duffort O, Peris A, Basomba A, Polo F: Isolation by mab based affinity chromatography of two Par j 1 isoallergens – Comparison of their physicochemical, immunochemical and allergenic properties. *Mol Immunol* 1993;30:1347–1354.
- 13 Costa MA, Colombo P, Izzo V, Kennedy H, Venturella S, Cocchiara R, Mistrello G, Falagiani P, Geraci D: cDNA cloning, expression and primary structure of Par j 1, a major allergen of *Parietaria judaica* pollen. *FEBS Lett* 1994;341:2–3.
- 14 Corbi AL, Pelaez A, Errigo E, Carreira J: Cross-reactivity between *Parietaria judaica* and *Parietaria officinalis*. *Ann Allergy* 1985;54:142–147.
- 15 Köhler G, Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975;256:495–497.
- 16 Goding JW: Antibody production by hybridomas. *J Immunol Methods* 1980;39:285–308.
- 17 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.
- 18 Kyhse-Andersen J: Electrophoretic transfer of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* 1984;10:203–209.
- 19 Schagger H, von Jagow G: Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 1987;166:368–379.
- 20 Harlow E, Lane D: Preparing protein A bead-antibody affinity columns – Direct coupling in high salt; in: *Antibodies – A Laboratory Manual*. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1988, pp 524–525.
- 21 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with Folin-phenol reagent. *J Biol Chem* 1951;193:265–275.
- 22 Wahl R, Geissler W, Maasch HJ: Comparison of classical Lowry, modified Lowry and a dye-binding assay for the estimation of protein in allergen extracts and influence of different parameters on the modified Lowry assay. *Biol Chem Hoppe Seyler* 1985;366:979–984.
- 23 Matsudeira P: Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* 1987; 262:10035–10038.
- 24 Petersen A, Becker WM, Schlaak M: Characterization of isoforms of the major allergen *Phl p* V by two-dimensional immunoblotting and microsequencing. *Int Arch Allergy Immunol* 1992;98:105–109.
- 25 Griffith IJ, Pollock J, Klapper DG, Rogers BL, Nault AK: Sequence polymorphism of Amb a I and Amb a II, the major allergens in *Ambrosia artemisiifolia* (short ragweed). *Int Arch Allergy Appl Immunol* 1991;96:296–304.