

Basic isoforms of Par o 1, the major allergen of *Parietaria officinalis* pollen

Coscia MR, Ruffilli A, Oreste U. Basic isoforms of Par o 1, the major allergen of *Parietaria officinalis* pollen.

Allergy 1995; 50: 899–904. © Munksgaard 1995.

M. R. Coscia¹, A. Ruffilli², U. Oreste¹

¹ Institute of Protein Biochemistry and Enzymology, CNR, Naples; ² International Institute of Genetics and Biophysics, CNR, Naples, Italy

We describe a group of basic isoforms of Par o 1 (cumulatively referred to as Par o 1b), purified by anion-exchange chromatography. The allergenic activity of Par o 1b was compared with that of the acidic isoform (Par o 1a) by RAST inhibition. Par o 1b showed a cathodic mobility in crossed immunoelectrophoresis. It was found to be homogeneous in SDS-PAGE and SE-HPLC (14.5 kDa), and heterogeneous in PAG-IEF, yielding five IgE-binding bands with pI ranging between 7.9 and 9.6. PAG-IEF individual components were isolated by cation-exchange HPLC. The N-terminal amino acid sequence of the main component (pI 8.8) was determined and found to be similar to that of Par o 1a.

Key words: cation-exchange HPLC; isoallergen; major allergen; *Parietaria*; polyacrylamide gel-isoelectrofocusing.

Umberto Oreste
Institute of Protein Biochemistry and Enzymology, CNR
Via Marconi 10
80125 Naples
Italy

Accepted for publication 25 July 1995

Pollinic extracts are very complex mixtures, including antigenic molecules (allergens) inducing an abnormal IgE antibody response in man (pollinosis). In Italy, the prevalence of pollinosis caused by *Parietaria* (pellitory) is at least 30% in the adult allergic population.

Parietaria is a genus of dicotyledonous weeds of the family Urticaceae. The most relevant species are *Parietaria officinalis* and *P. judaica*.

The major pollen allergen of *P. officinalis*, Par o 1, has been purified by our group. Its molecular mass, determined by SDS-PAGE, is 14 kDa, and the isoelectric point is 4.6. It is a glycoprotein with a protein-to-carbohydrate ratio of 100:21. The amino acid composition and the sequence of the first 12 N-terminal residues have been determined. Par o 1 contains dominant epitopes for human IgE and inhibits *Parietaria* specific RAST up to 85% (13). A cDNA sequence coding for an epitope common to Par o 1 and Par j 1 has been described (18).

Par j 1, the major allergen of the species *P. judaica*, has been isolated and characterized by several groups (2, 6). Cloning, expression, and deduced primary structure of Par j 1 have been recently reported (5). T-cell and antibody responses to *P. judaica* allergens have also been investigated (17).

The major allergens Par o 1 and Par j 1 are closely related, as demonstrated by the comparison of their physicochemical and allergenic features (4).

Like other proteins, some purified allergens consist of isoforms: molecules isolated from the same source displaying similar immunologic properties and closely related physicochemical structure (8, 12, 15). Described isoforms of allergens share antigenic and allergenic epitopes but differ in isoelectric point because of limited amino acid substitutions or varying degree of glycosylation (14). Recently, two isoallergens of Par j 1 have been separated by means of monoclonal antibodies, and their N-terminal amino acid sequences have been determined (1). We show that a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) band with an electrophoretic mobility slightly slower than that of Par o 1 corresponds to a group of basic isoforms of Par o 1 (Par o 1b).

Material and methods

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SDS-PAGE was performed according to Laemmli (10) under reducing conditions by boiling samples in 0.35 mol/l Tris-HCl buffer, pH 8.6, containing 6% w/v SDS and 10% v/v 2-mercaptoethanol; the separating gel contained a 10–15% polyacrylamide gradient. The electrophoretic run was performed for 5 h at 25 mA. The molecular weight standards used were

from Bio-Rad (broad range). Separated proteins were stained with Coomassie brilliant blue R-250.

