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Isolation and Characterization *in vitro* of an Allergen from Plane-Tree (*Platanus acerifolia*) Pollen¹

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Abstract. A plane-tree pollen allergen was obtained by ion exchange and gel filtration chromatography. It is a glycoprotein with a molecular weight of 22,000. By isoelectrofocusing, two isomers with a high cross-allergenicity were obtained. The results suggest that this fraction is not the only allergen but certainly the most active.

Introduction

During the last few years, many attempts have been made to purify and characterize allergenic substances. Aqueous extracts used for diagnosis represent the bulk of pollen proteins and are not purified. Antigen E [10] was identified in 1962 as the major allergen of ragweed, and grass pollen was also extensively studied [9, 11, 12]. Tree pollens have been less investigated [3, 5]. Plane-tree pollens are able to give spring pollinosis, and this paper describes the isolation of an allergenic component, its allergenicity being assayed by *in vitro* methods.

Materials and Methods

Whole allergenic extract (WAE). 50 g of ether-defatted pollen were extracted with phosphate

0.005 M buffer; the precipitate was discarded and the antigens in the supernatant were precipitated with ammonium sulfate at saturation. The precipitate was then dissolved in phosphate buffer and lyophilized. The nitrogen protein content was estimated by the Kjeldahl's micromethod.

Column chromatography. DEAE-Sephadex A-25 column (2.8 × 70 cm) was equilibrated with Tris-HCl 0.02 M (pH 7.6) NaCl 0.2 M buffer; a stepwise elution was performed by increasing the saline molarity to 0.4, 0.5, and 0.8 M. The fractions eluted at a flow rate of 30 ml/h were recovered by the absorbancy at 280 nm. G-75 (2.8 × 40 cm) and G-50 (1.6 × 100 cm) columns were equilibrated with Tris-HCl 0.02 M (pH 7.8) (NH₄)₂SO₄ 0.2 M buffer, at a flow rate of 10 ml/h.

Antisera against WAE. Two sets of rabbits were immunized with WAE (10 ng/ml) included in Freund's complete adjuvant. Starting 2 weeks later, they received a weekly injection in saline. One set was immunized during 2 months and the other one during 6 months. They were then bled and by immunoelectrophoresis, the same number of precipitin lines was seen between the two antisera.

Molecular weight determination. The method described by Andrews [2] was used. A Sephadex G-100 column was calibrated with cytochrome c

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(MW 12,400), trypsin inhibitor (MW 21,500), β -lactoglobulin (MW 36,500), ovalbumin (MW 45,000).

Isoelectrofocusing. The isolated fraction (10 mg) was electrofocused in a 110-ml column in a density gradient of 0–40% sucrose with carrier ampholyte covering the pH range 3.0–10. The pH was immediately measured in 2 ml collected fractions. The cross-allergenicity of these was tested by RAST inhibition.

Enzymatic hydrolysis. The allergenic fraction (10 mg/ml) was hydrolyzed with pepsin, pronase, chymotrypsin and neuraminidase for a day, at 37 °C. After hydrolysis, the allergenicity of the fractions was tested.

Allergic human sera. Sera were obtained from eight plane-tree allergic patients who elicited positive skin reactions when tested with aqueous plane-tree extracts (Stallergène 1/20). The sera were pooled.

In vitro testing of allergenic activity. The allergenicity of each fraction was tested by RAST direct technique [15]; the potency of the allergenicity was determined by RAST inhibition [7] and histamine release-inhibition [13] from human allergic leukocytes and mast cells from rats sensitized with WAE included in *Bordetella pertussis* vaccine. The ID_{50} was the concentration which caused 50% inhibition of RAST or histamine release.

Results

Whole allergenic extract. The specific extraction of 50 g defatted pollen yielded 1.5 g of water-soluble components, and it was assayed for allergenic activity. The ID_{50} values were determined by RAST and histamine release (table I) and were used as references for further purification.

