

Isolation and purification of a major allergen from *Parietaria officinalis* pollen

A. GIULIANI, C. PINI, S. BONINI*, N. MUCCI, L. FERRONI & G. VICARI

Laboratorio di Immunologia, Istituto Superiore di Sanità, and *I Clinica Medica, Università "La Sapienza", Roma, Italy

A major allergen of *Parietaria officinalis*, a species responsible for a large number of respiratory allergies in Mediterranean areas, has been identified and characterized. This allergen (Pol) was found in the fraction which precipitates between 70 and 100 % ammonium sulphate saturation. Pol showed a molecular weight of 15,000 daltons as determined by SDS-PAGE and HPLC. The pI of Pol was in the pH region 4-6, IEF showing four major bands. Two major bands were shown by CIE, CRIE and immunoblotting; major contaminants or aggregates were also revealed by the latter technique and by HPLC. Pol showed an allergic specific activity 2 times higher than the crude extract; moreover it was shown to be a major allergen since it inhibited 29 out of 30 sera from allergic patients sensitive to *P. officinalis*.

Key words: allergen; fractionation; pollen; purification.

Accepted for publication 22 January 1987

In recent years many studies have dealt with the isolation and chemical characterization of different pollens widely diffused and clinically relevant in North America and Europe. However, identification, isolation and characterization have been obtained for a few species of grasses only, such as Rye grass (*Lolium perenne*) (13, 14), Timothy grass (*Phleum pratense*) (18), and short Ragweed (*Ambrosia elatior*) (15, 16, 20, 23).

Parietaria is clinically relevant in Italy and in other Mediterranean countries due to its widespread distribution in these areas and to its long pollinating season. Among the different species (e.g. *P. officinalis*, *P. judaica*, *P. lusitanica*, *P. mauritanica*, etc.) the most common in Italy are *P. officinalis* and *P. judaica*, which have different distribution areas, the latter being widespread all over the country (3, 7), whereas *P. officinalis* is mainly present in Northern and Central Italy.

Up to now, both *Parietaria* species have been studied, but structure and composition of the allergenic components are better defined for *P. judaica* (4, 8). This paper reports biochemical and immunochemical data on *P. officinalis* pollen allergens.

MATERIAL AND METHODS

Preparation of pollen extract

Dried *P. officinalis* pollen was kindly provided by Bayropharm Italiana (Milano). The material was stored at 4°C under vacuum until used. The pollen extraction was carried out according to Marsh (21) with minor modifications. Briefly, ether-soluble fatty compounds were removed by washing pollen eight times with 1:5 (w/v) peroxide-free dry diethylether. According to Hussain et al. (12) the defatting method was chosen to improve the fractionation; indeed, isoelectric focusing performed on defatted and non-defatted material confirms that defatting does not introduce any change in the release of antigens from pollen. Defatted pollen was submitted to extraction in a 0.125 M ammonium bicarbonate buffer pH 7.5 containing 1 mM phenyl-methyl-sulphonyl-fluoride (PMSF) and 15 mM sodium azide for 20 h at 4°C. After extensive dialysis against 2 mM NH₄HCO₃, containing 15 mM NaN₃, and finally against water, the pollen extract was lyophilised and stored at 4°C until used.

Salt precipitation

Ammonium sulphate precipitation was carried out at different saturations overnight at 4°C. Precipitates and supernatants were extensively dialysed against water using Visking tubing (Union Carbide, Canada) with a cut-off of about 10,000 daltons, and then lyophilised.

Isoelectric focusing

Isoelectric focusing (IEF) was performed at 1200 V for 90 min at 4°C in a Multiphor apparatus (LKB, Bromma, Sweden) in 2 mm thin layer polyacrylamide gels containing 2 % (v/v) LKB ampholine, pH 3.5–10. Gel preparation, run conditions, and staining procedure were performed according to LKB instructions.

Chemical determinations

Proteins were determined according to Bradford (2). The binding of Blue Coomassie G-250 to the proteins was measured at 595 nm, using bovine serum albumin as reference.

Total sugars were evaluated by the phenol-sulphuric acid method (6), using mannose as reference, and readings were performed at 490 nm.

Sera

Antibodies against *P. officinalis* whole extract were raised in rabbits essentially according to

Mayer & Walker (22); the animals were injected with 1 ml of a 1:1 emulsion of pollen extract (2 mg/ml saline) in complete Freund's adjuvant. The injection was repeated after 1 month and for each month later up to a period of 6 months.