Polyacrylamide gel isoelectrofocusing

Polyacrylamide gel isoelectrofocusing (PAG-IEF) was performed, according to Wrigley (20), with a 7.5% polyacrylamide gel containing 2.3% Bio-Lyte 3–10 and 0.9% Bio-Lyte 7–9 ampholytes. An amount of 40 µg of sample was focused for 14 h at 2 W. The apparatus was refrigerated at -2°C . Proteins of known pI (Bio-Rad) were used as markers. Gels were stained with Coomassie brilliant blue G-250 according to Reisner et al. (16).

Immunoblotting

The proteins separated by SDS-PAGE were transferred onto nitrocellulose sheets (BA 83 Schleicher & Schuell) as described by Towbin et al. (19) at 20 mA for 14 h, in 25 mmol/l Tris, 192 mmol/l glycine, pH 8.3, containing 20% v/v methanol.

The electrofocused proteins were transferred onto nitrocellulose passively.

In both blotting procedures, the remaining reactive sites of nitrocellulose were blocked with 50 mmol/l Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 3% w/v BSA, and 0.3% v/v Tween 20, for 1 h at 37°C . The sheets were then incubated for 16 h at 4°C with a pool of sera from 20 allergic patients (or a pool of sera from 20 healthy people), diluted 1:5 with 50 mmol/l Tris-HCl buffer, pH 8.0, containing 150 mmol/l NaCl, 0.05% M NaN_3 , and 0.3% v/v Tween 20. Finally, bound IgE was revealed by incubation with ^{125}I -labeled antihuman IgE (6.8 µCi/µg, Pharmacia, preadjusted to 130×10^4 cpm/ml), overnight at 4°C . Fuji NIF x-ray films were exposed, with intensifying screens, to the sheets for 1 week at -80°C .

Carbohydrate detection

The presence of carbohydrate residues on proteins blotted onto nitrocellulose sheets was detected with the DIG Glycan Detection Kit (Boehringer Mannheim Biochemica).

Crossed immunoelectrophoresis

Crossed immunoelectrophoresis (CIE) was essentially performed as described by Løwenstein (11): 25 µg of sample was applied; first-direction electrophoresis was carried out for 45 min at 8.6 V/cm; the second one for 14 h at 2.8 V/cm in anodic and cathodic gels containing respectively 21 and 31 µl of rabbit and anti-*P. officinalis* pollen extract antiserum per cm^2 . The running buffer was 0.1 mol/l Tris-

barbital, pH 8.6. Immunoprecipitates were stained with Coomassie brilliant blue R-250.

Anion-exchange chromatography

Anion-exchange chromatography was performed on diethylaminoethyl cellulose (DE-52, Whatman), eluting the sample (0.4 mg of the pollinic fraction obtained by gel-filtration on Sephadex G-50) through a 0.6×48 cm column at a flow rate of 18 ml/h. Unbound proteins were recovered by using 5 mmol/l Tris-HCl buffer, pH 7.8, as eluent; to recover the bound fraction, two gradients of increasing NaCl concentration (0.0–0.2 and 0.2–2.0 mol/l) were run sequentially.

Cation-exchange chromatography

Cation-exchange chromatography was performed by using an HPLC apparatus. The sample (26 µg of Par o 1b) was eluted through an HPLC MA7C column (0.78×50 cm, Bio-Rad) with 0% B for 5 min followed by a linear gradient of 0–50% B in 30 min, at a flow rate of 1.5 ml/min. The buffers used were as follows: A: 20 mmol/l Tris-HCl, pH 6.4; B: 20 mmol/l Tris-HCl, pH 6.4, containing 500 mmol/l NaCl. The absorbance profile was determined at 280 nm (0.032 AUFS). Bio-Rad pI markers (cytochrome C, pI 9.6; ribonuclease A, pI 7.8, myoglobin, pI 7.0) were used to evaluate the isoelectric point of separated peaks.

Size-exclusion HPLC

SE-HPLC was performed by eluting 13 µg of sample on a Protein Pak TM60 column (0.78×30 cm; packing material: bonded silica gel, particle size: 10 µ; Waters-Millipore), for separation in the molecular mass range 1–20 kDa. The mobile phase, which is 0.1 M phosphate buffer, pH 7.0, was used at a constant flow rate of 0.3 ml/min. Myoglobin (17.2 kDa), ribonuclease A (13.4 kDa), and cytochrome C (12.3 kDa) were used as molecular weight markers. Absorbance was detected at 280 nm (0.032 AUSF).