Chromatography on DEAE-Sephadex. The bulk of total proteins was chromatographed through a DEAE-Sephadex column equilibrated with Tris-HCl 0.025 M (pH 7.8) saline 0.2 M buffer; the elution was obtained by increasing molar concentrations of saline. Five fractions were recovered

Table I. Inhibition of RAST and histamine release from mast cells by fractions obtained from plane-tree extracts

Fractions	ID_{50} determined by		
	RAST	allergen-induced histamine release	
		rat mast cells	human leukocytes
WAE	50	50	20
DEAE fraction III	25	33	18
DEAE fraction IV	18	21	15
DE IV 1–1	0.1	0.54	0.33

(fig. 1); two of these showed the highest allergenic activity by direct RAST. As the other ones lacked specific allergenic activity, it was concluded that they did not contain pollen allergens. The ID_{50} of both active fractions was about the same in RAST; as fraction IV developed the strongest reaction on allergen-induced histamine release (table I), it was investigated in further studies.

Chromatography on Sephadex G-75. 80 mg of fraction IV were applied to a Sephadex G-75 column and eluted with Tris-HCl 0.05 M (pH 7.8) $(NH_4)_2SO_4$ 0.2 M buffer; the allergenic activity was essentially recovered in fraction IV–I (fig. 2); its ID_{50} was 20-fold fewer than that of DE IV (table I).

Chromatography on Sephadex G-50. 20 mg were applied to a Sephadex G-50 column; two fractions were eluted with the same buffer as above. The whole allergenic activity was concentrated in the first peak (fig. 3). Its ID_{50} was 200-fold stronger than that of the WAE (table I). By immunoelectrophoresis, it only gave one precipitin line against rabbit antisera (fig. 4). From 50 g of

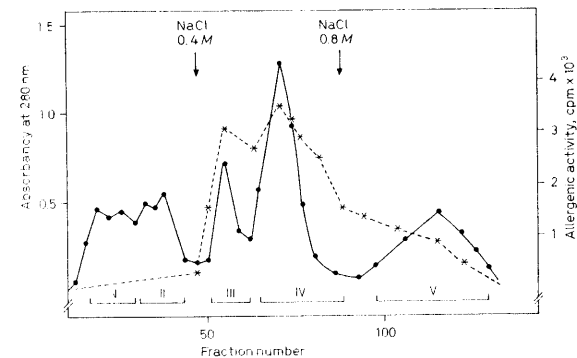


Fig. 1. Chromatography on DEAE-Sephadex column of 1.5 g WAE in 10 ml of TRIS HCl 0.02 M (pH 7.6) saline 0.2 M buffer. Vol/tube = 3.6 ml; flow rate = 30 ml/h. The arrows indicated the increase in saline molarity. ● = Absorbance at 280 nm; * = allergenic activity.

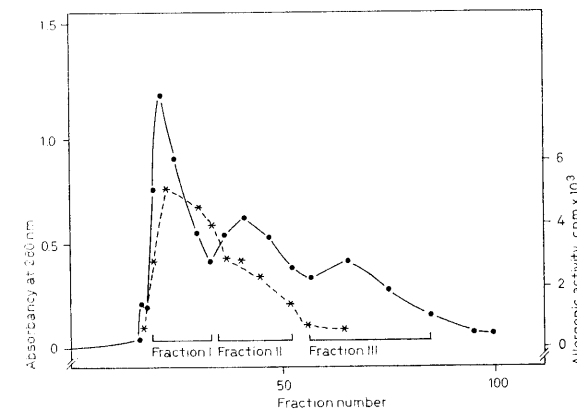


Fig. 2. Chromatography on Sephadex G-75 column of 80 mg DE IV in 8 ml buffer. Flow rate = 10 ml/h; vol/tube = 1.1 ml; ● = absorbance at 280 nm; * = allergenic activity.