The presence of specific antibodies was tested by rocket immunoelectrophoresis (RIE) and by crossed-immunoelectrophoresis (CIE), and the optimal bleeding was chosen for all experiments.

Human sera were obtained from 30 patients sensitive to *P. officinalis* only as proven by skin test and RAST. Highly positive sera were pooled, aliquoted and stored at -30°C until used.

RIE, CIE, and CRIE

Rocket immunoelectrophoresis, crossed immunoelectrophoresis and crossed radioimmuno-electrophoresis (CRIE) were performed in an LKB apparatus, according to Löwenstein (19).

Polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a vertical slab gel in a continuous gradient ranging from 5 to 20 %, according to Laemmli (17). The run was carried out in a refrigerated system and the gel was stained with 0.05 % Coomassie Brilliant Blue in water:methanol:acetic acid (50:40:10).

The blotting was performed according to Towbin et al. (24) in a BIO-RAD apparatus.

Table 1
Mean yield, protein content and allergenic activity of fractions obtained by ammonium sulphate precipitation of three batches of *P. officinalis* pollen extract

		Dry weight mg (range)	Protein percent mg (range)	RAST inhibition µg prot/ml (range)
Whole extract		200	18	1.01 (0.4–1.6)
70 % *	PPT	55.4 (54.7–56)	34.2 (32.5–36)	7.2
	SUP	97.2 (94–101)	3.7 (3.2–4.1)	0.84 (0.26–1.36)
70–100 % **	PPT	11.6 (11–12.3)	16 (14–18)	0.46 (0.12–0.84)
	SUP	39.9 (39.8–40)	0.56 (0.33–0.8)	1.6

* 70 % ammonium sulphate precipitation.
** 100 % ammonium sulphate precipitation of the 70 % SUP.

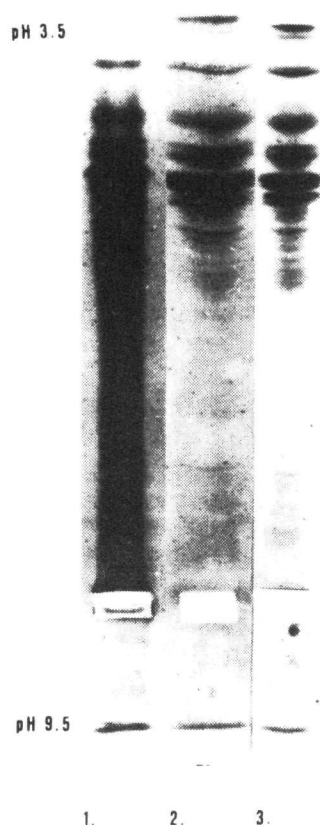


Fig. 1. Isoelectrofocusing of different steps from *P. officinalis* pollen extract purification. 1. Whole extract; 2. 70% SUP; 3. 70–100% PPT. 40 µg of protein were run at 1200 V for 90 min at 4°C. pH range 3.5–10. After running, the proteins were fixed with 50% TCA for 1 h and stained overnight with 0.3% Coomassie Brilliant Blue G 250 dissolved in ethanol: acetic acid: water (2.5: 0.8: 10). Destaining was performed with water.

Allergenic components were detected by incubation with the pool of sera from *P. officinalis*-sensitive patients and ^{125}I -anti-human IgE (Pharmacia, Uppsala, Sweden).

RAST inhibition

RAST inhibition was performed according to Yman et al. (25). The fractions were tested at 10-fold serial dilutions ranging from 1 mg/ml (dry weight) to 0.1 µg/ml. Data were expressed

in terms of protein concentration of fraction which produced 50% inhibition.

High pressure liquid chromatography

Gel filtration was performed at room temperature in a high pressure liquid chromatography (HPLC) system consisting of a Waters Model 590 pump. An LKB 2135 Ultropac Column (Blue Column) TSK-G2000 (7.5 × 600 mm) was used. The column was equilibrated with 0.05 M phosphate buffer, pH 7 and the eluate was monitored at 280 nm using an LKB Uvicord apparatus.

RESULTS

Ten grams of dry pollen gave a crude extract which, after extensive dialysis and lyophilisation, contained approximately 1 g of dry weight material with a protein content of 18 per cent.