Radioallergosorbent test inhibition

Inhibition of the radioallergosorbent test (RAST) was performed as described by Gleich et al. (7). The solid phase was prepared by covalently binding Par o 1a or Par o 1b or *P. officinalis* extract proteins to the cellulose (3). Bound IgE was revealed by incubating each disk with 25 µl of ^{125}I -labeled antihuman IgE (5×10^3 cpm/disk). The serum pool used as reagenic serum, obtained from 20 patients allergic to *Parietaria*, bound 40.1 or 32.4 or 42.7% of the added cpm when *P. officinalis* extract or Par o 1a or

Par o 1b was used as solid phase; the control pool, obtained from 20 nonallergic individuals, bound 2.5 or 6.2 or 3.7% of the added cpm when the above stated solid phases were used. Purified allergens, whose protein content had been determined by the Bio-Rad protein assay kit, were used as inhibitors. Results were calculated according to the formula:

$$[1 - (b - c)/(a - c)] \times 100$$

where a = cpm bound by noninhibited serum, b = cpm bound by inhibited serum, and c = cpm bound by the control pool. Each determination was performed in duplicates and the values reported are means. I 50% (protein concentration required to obtain 50% RAST inhibition) was calculated by plotting percent inhibition against protein concentration.

N-terminal sequence analysis

Automated repetitive Edman degradation was performed on a pulsed-liquid phase Sequencer model 477A from Applied Biosystem, equipped with a 120A analyzer for the on-line detection of phenylthiohydantoin amino acid.

Results

P. officinalis pollen extract includes an IgE-binding fraction, obtained by Sephadex G-50 gel filtration (13). The allergenic activity of this fraction was determined by RAST inhibition and was found to be 24-fold higher than that of the whole extract. In SDS-PAGE, performed under reducing conditions, this fraction showed a doublet of very close bands with the apparent molecular masses of 14.5 and 14.0 kDa (Fig. 1, lane b); both components specifically bound human IgE in immunoblotting. The major allergen purified from the faster band, previously referred to as Par o 1 (13), is designated as Par o 1a.

The doublet components were separated through a DE-52 column by elution with an ionic strength gradient as described in Material and methods; the unbound fraction contained the slower migrating band of the SDS-PAGE doublet, referred to as Par o 1b (Fig. 1, lane a), while the retained fraction contained mainly the faster band (Fig. 1, lane c). Both doublet components, blotted onto nitrocellulose, were positive at the DIG glycan detection test.

The homogeneity of Par o 1b was verified with respect to either the molecular mass or the isoelectric point. In SDS-PAGE, under reducing conditions, Par o 1b yielded a single band (Fig. 1, lane a). This result was confirmed also under native conditions. In fact, it gave a single symmetric peak in SE-HPLC,

