

PURIFICATION OF *ART v I*, A RELEVANT ALLERGEN
OF *ARTEMISIA VULGARIS* POLLEN

FRANCISCO DE LA HOZ,*† FLORENTINO POLO,* JAIME MOSCOSO DEL PRADO,* JAVIER G. SELLÉS,‡
MANUEL LOMBARDEO* and JOSÉ CARREIRA*

*Alergia e Inmunología Abelló S. A., Miguel Fleta 19, 28037 Madrid, Spain and ‡Ciudad Sanitaria Virgen
de la Arrixaca, Murcia, Spain

(First received 21 July 1989; accepted in revised form 9 January 1990)

Abstract—An allergenic protein from *Artemisia vulgaris* pollen has been purified to homogeneity. Its molecular weight in native conditions is 47,000. The purified allergen, hereafter denominated *Art v I*, is a monomeric protein. It is a clinically relevant allergen since, at least, 70% of the individuals allergic to *Artemisia vulgaris* pollen have specific IgE in serum and in mast cells, demonstrated by ELISA and skin prick tests, respectively.

INTRODUCTION

Artemisia genus is constituted by various species which belong to the family Compositae, and, among them, *Artemisia vulgaris* is of major importance. It is widely spread, especially, in the temperate and humid zones of the northern hemisphere and along the mediterranean basin (Futin *et al.*, 1976).

The incidence of allergic disease caused by the pollen of this species is quite high, being accepted that between 10 and 14% of pollinic patients suffer from allergy to it (Cornillon *et al.*, 1972; Duchaine and Spapen, 1961; Spieksma *et al.*, 1980). Pollen of *Artemisia vulgaris* has been considered the most important cause of pollinosis at the end of summer and beginning of autumn, in western Europe, with the precision that in Central Europe pollinization occurs at the end of July and through August while in the mediterranean areas it takes place, mostly, in September and beginning of October (Spieksma *et al.*, 1980).

Previous studies showed that, at least, 9 allergens could be demonstrated in the soluble fraction of *Artemisia vulgaris* pollen. The most important were found at pIs 4.2–4.7 and 8–9, while proteins with pIs between 5 and 8 did not exhibit significant allergenic activity. The inhibition capacity between acidic and basic groups was less than 50%, but it was very high when allergens of the same group were compared by RAST inhibition (Hoz, de la *et al.*, 1990).

Using crossed-radioimmuno-electrophoresis others (Ipsen *et al.*, 1985; Smestad Paulsen *et al.*, 1985; Diener *et al.*, 1988) had previously shown that this pollen contained between 7 and 10 allergens.

The present paper describes the purification of an allergenic protein to homogeneity. Its immunochemical and biochemical characteristics as well as its

capacity to bind individual serum IgE, both *in vivo* and *in vitro*, are presented and discussed.

MATERIALS AND METHODS

Source material

Pollen of *Artemisia vulgaris* was purchased from Allergon AB, Sweden. The batch used throughout this work contained less than 3% of non-pollen contaminants and less than 0.5% of foreign pollens.

Preparation of extract

Pollen was extracted with 50 mM ammonium bicarbonate pH 8.5 at a 10% (w/v) ratio. After stirring for 60 min at room temperature, soluble components were separated from the insoluble ones by centrifugation at 27,000 *g* for 20 min at 4°C. The supernatant was dialyzed against distilled water, centrifuged again as indicated, filtered through a 0.22 μ m pore size membrane and lyophilized.

Protein determination

Protein concentration was measured according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis and isoelectric focusing

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as indicated by Laemmli (1970) with an acrylamide gradient concentration of 10–18%, and the protein bands were stained with Coomassie brilliant blue.

Sometimes an approximate quantitation of Coomassie stained protein bands was performed using a Bio-Rad Video Densitometer Model 620, and when possible, a purified marker was used as reference.

†Author to whom correspondence should be addressed.

Analytical isoelectric focusing (IEF) was carried out in agarose plates from FMC Bio-Products, according to the manufacturer's protocol, using a pH gradient from 3.5 to 10.