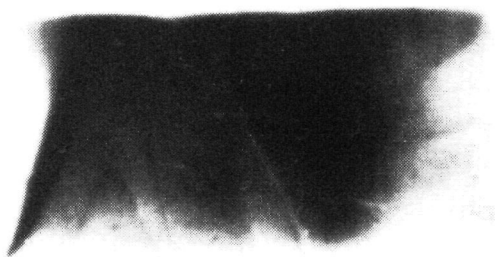
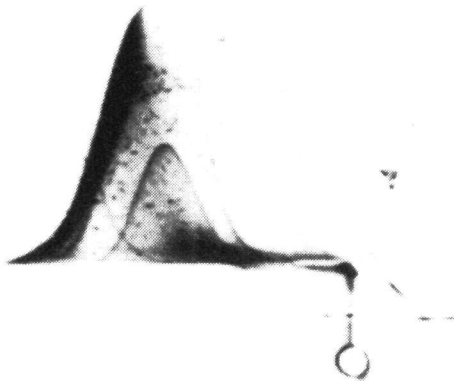
Aliquots of 200 mg of this material were submitted to ammonium sulphate precipitation at three final saturations, namely 50%, 70% and 90%. The obtained fractions were tested for specific inhibiting activity and electrophoretic pattern. The supernatant of the 70% (70% SUP) final saturation displayed the highest specific activity in RAST inhibition and the lowest number of components in IEF and CIE. Therefore, this condition was subsequently chosen as the first step in the purification procedure of the pollen extract.

Further purification and concentration of the 70% supernatant fraction was performed by submitting it to a precipitation at ammonium sulphate 100% saturation (70–100% PPT).

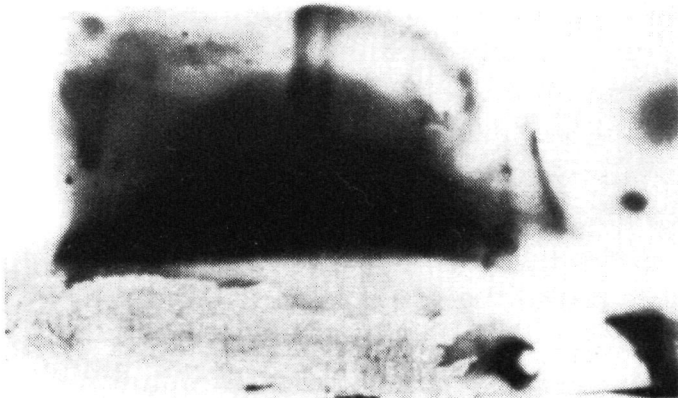
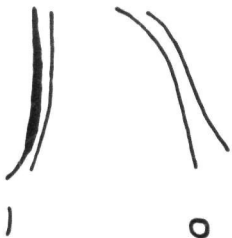
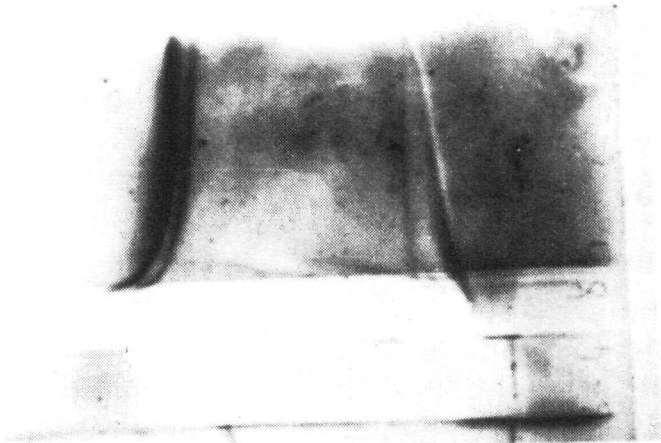
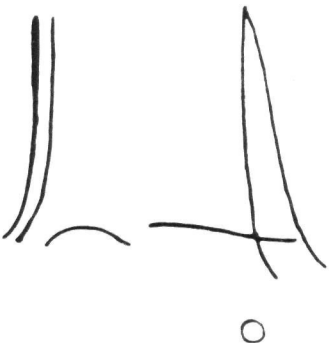
Table 1 reports the mean yield for three batches of allergen obtained from aliquots of 200 mg (dry weight) of pollen extract. A mean protein concentration of about 4% and a speci-