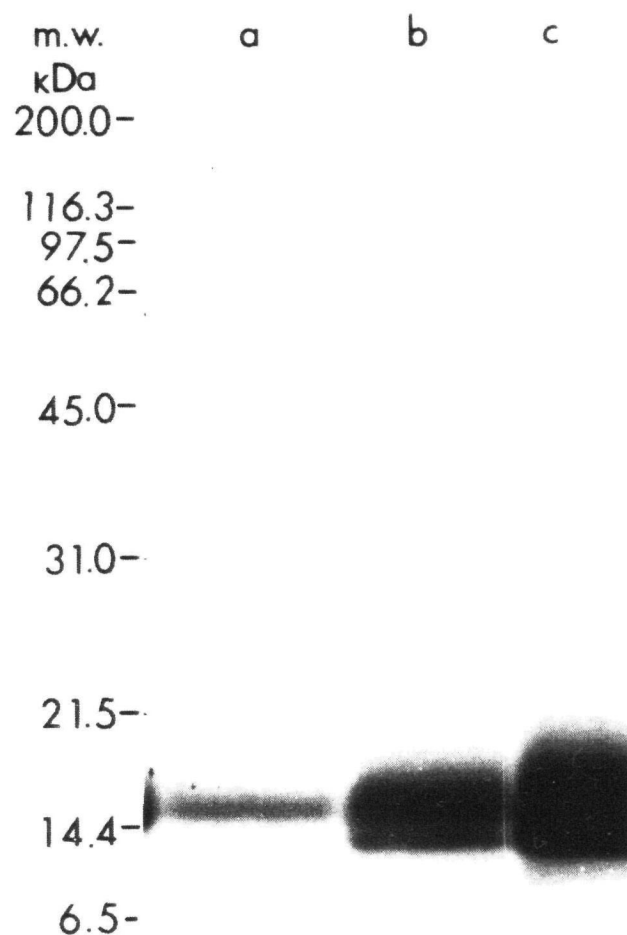


Fig. 1. SDS-PAGE analysis of the fractions eluted by anion-exchange chromatography. Lane a) unbound fraction (Par o 1b) (79 µg); b) pollinic fraction applied on column (described in Material and methods) (198 µg); c) retained fraction (364 µg).

performed on a calibrated column (Fig. 2A); the molecular mass values determined in both experimental conditions matched (about 14.5 kDa). In contrast, microheterogeneity was found with respect to the isoelectric point. At least five bands of pI, ranging from 7.9 to 9.6, were observed in PAG-IEF. The IgE-binding activity of all PAG-IEF-separated components was verified by immunoblotting (Fig. 2C).

CIE analysis of Par o 1b revealed the presence of at least three cathodic arcs (Fig. 2B), whereas Par o 1a is known to give only one anodic arc (13).

Specific allergenic activities of Par o 1a and Par o 1b were compared by RAST inhibition using *P. officinalis* whole extract as solid phase; the protein concentrations required to give 50% inhibition (I 50%) were found to be similar for both isoallergens: I 50% was 4.8 µg/ml for Par o 1b and 4.6 µg/ml for Par o 1a (Fig. 3A). These values are 23- and 24-fold higher than that of the extract. The high values of inhibition reached confirmed that both isoallergens share most of the IgE-binding epitopes of the extract. To investigate the antigenic relationship between Par o 1 isoforms, we tested the ability of