Immunodetection

Electrotransfer of separated polypeptides was carried out according to Towbin *et al.* (1979), followed by immunodetection of IgE-binding polypeptides using a human serum pool from 33 *Artemisia vulgaris* sensitive patients and Hamlet[®]-¹²⁵I (Alergia e Immunologia Abelló), an iodinated mouse anti-human IgE monoclonal antibody as reported elsewhere (Corbi and Carreira, 1984).

Dot immunoblotting

Fractions from the Ultrogel AcA 44 column, with capacity to bind specific human IgE, were detected by dot immunoblotting (Hawkes *et al.*, 1982) following the protocol and basic equipment of Bio-Rad, Bulletin 1141. Five microliter samples were applied per fraction and after washing, the nonspecific antibody binding sites were blocked with 3% BSA. The membrane was washed again, and the further detection of allergens was accomplished as indicated in the previous paragraph for polypeptides separated by SDS-PAGE and electrotransferred.

Immuno-electrophoresis

Crossed immuno-electrophoresis (CIE) was performed as recommended by Weeke (1973) using a rabbit serum pool from four animals made up of 10 successive bleedings. All rabbits were injected with 2 mg of protein of the whole pollen extract in Freund's Complete Adjuvant for the first time, and then, with the same amount of protein in Freund's Incomplete Adjuvant every 2 weeks. After the seventh injection the serum titer of all animals was considered to be adequate by radial immunodiffusion, and since then, 10 bleedings were carried out alternating weekly bleedings and injections.

For crossed radioimmuno-electrophoresis (CRIE) (Weeke and Lowenstein, 1973) we employed a human serum pool from 33 patients who had demonstrated clinical hypersensitivity with strong cutaneous reactions and high levels of specific IgE to the extract of *Artemisia vulgaris* pollen.

The ¹²⁵I anti-human IgE used was the Hamlet[®] from Alergia e Immunologia Abelló, and the total radioactivity applied per plate was 250,000 cpm and the autoradiography duration was 96 hr.

Ammonium sulfate precipitation

Seventy-five milligrams of pollen extract protein was resuspended in 50 ml of distilled water, and then, solid ammonium sulfate was added gradually under stirring at 4°C until 55% (2.66 M) saturation was reached. Precipitated proteins were recovered after centrifugation at 27,000 g for 15 min at 4°C, resus-

pended in 50 ml of PBS, exhaustively dialyzed against distilled water and, finally, lyophilized.

Gel filtration

Those proteins precipitated with ammonium sulfate at 55% saturation, and exhaustively dialyzed, were filtered through a 1.6 × 100 cm Ultrogel AcA 44 column, at 4°C, which had been equilibrated with 50 mM Tris-HCl pH 7.5. The sample was run in the same buffer at a flow rate of 6 ml/hr and 1.6 ml fractions were collected. Protein elution was checked by measuring the absorbance at 280 nm and by SDS-PAGE. Central fractions of an individual peak containing *Art v I* were pooled, dialyzed and lyophilized.

Gel filtration on Spherogel TSKG 3000 SW column (21.5 × 300 cm) was carried out using an HPLC equipment from Waters. The mobile phase consisted of 100 mM Na₂SO₄-20 mM NaH₂PO₄, pH 6.8, and the sample was eluted at a constant flow rate of 1 ml/min. Sample elution was monitored at 280 and 214 nm. The column was calibrated by injecting a gel filtration standard from Bio-Rad, containing thyroglobulin (mol. wt. 670 kD), gamma globulin (150 kD), ovalbumin (44 kD) and myoglobulin (17 kD).

Immunoassays

Detection of specific human IgE was carried out by radioimmunoassay, linking the whole extract to cyanogen bromide activated paper discs (Ceska *et al.*, 1972). Individual sera from *Artemisia vulgaris* sensitive patients was added to the discs and, after incubation and washing, Hamlet[®]-¹²⁵I (Sanchez-Madrid *et al.*, 1984), an anti-human IgE monoclonal antibody, was also added. Radioactivity bound to the discs indicated the presence of specific IgE.