Fig. 2. Crossed immunoelectrophoresis and crossed radio immunoelectrophoresis of *P. officinalis* whole extract and 70–100% PPT. a) Whole extract. CIE (upper) and CRIE (lower). b) 70–100% PPT. CIE (upper) and CRIE (lower). On the left the drawing of the respective precipitation pattern. CIE: in the first dimension 90 µg of protein were run at 10 V/cm for 75 min. Temperature was 10°C. The second dimension was carried out against the antibody-containing gel at 2 V/cm for 18 h at 16°C. CRIE: CIE plate was incubated with *Parietaria*-sensitive patient serum pool, washed and incubated with ^{125}I -anti-human IgE. The gel was then autoradiographed at -80°C for 15 days.

a



b



fic mean activity of 0.84 $\mu\text{g/ml}$ were detected in the supernatant.

When this fraction was submitted to 100% precipitation all the protein was recovered in the precipitate which displayed a mean value of 16% of proteins and an inhibiting activity of 0.46 $\mu\text{g/ml}$.

Despite the fact that only a twofold increase in the specific activity could be obtained in the fraction, a strong concentration of the active material, together with a significant loss of pigments, sugars (19% vs 6%) and small molecules, could be achieved by this procedure.

Fig. 1 shows the results obtained when the two fractions (70% SUP and 70-100% PPT) were analysed by IEF. The high heterogeneity of the whole extract is reduced in a more narrow region between pH 4 and 6.

Fig. 2 shows CIE and CRIE of the crude extract and of the 70-100% PPT. At least eight precipitin arcs are shown in the crude extract, all reactive with both rabbit antiserum and human patient sera, whereas two precipitin arcs only can be detected in 70-100% PPT.

The SDS-PAGE patterns of the crude extract and of the 70-100% PPT obtained both after protein staining and immunoblotting are shown in Fig. 3. Protein staining of 70-100% PPT reveals only two large bands almost confluent with a molecular weight close to 15,000 daltons. These bands cannot be easily identified in the whole extract, possibly because of their low concentration in the starting material. Immunoblotting shows the presence of two additional bands of higher molecular weight, suggesting the presence of aggregates or other components.

The presence of more than one component was confirmed when 70-100% PPT was submitted to HPLC (Fig. 4). A major peak with a molecular weight of 15,000 daltons was present. Traces of components of different molecular weight could also be detected. The major peak had a specific activity of 0.21 $\mu\text{g protein/ml}$ which was approximately twofold higher as compared to the 70-100% PPT.

The reactivity of the purified fraction with individual patient sera was studied by RAST inhibition experiments; 29 of the 30 sera were strongly inhibited by the fraction. ($91 \pm 0.8\%$ of