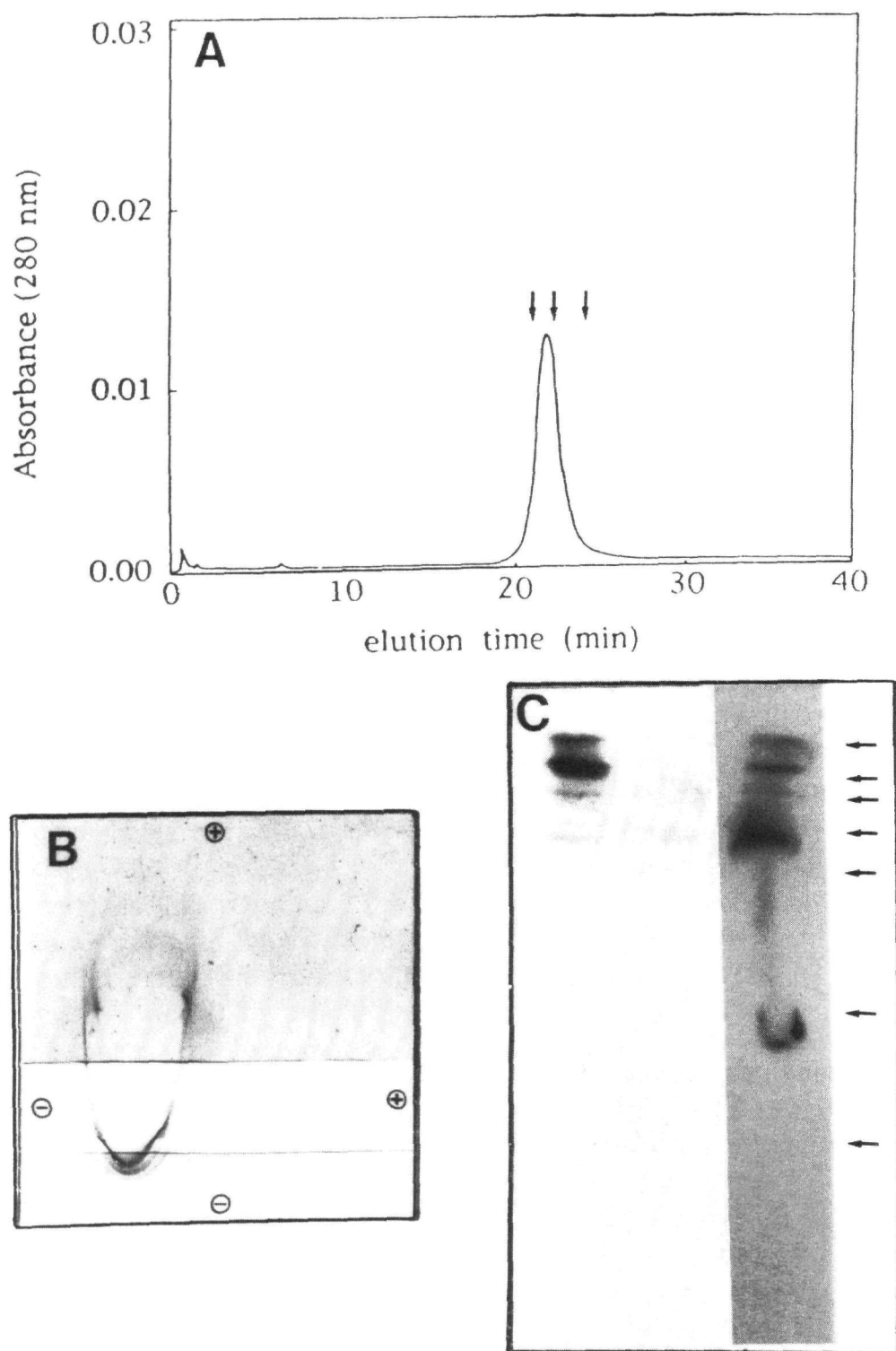


Fig. 2. Analysis of Par o 1b. A) SE-HPLC profile (applied sample: 13 μ g); arrows indicate elution times of molecular weight markers (from left: myoglobin, 17.2 kDa; ribonuclease A, 13.4 kDa; cytochrome C, 12.3 kDa). B) CIE (applied sample: 25 μ g). C) PAG-IEF (applied sample: 40 μ g) – on left, CBB staining; on right, immunoblotting of focused bands; arrows indicate pI values of markers (from top: 9.6, 8.6, 8.4, 8.2, 7.5, 6.5, 5.1).

Par o 1b (Par o 1a) to inhibit the binding of specific human IgE to solid-phase Par o 1a (Par o 1b). Fig. 3B shows extensive cross-reactivity between Par o 1 isoforms.

Individual Par o 1b isoallergens were separated by cation-exchange HPLC, as described in Material and methods. Five peaks were obtained and their pI, estimated by reference to markers, were 7.9, 8.2, 8.4, 8.8, and 9.6, respectively. These values matched those determined by PAG-IEF. The allergenic activity of each individual peak was confirmed by RAST inhibition with *P. officinalis* extract as solid phase (Fig. 4).

Automatic Edman degradation of the main peak (Par o 1b, pI 8.8) was performed to determine N-terminal amino acid sequence. This is reported in Fig. 5, in comparison with that of Par o 1a.

Discussion

We have shown that Par o 1, the major pollen allergen of *P. officinalis*, consists of isoallergens with similar molecular mass but different isoelectric point. We designated the acidic isoform (pI 4.6), previously purified from the lower band of the SDS-PAGE doublet, as Par o 1a, and the basic isoallergen com-