For the purified allergen, an enzyme immunoassay was used in which the allergen was coated to polystyrene microtiter wells (Dynatech). A quantity of 100 µl individual patient's sera was added, incubated for 2 hr at room temperature and washed with PBS 0.05% Tween-20. Then 100 µl Hamlet[®]-β-galactosidase, an enzyme-labelled anti-human IgE monoclonal antibody from Alergia e Immunologia Abelló, was applied to the wells and incubated for 1 hr. After washing, 100 µl of 300 mM, pH 7.25, orthonitrophenyl-β-D-galactopyranoside in 70 mM Hepes, 150 mM NaCl, 10 mM MgCl₂ was added, and after 2 hr at 37°C the enzymatic reaction was stopped with 100 µl of 1 M Na₂CO₃. Finally, the absorbance was read at 405 nm, using a 620 nm reference filter. IgE values were calculated by interpolation with a standard curve against *L. perenne* (Hamlet[®]-IgE).

Skin prick test

Purified allergen was tested in 34 allergic patients with clear hypersensitivity to *Artemisia vulgaris* pollen as demonstrated by the patient's clinical history, the cutaneous positive reaction to commercially

available *in vivo* diagnostics and the presence of serum IgE specific to *Artemisia vulgaris* pollen proteins, using commercial diagnostic kits. The concentrations tested were 2.5 and 0.5 µg of purified allergen/ml. As control a standardized extract with an activity of 100 BU/ml was used simultaneously. The activity of 100 BU/ml corresponds to an extract concentration which produces in the population of patients allergic to *Artemisia vulgaris* a geometric mean weal of 75 mm² (Lombardero *et al.*, 1986). An extract at 100 BU/ml contains, ca. 2.5 µg/ml of this allergen, as could be observed comparing the densitometric tracings of the SDS-PAGE analysis of the crude extract and the purified *Art v I*.

RESULTS

Purification of *Art v I*

The lyophilized supernatant obtained following ammonium bicarbonate extraction of *A. vulgaris* pollen yielded about 20 mg of protein per gram of pollen, and the sediment recovered after precipitating this extract with 2.66 M ammonium sulfate contained about 8% of the whole extract protein. This sediment was exhaustively dialysed against distilled water and a sample was applied to an Ultrogel AcA 44 column, which, on elution, produced the profile shown in Fig. 1. All elution fractions, starting with the void volume, were analysed by dot immunoblotting (Fig. 2) and those which showed capacity to bind specific human IgE (dots 26-33 in Fig. 2 corresponding to shaded peak in Fig. 1) were pooled. Approximately 26% of the total protein applied on top of the column was recovered in this pool. Finally, an Ultrogel

filtered sample was chromatographed through an HPLC Spherogel column rendering the elution profile shown in the insert of Fig. 1, where a yield of 76% of the applied protein was found in the main peak. After this third purification step 1.6% of the total extract protein was shown to be recovered as *Art v I*.

Some characteristics of *Art v I*

SDS-PAGE analysis of the crude extract from *A. vulgaris* pollen as well as of samples from the three purification steps is presented in Fig. 3. Ammonium sulfate precipitated material (lane B) contained several protein bands, being the one with mol. wt 60,000 D the most important, which on the basis of densitometric determinations, after Coomassie blue staining, represents about the 70% of total protein in the gel. Pooled fractions from the Ultrogel column (lane C) contained the 60,000 D protein, apparently, free from other contaminant bands, in spite of the large amount of protein applied to the gel (50 µg). However, as visualized in the next figure a slight contamination was still present, which disappeared after the third purification step (lane D).

Figure 4 shows the immunoblotting of those polypeptides separated in Fig. 3. At least, 10 polypeptides bound specific human IgE in the crude extract (lane A'), 7 in the ammonium sulfate precipitated material (lane B'), 2 in the Ultrogel purified fractions (lane C') and only one of 60,000 D after the last purification step (lane D').