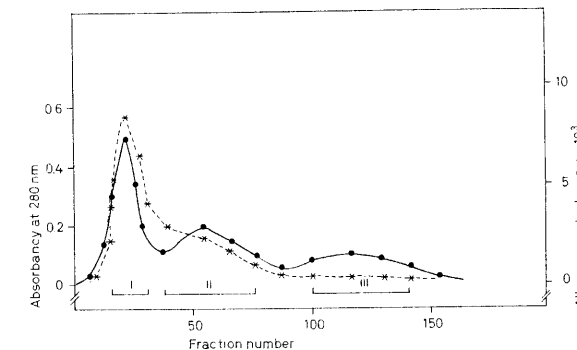


Fig. 3. Chromatography of 20 mg of fraction DE IV 1 in 2 ml buffer on a Sephadex G-50 column. Flow rate = 10 ml/h; vol/tube = 0.8 ml; ● = absorbance at 280 nm; * = allergenic activity.



Fig. 4. Immunoelectrophoresis of DE IV 1-1 in agar gel; anode on the right. The upper well contained the fraction (10 mg/ml), the lower contained WAE (10 mg/ml).

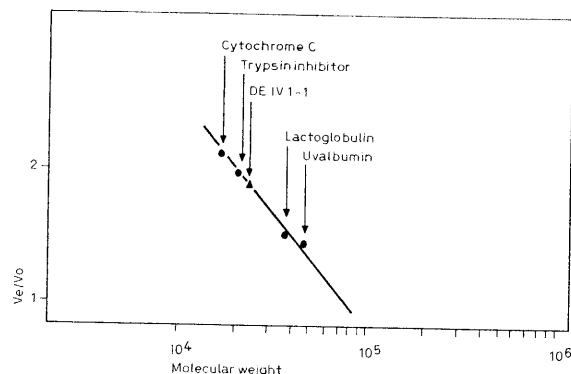


Fig. 5. Molecular weight determination on a Sephadex G-100 column. Flow rate = 5 ml/h; vol/tube = 1.2 ml.

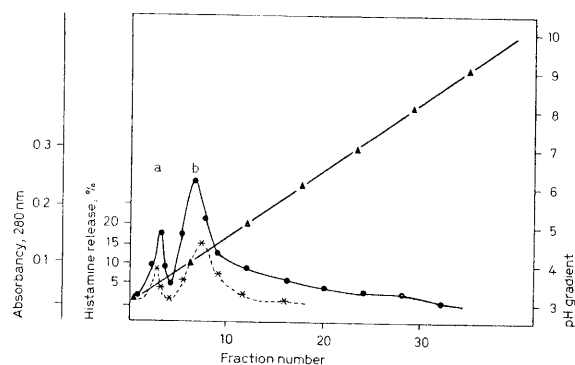


Fig. 6. Isoelectrofocusing of DE IV 1-1 in ampholyte gradient pH 0-10. Vol/tube = 2 ml; ● = absorbance; ▲ = pH gradient; × = percent of histamine release.

dry pollen, only 2 mg of active allergen were obtained.

Molecular weight determination. The molecular weight of the fraction DE IV 1-1 was determined on a Sephadex G-100 column, previously calibrated. It was found to be equal to 22,000 daltons (fig. 5). The carbohydrate content was estimated to be 5.9%.

Isoelectrofocusing. The results are shown in figure 6. Two protein bands were

Table II. Inhibition of RAST by fractions of plane-tree extract coupled to paper discs (cross-allergenicity)

	Percent inhibition of RAST obtained with fraction b Coupled to paper discs
Fraction b, 100 µg/ml	100
Fraction a, 100 µg/ml	93
Fraction a, 10 µg/ml	68

Table III. Inhibition of RAST and histamine release from mast cells by fractions of plane tree after enzymatic action

Treatment of fraction	Percent inhibition with 100 µg N of DE IV 1-1	
	RAST	allergen-induced histamine release
Control	92	90
Pepsin	15	12
Pronase	9	0
Chymotrypsin	75	69
Neuraminidase	86	79

observed: a minor component with pI 3.8 and a major one with pI 4.2. The allergenic activity was contained in the two fractions using histamine release from rat mast cells. After concentration, fraction a was tested for RAST inhibition against fraction b coupled to paper discs. The results showed that fraction a inhibited allergenic activity of the b allergen (table II).