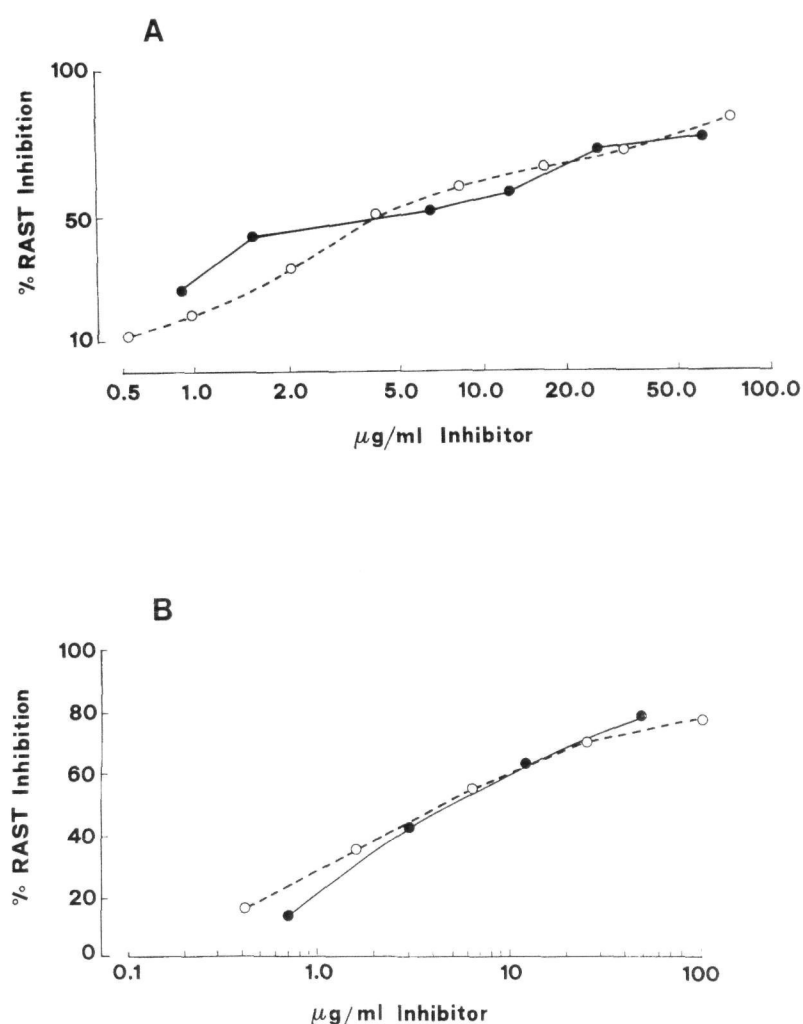


Fig. 3. Cross-reactivity between Par o 1a and Par o 1b. A) RAST inhibition of IgE binding to solid-phase *P. officinalis* extract proteins by solution-phase Par o 1a (●—●) or Par o 1b (○—○). B) RAST inhibition of IgE binding to solid-phase Par o 1a by solution phase Par o 1b (○—○) and to solid-phase Par o 1b by solution-phase Par o 1a (●—●). Details in Material and methods.

plex, corresponding to the higher band, as Par o 1b. The above proposed nomenclature is also justified

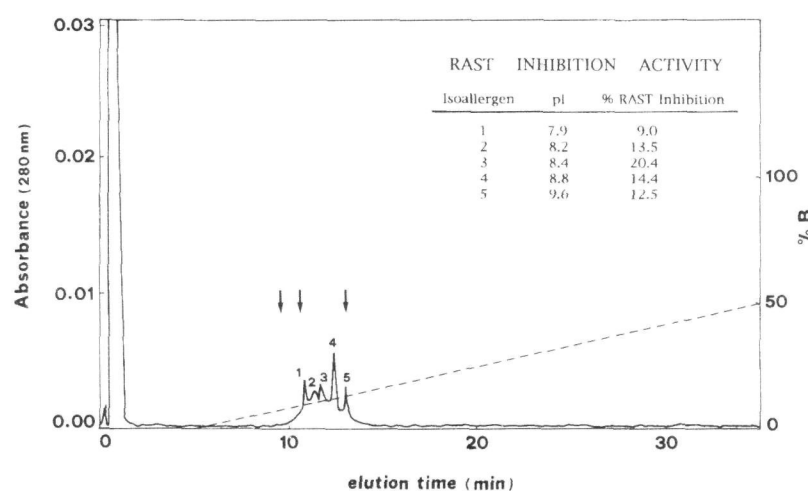


Fig. 4. Resolution of Par o 1b isoallergens by cation-exchange HPLC. Dashed line indicates gradient shape (0% B for 5 min, 0–50% B in 30 min; buffers: A) 20 mmol/l Tris-HCl, pH 6.4; B) 20 mmol/l Tris-HCl, pH 6.4, containing 500 mmol/l NaCl). Arrows indicate elution times of myoglobin (pI 7.0), ribonuclease A (pI 7.8), and cytochrome C (pI 9.6) in sequence from left. At top on right, RAST inhibition activity of each numbered peak is reported.