Analytical isoelectric focusing of *Art v I* is presented in Fig. 5. In comparison with the multiple bands of the whole extract (lane A) purified allergen (lane B) displays only a neat band with pI 4.4.

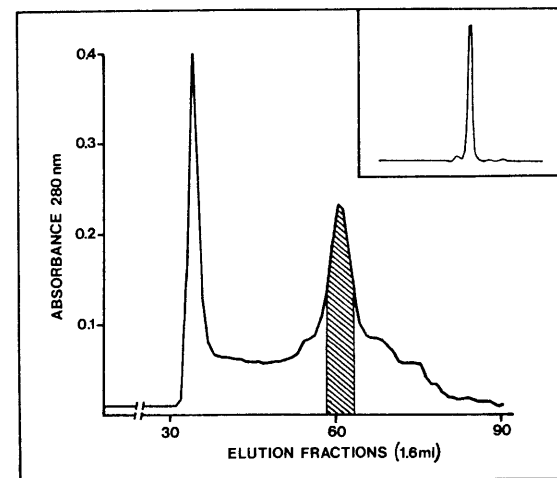


Fig. 1. Elution profile of ammonium sulfate precipitated *Art v I* through Ultrogel AcA 44. Five milligrams of protein was applied to the column. The shaded peak indicates the column elution of *Art v I*. The insert shows the elution profile of 2 mg Ultrogel purified protein after HPLC filtration on a Spherogel TSKG 3000 SW. Other details in Materials and Methods.

1 - 10
11 - 20
21 - 30
31 - 40
41 - 50
51 - 60

Fig. 2. Dot immunoblotting of elution fractions from the Ultrogel column. Dots 1-60 correspond to fractions 30-90 in Fig. 1. A quantity of 250,000 cpm was applied for all dots and the iodinated membrane was exposed to an X-ray film for 48 hr.

CRIE examination of crude extract (Fig. 6) demonstrated that, at least, 10 pollen proteins had capacity to bind specific IgE; 7 anodic and 3 cathodic. This shows that less than one third of all proteins (34) precipitated in CIE experiments (results not included) had allergenic activity. In contrast (Fig. 6) both CIE

Mw
Kd

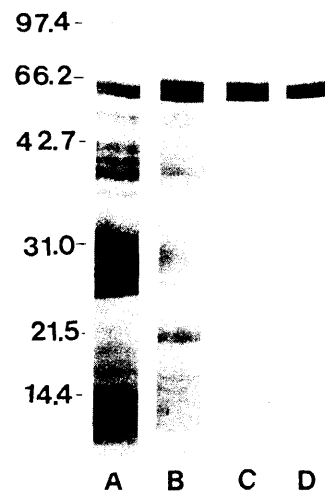


Fig. 3. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The acrylamide concentration was 10-18% gradient. (A) A quantity of 125 µg of the crude extract. (B) A quantity of 75 µg of ammonium sulfate precipitated protein. (C) A quantity 50 µg of Ultrogel pooled fractions. (D) A quantity of 50 µg of HPLC purified *Art v I*. All samples were stained with Coomassie brilliant blue.

Mw
Kd

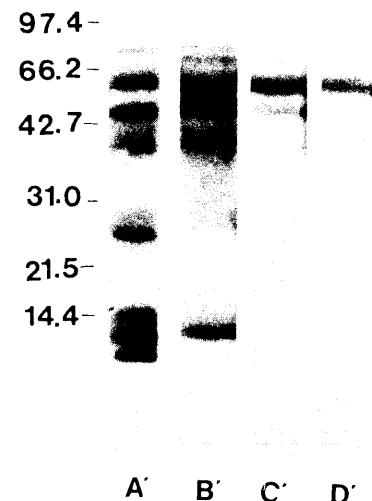


Fig. 4. Immunoblotting of polypeptides separated in Fig. 3. A', B', C', D' correspond to A, B, C, D in that figure. A quantity of 125,000 cpm was applied per lane and the autoradiography duration was 20 hr.

pI
9.5-

7.0-

5.0-

3.5-

A B

Fig. 5. Analytical isoelectric focusing on agarose gel of the whole extract (a) and purified *Art v I* (B). A quantity of 70 µg of whole extract and 20 µg of purified allergen was applied to the respective gels.