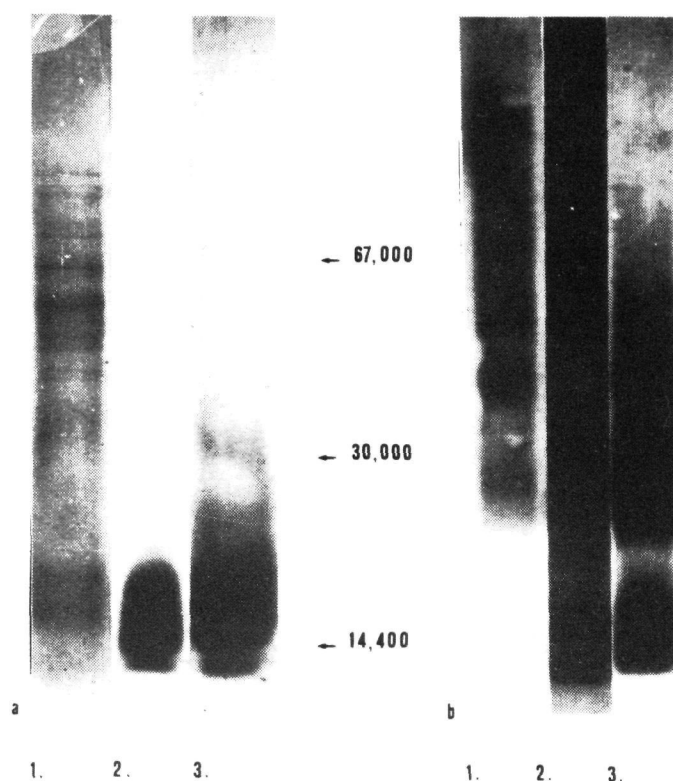


Fig. 3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (a) and immunoblotting (b) of *P. officinalis* whole extract and fractions. 1. Whole extract; 2. 70% SUP; 3. 70-100% PPT. SDS-PAGE was performed in reducing conditions at 20 mA for 2 h and at 40 mA for 3 additional hours. Blotting was performed overnight at 150 mA constant current. Paper (9×16 cm) was soaked in 25 ml of the patient sera diluted 1:10 in saline and specific IgE were revealed after incubation with 800,000 cpm of ^{125}I -anti-human-IgE. The X-ray film was developed after 8 days.

maximal inhibition). The results are shown in Fig. 5.

DISCUSSION

The present paper reports on the isolation and purification of a major allergen of *Parietaria officinalis*, a species responsible for a large number of respiratory allergies in Mediterranean areas. The purified fraction, hereafter termed Pol, is obtained by 100% ammonium sulphate precipitation of the 70% ammonium sulphate supernatant of an aqueous *P. officinalis* extract. The molecular weight of Pol, as determined both by SDS-PAGE in reducing conditions and by HPLC in native conditions, is close to 15,000 daltons. The pI of Pol falls in the pH region 4-6, IEF showing four major

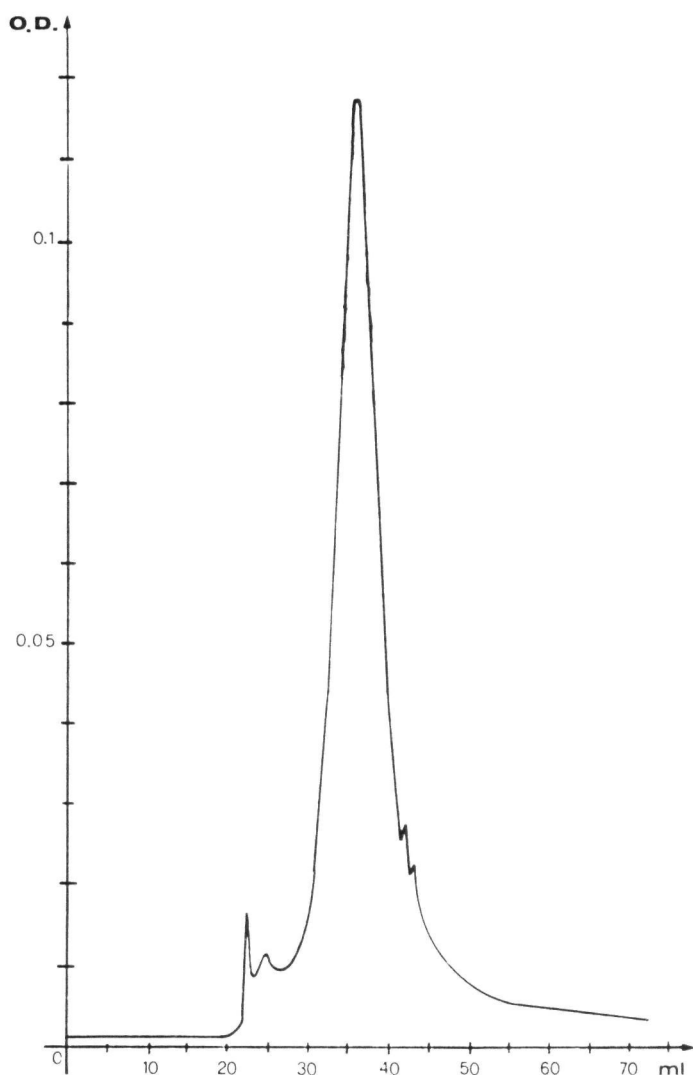


Fig. 4. Gel filtration of 70–100% PPT. The run was performed in TSK G2000 HPLC column 7.5×600 mm, 0.05 M phosphate buffer pH 7. 70–100% PPT protein: 400 μ g flow rate: 0.5 ml/min.

bands. CIE and CRIE of the purified fraction demonstrated the presence of two precipitin arcs; analysis of the same fraction by immunoblotting after SDS-PAGE confirmed the presence of these two active bands with molecular weight of 15,000 daltons together with additional bands of higher molecular weight, undetectable after Coomassie staining of the gel.