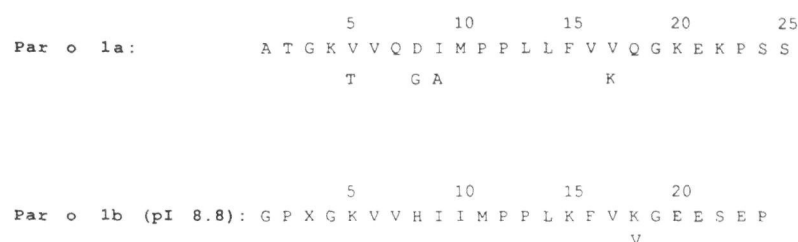


Fig. 5. N-terminal amino acid sequences of Par o 1a and Par o 1b (pI 8.8). When two amino acid residues were found in equal molar ratio, both were reported at same position.

by the immunologic behavior of these molecular species (9). In fact, Par o 1b is able to inhibit the binding of IgE from allergic patient sera to solid phase Par o 1a and vice versa. Furthermore, Par o 1a and Par o 1b isoallergens inhibit, at very similar protein concentration, 50% of IgE binding to solid-phase *P. officinalis* pollen extract antigens.

Par o 1b consists of a complex of molecules differing in isoelectric point, as demonstrated by PAG-IEF and cation-exchange HPLC. We also have evidence from PAG-IEF that, besides Par o 1a (pI 4.6), other acidic isoallergens are present in the polinic extract (unpublished data).

Differences in degree of glycosylation and in carbohydrate or amino acid composition could account for the observed molecular heterogeneity. However, the present amino acid sequence data, limited to the N-terminal region, do not allow us to ascribe this heterogeneity to difference in amino acid composition. On the other hand, the wide pI range found for Par o 1 isoforms (4.6–9.6) suggests that the carbohydrate moiety may be a determinant in this context. Enzymatic or chemical deglycosylation could be useful to clarify the role of carbohydrates in determining the isoelectric point. In addition, we plan to identify sugar residues of individual Par o 1 isoforms by using a panel of different lectins.

As IgE-binding was detected with a pool of sera from patients allergic to *Parietaria* pollen, only the epitopes recognized by IgE present, in high titer, in the majority of individual sera were identified. The observed cross-reactivity between Par o 1a and Par o 1b can be attributed to sharing of these epitopes. We do not exclude the presence of isoform-specific epitopes. Furthermore, we plan to perform PAG-IEF immunoblotting with an appropriate number of individual sera to settle this point.

Recently, the presence of isoforms has been reported also for the major allergen of the *P. judaica* species. Ayuso et al. (1) observed that Par j 1 migrates as a doublet in SDS-PAGE and showed that the individual components (the slower termed Par j 1a, and the faster Par j 1b) were cross-reactive by ELISA inhibition. The use of different SDS-PAGE systems may account for the discordance

found in the molecular mass values of Par o 1 and Par j 1 allergens.

The sequence of the first 12 N-terminal residues of Par o 1a has been previously determined (13). We report here the sequence of 25 residues of Par o 1a together with the 1–24 sequence of Par o 1b (pI 8.8): 13 residues out of 24 determined were found to be identical if the Par o 1b (pI 8.8) sequence alignment was offset backward by one residue. The limited sequence data available at present do not allow us to find a higher degree of homology between the identified isoforms. However, the complete sequence analysis would clarify this point.

Our data can be compared to those reported for Par j 1: 17 N-terminal amino acid residues of Par j 1a and 19 of Par j 1b (1) and the complete sequence of Par j 1 deduced from cDNA (5). Par o 1a shows high homology with Par j 1a (94% identity), but lower homology with the sequence deduced from cDNA. In addition, in comparison of Par o 1b (pI 8.8) with Par j 1b, only three out of 19 residues were found to be different (84% identity). This discordance might be attributable to species variability and/or to different degrees of purity of the isoforms.

In conclusion, the presence of isoforms of *Parietaria* allergens has been further documented and some of them have been isolated; a deeper investigation of their immunologic features could be useful in the analysis of individual immune responses to *Parietaria* pollen.