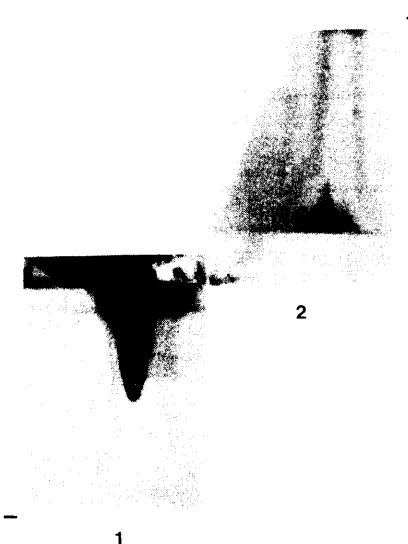


Fig. 6. Crossed radioimmuno-electrophoresis of the crude extract of *Artemisia vulgaris* pollen. Thirty micrograms of protein was applied per plate.

Table 1. Cutaneous reactivity to *Artemisia vulgaris* pollen extract and two concentrations of purified *Art v I* in 34 allergic patients

Sample concentrations	Geometric mean of weal area (mm ²)	Positivity ^a (%)
Whole extract, 100 BU/ml ^b	73	97
<i>Art v I</i> 2.5 µg/ml	45	73
<i>Art v I</i> 0.5 µg/ml	29	70

^aWe considered positives those weals with a size equal or larger than 12 mm², equivalent to 3.9 mm in diameter.

^b100 BU/ml represents the concentration of the extract that produces in the patient population a mean weal of 75 mm² by skin prick test.

and CRIE of purified *Art v I* demonstrated a unique precipitation arc.

Two other characteristics are worth mentioning. *Art v I* is a monomeric protein with a molecular weight of 60,000 D under dissociating conditions, but which undergoes no change when adding reducing agents (results not shown) indicating absence of interchain S-S bridges. On the other hand, using Bio-Rad standard proteins we calculated a molecular weight for *Art v I* of 47,000 D in native conditions (see insert Fig. 1).

Clinical relevance of *Art v I*

In order to show the clinical relevance of *Art v I* the cutaneous reactivity to the whole extract and the purified allergen was studied in 34 selected patients as well as the presence of specific serum IgE to these same samples. In Table 1 it is indicated that 73% of patients were positive to *Art v I* using an allergen concentration of 2.5 µg/ml which is roughly the content of *Art v I* in the whole extract at 100 BU/ml. It is obvious that other allergens contribute to the whole extract activity as can be gathered by the different weal sizes (73 mm² for the whole extract and 45 mm² for *Art v I*), but it is also pertinent to add that 6 patients (17%) displayed very similar weals for both samples, indicating the relevance of *Art v I* in these cases.

Determination of serum specific IgE in the same patients gave analogous results (Table 2). Even rejecting as negatives those sera with RAST class 1, 68% of patients demonstrated high levels of specific IgE to *Art v I*, a percentage similar to that obtained from the prick test studies.

When comparing *in vivo* and *in vitro* results for *Art v I* a correlation coefficient of 0.36 ($P < 0.05$) was found.

Table 2. Determination of serum specific IgE to *Artemisia vulgaris* pollen extract and purified *Art v I* in 34 allergic patients

Samples	RAST class					Positivity ^a %
	4	3	2	1	0	
Whole extract ^b	5	20	7	1	1	94
<i>Art v I</i> ^b	6	8	9	6	5	68

^aWe considered positives only those sera with RAST class 2 or superior.

^bFigures indicate the number of patients with the above RAST class.