Enzymatic hydrolysis. The allergenic component was submitted to enzymatic hydrolysis and then tested for allergenic activity. This was abolished by pronase digestion and greatly diminished by pepsin attack. Little degradation was obtained with chymotrypsin and neuraminidase. These results suggest that the allergen contained a protein moiety (table III).

Discussion

The results described in this paper show the first attempts to isolate a major allergen from plane-tree pollen. By ion exchange and gel filtration chromatography a fraction was obtained which gave an homogenous peak and a single precipitin line. By isoelectrofocusing, two fractions were found with allergenic activity and by RAST inhibition a high degree of cross-allergenicity was obtained. This suggests that they represent the same molecule with two different ionised isomers in ampholyte solution. The allergenic activity was destroyed by proteolytic enzymes, the most effective being pronase and then pepsin. Allergenic activity was not destroyed by DNase and RNase. It is possible that enzymatic attacks may be produced during RAST, but it is unlikely because sera of patients contained natural protease inhibitors, and for pepsin the activity was destroyed at basic pH values. The protein characterized here was associated with a sugar moiety, the molecular weight being equal to 22,000.

Atopic allergens can be grouped into a distinct class of active substances with definite properties [4]. Many of them possess similar physicochemical properties (e.g., acidity) [1, 4].

Whole pollen extracts are characterized by a heterogeneity in their protein content, many of which are not allergenic. This is true for ragweed as well as for grass pollen [9, 10, 12, 14]. In our work, one component was purified because it developed the greatest activity on sensitized cells, and it is possible that fraction III contained another allergenic determinant. Nevertheless, other nonallergenic proteins can interfere in coupling to the activated paper discs and give

false positive reactions in RAST, and this could explain why the same results were obtained for both fractions in RAST.

Specific desensitization of allergic patients with modified allergens represents an important field of application for purified allergens [8].

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Reaginic Antibody Production to *Ascaris suum* Allergen, ASC-1

I. The Function of Glutaraldehyde-Polymerized Antigen in the Induction of Reaginic (IgE) Antibodies in the Rat¹

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Abstract. Rats immunized with a purified *Ascaris suum* allergen (Asc-1) or with its dinitrophenylated derivative (DNP-Asc-1) produced high levels of reaginic (IgE) antibodies. A second injection of antigen given 30 days later did not result in an anamnestic IgE antibody response. Immunization of adult-thymectomized, lethally-irradiated and bone-marrow reconstituted (ATxB) rats with soluble Asc-1 or DNP-Asc-1 failed to stimulate reaginic antibody production. The administration of glutaraldehyde-polymerized antigen induced in some but not all ATxB rats, low but detectable levels of IgE antibodies. These levels increased following a second injection of nonpolymerized antigen in Al(OH)₃ gels. Priming of animals with polymerized carrier and *Bordetella pertussis* did not stimulate a primary anticarrier IgE response but led to an enhanced antihapten IgE response following the administration of soluble DNP-Asc-1 in Al(OH)₃. The results are consistent with the notion that a sharply reduced but clearly functional T-derived helper cell population could be triggered by the polymerized but not by the soluble form of the immunogen.

Introduction

The antigen-induced stimulation of the reaginic (IgE) antibody response in most animal models depends on a number of factors such as the nature and amount of immunogen administered, the type of adjuvant, the route of immunization as well as the genetic background of the host.

Previous studies on reaginic antibody production have indicated that - as for all thymus lymphocyte-dependent responses -

the collaboration of at least three cell types was required for the development of an optimal response: the thymus-derived (T) lymphocyte, the bone marrow-derived (B) lymphocyte and a cell endowed with the property to adhere to plastic or glass surfaces, presumably macrophages [15, 17, 26, 37].

It has been known for many years that normal, nonatopic humans [10], as well as experimental animals [30, 31, 38], develop high levels of reaginic antibodies following the administration of crude soluble extracts obtained from *Ascaris suum*.

When rats were immunized with dinitro-

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