Several biochemical and immunochemical studies have been performed on allergens from *Parietaria* spp. but a complete purification has been reported for *P. judaica* only. An active polypeptide with a molecular weight of 10,000 daltons has been described by Feo et al. (8) and by Corbi & Carreira (4) for *P. judaica*. *P. officinalis* pollen extract has been studied by

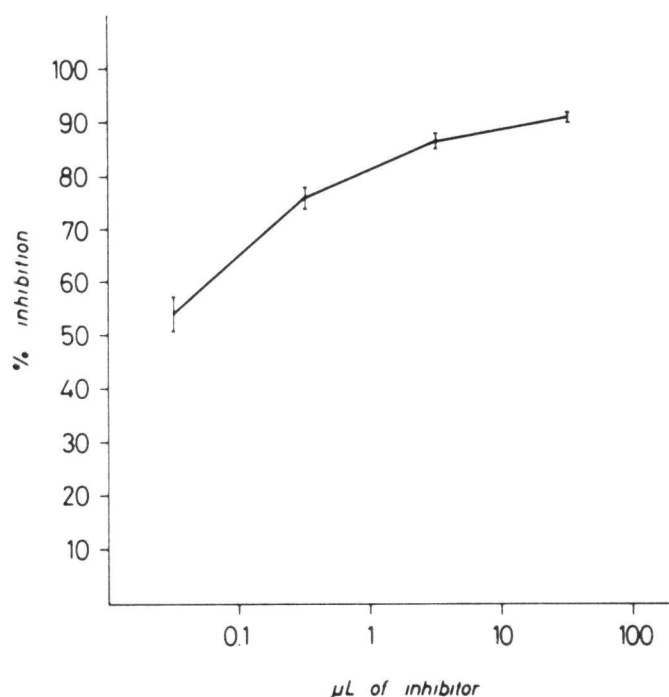


Fig. 5. Behaviour of individual sera tested with purified allergen Pol in RAST inhibition. Each point represents the mean value ($\pm$ SE) of 29 sera.

Geraci et al. (10). Four fractions with allergenic activity and a molecular weight ranging from 12,000 to 42,000 daltons were obtained. These fractions were present in both the non-dialysable and the dialysable material.

This paper describes a procedure for obtaining a purified fraction with well-defined biochemical and immunochemical properties. This antigen shows biochemical and immunochemical properties (MW, pI and CIE pattern) similar to those of the allergens Pj10 described by Corbi & Carreira (4) and Pj2 described by Feo et al. (8). Indeed, recent data have been presented on cross-reactivity between *P. officinalis* and *P. judaica* extracts (1, 5). Several bands in CIE and CRIE were reported by Geraci et al. (11). It is conceivable that several allergens are present in *Parietaria* spp. pollens, as recently reported by Ford et al. (9) for *P. judaica* by using the immunoblotting technique. From the same paper it is also evident that the major allergen displays an MW and an immunoelectrophoretic pattern almost identical to our purified antigen, a finding which further confirms the similarity between the two species. Moreover, in agreement with the above authors

(9), our results suggest that Pol is a major allergen since 29 of 30 of the sera from *P. officinalis*-sensitive subjects were strongly inhibited by it in RAST inhibition.

The availability of purified allergens greatly facilitates studies on cellular and humoral immunity in allergic patients. Purified allergenic fractions may also be useful for standardization of extracts, which is a primary requirement for an adequate diagnosis and the immunotherapy of atopy.

ACKNOWLEDGEMENTS

The authors thank Mr. G. Lanzi for his technical assistance in the HPLC analysis and Mr. L. Bernardini for the photographic assistance. Special thanks to Mrs. D. Campanile and Mr. G. Rizzottolo for their laboratory assistance.