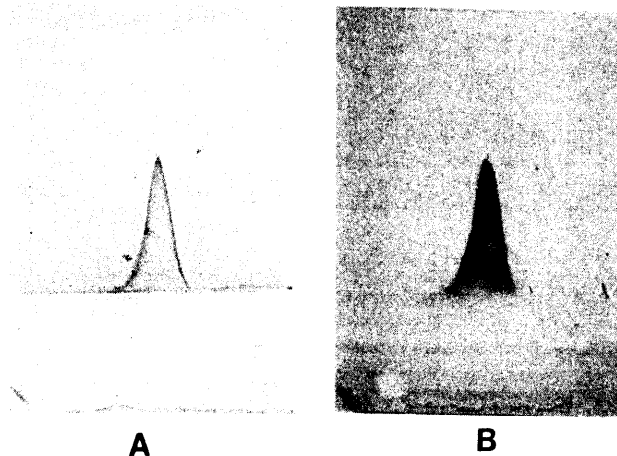


Fig. 7. Crossed immunoelectrophoresis (A) and crossed radioimmuno-electrophoresis (B) of HPLC purified *Art v I*. Thirty micrograms of pure allergen was applied per sample. Rabbit antiserum used in the second dimension was raised against the whole extract of *Artemisia vulgaris* pollen.

DISCUSSION

In a previous paper (Hoz, de la *et al.*, 1990) we described the identification of two main groups of allergens in the extract of *Artemisia vulgaris* pollen, one acidic and one basic. Proteins with pIs between 5 and 8 were basically inactive. Among the various proteins of the first group we localized a very important acidic allergen which, in addition, was easily identified in SDS-PAGE, and we set up to purify and characterize it.

The three step purification reported in the present paper has proved to be efficacious, as it yielded a good recovery of a very pure protein. The homogeneity of this purified allergen could be clearly evidenced by SDS-PAGE, IEF and CIE-CRIE techniques. One apparent exception could be the appearance of a doublet in SDS-PAGE which, although not self evident in the photograph of Fig. 3, it is always visible in the original gels. The explanation for this doublet could be found in the sugar composition of the protein molecule. However, those sugars should be neutral since the allergen does not show charge heterogeneity in analytical IEF. In support of this explanation we can add that the purified allergen contains, at least, a 6% of neutral sugars (results not shown).

Purified *Art v I* is a native protein with molecular weight of 47,000 D, as determined by gel filtration chromatography. This determination contrasts, to some extent, with that obtained under dissociating and reducing conditions, namely 60,000 D. However, this discrepancy is easily understood considering that the physical environment of either method is very different, and therefore, the results have not necessarily to be coincident, specially for a glycoprotein. *Art v*

I is a monomeric protein without subunits and without interchain disulphide bridges. It is one of the largest monomeric allergens so far purified (Duffort *et al.*, 1987; Ayuso *et al.*, 1988; Lowenstein and Osterballe, 1986). This protein is the most acidic allergen of *Artemisia vulgaris* pollen, it has very strong cross reactivity with other acidic allergens and very weak with the basic ones (Hoz, de la *et al.*, 1990).

Art v I is a clinically relevant allergen. At least, 70% of the individuals allergic to *Artemisia vulgaris* pollen, have IgE specific to this allergen, both, in serum and bound to mast cells. However 15% of patients with high sensitivity to the extract *in vivo* and *in vitro* did not respond to *Art v I*, while only in one case (3%), the response was higher for *Art v I* than for the whole extract. Furthermore, the allergenic activity of this allergen is roughly less than 50% of the activity of the whole extract (Hoz, de la *et al.*, 1990), although it is difficult to determine with accuracy taking into account its cross-reactivity with other allergens of the same species not yet purified.

We may conclude that *Art v I* is of major importance among individuals allergic to *Artemisia vulgaris*. Probably it is not the most important allergen of *Artemisia vulgaris* pollen (results in preparation), but it has to be present in appropriate concentrations, in materials for clinical use both for diagnosis and immunotherapy.

REFERENCES

- Ayuso R., Polo F. and Carreira J. (1988) Purification of *Par j 1* the major allergen of *Parietaria judaica* pollen. *Molec. Immun.* **25**, 49–56.
- Ceska M., Eriksson R. and Varga J. (1972) Radioimmunosorbent assay of allergens. *J. Allergy clin. Immun.* **49**, 1–9.