Acknowledgments

We would like to thank Dr G. Sacerdoti for providing us with sera from allergic patients and Mr V. Carratore for determining the N-terminal amino acid sequences. This investigation was supported by CNR (Grant AS Bilaterale 088253) and by UE (Grant BIO2/CT92/0363).

References

1. AYUSO R, CARREIRA J, LOMBARDERO M, et al. Isolation by mAb-based affinity chromatography of two *Par j* I isoallergens. Comparison of their physicochemical, immunochemical and allergenic properties. *Mol Immunol* 1993; 30: 1347–54.
2. AYUSO R, POLO F, CARREIRA J. Purification of *Par j* I, the major allergen of *Parietaria judaica* pollen. *Mol Immunol* 1988; 25: 49–56.
3. CESKA M. Radioimmunoassay of IgE using paper discs. *Methods Enzymol* 1981; 73: 646–56.
4. CORBI AL, PELAEZ A, ERRIGO E, CARREIRA J. Cross-reactivity between *Parietaria judaica* and *P. officinalis*. *Ann Allergy* 1985; 54: 142–7.
5. COSTA MA, COLOMBO P, IZZO V, et al. cDNA cloning, expression and primary structure of *Par j* I, a major allergen of *Parietaria judaica* pollen. *FEBS Lett* 1994; 341: 182–6.
6. FEO S, COCCHIARA R, GERACI D. Allergens of *P. judaica* pollen. I. Purification and characterisation of a hapten and a low molecular weight allergenic peptide. *Mol Immunol* 1984; 21: 25–36.
7. GLEICH G, LARSON J, JONES R. Measurement of the potency of allergic extracts by their inhibitory capacities in the radioallergosorbent test. *J Allergy Clin Immunol* 1974; 53: 158–69.
8. 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.
9. KING TP, HOFFMAN D, LÖWENSTEIN H, MARSH DG, PLATTS-MILLS TAE, THOMAS W. Allergen nomenclature. *Int Arch Allergy Appl Immunol* 1994; 105: 224–33.
10. LAEMMLI U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680–85.
11. LÖWENSTEIN H. Quantitative immunoelectrophoretic method as tool for the analysis and isolation of allergens. *Prog Allergy* 1978; 25: 1–62.
12. NAYAK BN, KISIL FT. NH₂-terminal amino acid sequences of *Poa p* Ia and *Poa p* Ib allergens in Kentucky bluegrass pollen. *J Allergy Clin Immunol* 1991; 87: 325–31.
13. ORESTE U, COSCIA MR, SCOTTO D'ABUSCO A, SANTO-NASTASO V, RUFFILLI A. Purification and characterisation of *Par o* I, major allergen of *Parietaria officinalis* pollen. *Int Arch Allergy Appl Immunol* 1991; 96: 19–27.
14. POLO F, AYUSO R, CARREIRA J. Studies on the relationship between structure and IgE-binding ability of *Parietaria judaica* allergen I. *Mol Immunol* 1991; 28: 169–75.
15. RAFNAR T, GRIFFITH IJ, KUO M, BOND JF, ROGERS BL, KLAPPER DJ. Cloning of *Amb a* I (antigen E), the major allergen family of short ragweed pollen. *J Biol Chem* 1991; 266: 1229–36.
16. REISNER AH, NEMES P, BUCHOLTZ C. The use of Coomassie brilliant blue G-250 perchloric acid solution for staining in electrophoresis and isoelectric focusing on polyacrylamide gels. *Anal Biochem* 1975; 64: 509–16.
17. SALLUSTO F, QUINTIERI F, PUGLIESE O, REALE G, PINI C, DI FELICE G. T-cell and antibody response to *Parietaria judaica* allergenic fractions in atopic and nonatopic individuals. *Allergy* 1993; 48: 37–44.
18. SCOTTO D'ABUSCO A, MENNA T, DE SANTO C, COSCIA MR, ORESTE U, RUFFILLI A. A cDNA sequence encoding a dominant epitope of *Par o* I. *Molecular biology and immunology of allergens*. Boca Raton, FL: CRS Press, 1993: 251–3.
19. TOWBIN H, STAHELIN T, GORDON J. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1980; 76: 365–70.
20. WRIGLEY C. Gel electrofocusing. *Methods Enzymol* 1971; 22: 559–64.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.