REFERENCES

1. Bengtsson, A., Colfsen, W., Einarsson, R.: Characterization of allergens and patient sera by a nitrocellulose immunoprint technique. *Int. Arch. Allergy Appl. Immunol.* **78**, 139-144, 1985.
2. Bradford, M. M.: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254, 1976.
3. Charpin, J., Surinyach, R. & Frankland, W.: *Atlas Europeen des pollens allergisants*. Sandoz, Paris, 1974.
4. Corbi, A. L. & Carreira, J.: Identification and characterization of *Parietaria judaica* allergens. *Int. Arch. Allergy Appl. Immunol.* **74**, 318-323, 1984.
5. Corbi, A. L., Pelaez, A., Errigo, E. & Carreira, J.: Cross-reactivity between *Parietaria judaica* and *Parietaria officinalis*. *Ann. Allergy* **54**, 142-147, 1985.
6. Dubois, M., Gilles, K. A., Hamilton, J. K., Roberts, P. A. & Smith, F.: Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**, 350-354, 1956.
7. Errigo, E., Monti, R. & Battista, A.: Indagini sulla pollinosi da *Parietaria* in Italia. *Folia Allergol. Immunol. Clin.* **29**, 155-163, 1982.
8. Feo, S., Cocchiara, R. & Geraci, D.: Allergens of *Parietaria judaica* pollen. I. Purification and chemical characterization of a hapten and a low molecular weight allergenic peptide. *Molec. Immunol.* **21**, 25-36, 1984.
9. Ford, S. A., Baldo, B. A., Geraci, D. & Boss, D.: Identification of *Parietaria judaica* pollen allergens. *Int. Arch. Allergy Appl. Immunol.* **79**, 120-126, 1986.
10. Geraci, D., Oreste, U. & Ruffilli, A.: Purification and characterization of allergens from *Parietaria officinalis* pollen. *Immunochemistry*, **15**, 491-498, 1978.
11. Geraci, D., Billesbolle, K. B., Cocchiara, R., Löwenstein, H. & Ipsen, H.: Immunochemical characterization of antigens of *Parietaria judaica* pollen. *Int. Arch. Allergy Appl. Immunol.* **78**, 421-428, 1985.
12. Hussain, R., Norman, P. S. & Marsh, D. G.: Rapidly released allergens from short ragweed pollen. *J. Allergy Clin. Immunol.* **67**, 217-222, 1981.
13. Johnson, P. & Marsh, D. G.: The isolation and characterization of allergens from the pollen of rye grass (*Lolium perenne*). *Eur. Polymer. J.* **1**, 63-77, 1965.
14. Johnson, P. & Marsh, D. G.: Allergens from common rye grass pollen (*Lolium perenne*). I. Chemical composition and structure. *Immunochemistry* **3**, 91-100, 1966.
15. King, T. P., Norman, P. S. & Lichtenstein, L. M.: Isolation and characterization of allergens from ragweed pollen. IV. Biochemistry **6**, 1992-2000, 1967.
16. Klapper, D. G., Goodfriend, L. & Capra, J. D.: Amino acid sequence of ragweed allergen Ra3. *Biochemistry* **19**, 5729-5734, 1980.
17. Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685, 1970.
18. Löwenstein, H.: Isolation and partial characterization of three allergens of timothy pollen. *Allergy* **33**, 30-41, 1978.
19. Löwenstein, H.: Quantitative immunoelectrophoretic methods as a tool for the analysis and isolation of allergens. *Prog. Allergy* **25**, 1-62, 1978.
20. Löwenstein, H. & Marsh, D. G.: Antigens of *Ambrosia elatior* (Short ragweed) pollen. *J. Immunol.* **126**, 943-948, 1981.
21. Marsh, D. G., Haddad, Z. H. & Campbell, D. H.: A new method for determining the distribution of allergenic fractions in biological materials: its application to grass pollen extracts. *J. Allergy* **46**, 107-121, 1970.
22. Mayer, R. J. & Walker, J. H.: *Immunochemical methods in the biological sciences: enzymes and proteins*. Academic Press, London, 1980.
23. Mole, L. E., Goodfriend, L., Lapkoff, C. B., Kehoe, J. M. & Capra, J. D.: The amino acid sequence of allergen Ra5. *Biochemistry* **14**, 1216-1220, 1975.
24. Towbin, H., Staehlin, T. & Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci.* **76**, 4350-4354, 1979.
25. Yman, L., Ponterius, G. & Brandt, R.: RAST-based allergen assay methods. *Dev. Biol. Stand.* **29**, 151-165, 1975.

Address:

Anna Giuliani

Laboratorio di Immunologia

Istituto Superiore di Sanità

Viale Regina Elena 299-Roma

Italy

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.