- Corbi A. L. and Carreira J. (1984) Identification and characterization of *Parietaria judaica* allergens. *Int. Archs Allergy appl. Immun.* **74**, 318–323.
- Cornillon J., Bernard J.-P., Gucho E. and Touraine R. (1972) Les pollinoses estivo-automneales. *Rev. Franc. Allergol.* **12**, 131–135.
- Diener Chr., Frenzel K., Wiebicke K., Schlenvoigt G., Fuster R. and Jager L. (1988) Allergen analyse von Beifu pollen (*Artemisia vulgaris*) mittels gekreuzter radioimmunoelektrophorese (CRIE) und RAST-Hemmung. *Allerg. Immunol.* **34**, 3–9.
- Duchaine J. and Spapen R. (1961) L'allergie respiratoire au pollen des composées en Europe, étudiée par les épreuves de provocation. *Int. Archs Allergy* **18**, 121–124.
- Duffort O., Carreira J. and Lombardero M. (1987) Characterization of the main IgE-binding components of cat dander. *Int. Arch Allergy appl. Immun.* **84**, 339–344.
- Futin F. G., Person K. and Gutermann W. (1976) *Artemisia*. In *Flora Europea*, Vol. 4B (Edited by Fritin F. G., Heywood V. tt., Burges N. A., Moore D. M., Walters S. M. and Webb D. A.), pp. 178–186. University Press, Cambridge.
- Hawkes R., Niday E. and Gordon J. (1982) A dot-immunobinding assay for monoclonal and other antibodies. *Analyt. Biochem.* **119**, 142–147.
- Hoz (de la) F., Lombardero M. and Carreira J. (1990) Biological activity and identification of the main allergens of *Artemisia vulgaris* pollen extract. *Allergologie*. (In press).
- Ipsen H., Formgren H., Lowenstein H. and Ingemann L. (1985) Immunochemical and biological characterization of a mugwort (*Artemisia vulgaris*) pollen extract. *Allergy* **40**, 289–294.

- Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature, Lond.* **227**, 680–685.
- Lombardero M., Gonzalez R., Duffort O., Juan F., Ayuso R., Ventas P., Cortés C. and Carreira J. (1986) Evaluación de la actividad biológica total y composición alérgica de extractos alérgicos. *Allergol. et Immunopathol.* **14**, 189–198.
- Lowenstein H. and Osterballe O. (1986) Standardized grass pollen extracts. *Clin. Rev. Allergy* **4**, 405–423.
- Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurements with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.
- Sanchez-Madrid F., Morago G., Corbi A. L. and Carreira J. (1984) Monoclonal antibodies to three distinct epitopes on human IgE: their use for determination of allergen specific IgE. *J. Immun. Meth.* **73**, 367–378.
- Smestad Paulsen B., Flo L., Nesje G. and Wold J. K. (1985) Allergens in pollen from mugwort (*Artemisia vulgaris*) I. Partial characterization of allergen preparations from mugwort pollen with emphasis on the carbohydrate moiety. *Int. Archs Allergy appl. Immun.* **78**, 206–212.
- Spiekma F. Th. M., Charpin H., Nolard N. and Stix E. (1980) City spore concentrations in the European Economic Community (EEC). IV summer weed pollen (Rumex, Plantago, Chenopodiaceae, *Artemisia*) 1976 and 1977. *Clin. Allergy* **10**, 319–329.
- Towbin H., Staehelin T. and Gordon I. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. natn. Acad. Sci. U.S.A.*, **76**, 4350–4354.
- Weeke B. (1973) Crossed immunoelectrophoresis. *Scand. J. Immun.* **2**(Suppl. 1), 47–56.
- Weeke B. and Lowenstein H. (1973) Allergens identified in crossed radioimmuno-electrophoresis (CRIE). *Scand. J. Immun.* **2**(Suppl. 1), 149–153.