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Note

Purification of allergens by high-performance liquid chromatography

II*. Purification of the major allergen of the mite *Dermatophagoides pteronyssinus* (P_1)

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The pyroglypid mite *Dermatophagoides pteronyssinus* is the main cause of house dust-mediated allergy[1]. Up to 72% of seric IgE antibodies in dust allergic people are directed against a 24 000 dalton glycoprotein[2] associated with mite faecal particles[3]. This major allergen is present as a large proportion (10–20%) of the protein in mite cultures[2], and has been obtained in a highly purified form by time-consuming biochemical methods[2,4,5].

We report here, as the second paper in a series dealing with the rapid purification of allergens by high-performance liquid chromatography (HPLC), the one-step purification and immunological characterization of such major allergen.

EXPERIMENTAL

Mite cultures and allergen extraction

Dermatophagoides pteronyssinus (Troussart, 1897) was kindly supplied by Prof. A. Ishii (Miyazaki Medical College, Japan) and cultured in Roux bottles according to the method described by Miyamoto *et al.*[6].

A 1-g amount of whole culture was extracted with 10 ml of phosphate-buffered saline (PBS) containing 50 μg/ml of phenylmethyl sulphonylfluoride (PMSF) (Sigma, U.S.A.) for 30 min at 0°C. After centrifugation at 12 000 *g* for 15 min, the extract was filtered on a 0.45 μm Millipore membrane.

All protein determinations were carried out by the method of Lowry *et al.*[7].

Size exclusion HPLC

The HPLC equipment, columns and eluent were described in detail previously[8].

* For Part I, see ref. 8.

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RAST inhibition assay

Volumes of 50 μl of serially ten-fold diluted allergen solutions were incubated for 1 h at 37°C with 10 μl of an appropriate dilution of a pool of allergic sera. This mixture was then incubated with RAST paper discs of *D. pteronyssinus* for 3 h at room temperature. The discs were washed three times and 50 μl of Rabbit anti-human IgE labelled with ^{125}I (Pharmacia, Sweden) were added and incubated overnight. After washing, the discs were counted in a Packard Autogamma 500c gamma counter for bound radiactivity.

Solid-phase radioimmunoassay (SPRIA)

SPRIA was performed according to the method described in ref. 8.

Labelling and precipitation of the purified allergen

A 10-μg amount of HPLC-purified allergen was labelled with 1 mCi of ^{125}I (Amersham, U.K.) using the chloramine-T method[9]. The specific activity of the labelled allergen was 20000 cpm/ng.

For precipitation, serial two-fold dilutions of a pool of sera from patients containing IgG blocking antibodies were incubated with 100000 cpm of allergen for 1 h at 37°C. The complex was then precipitated with 100 μl of 10% *Staphylococcus aureus* (Cowan strain) for 30 min at room temperature.

The precipitates were washed twice with 1 ml of TNE + 0.1% Triton X-100 and counted.

Skin testing

Intradermal skin tests were carried out in suspected *D. pteronyssinus* allergic patients by using the classical prick test with serial ten-fold dilutions of the HPLC-purified allergen in 50% glycerol. The skin response was measured after 20 min. The positive control was 0.1% histamine hydrochloride and the negative control PBS.

RESULTS

Allergen purification

Using the purification conditions employed previously[8], *i.e.*, Waters Protein Pack 125 columns, phosphate-buffered saline (pH 7.4) as eluent and a flow-rate of 1 ml/min, the major allergen of *Dermatophagoides pteronyssinus* can be purified in an one-step scheme.

Fig. 1 (top) shows the chromatogram of the crude extract from a whole culture of mites. When each fraction was tested by SPRIA against three selected strong allergic sera, class 4 RAST, the antigenic activity was reproducibly found associated with fractions 8–10.

The lower molecular weight fractions that strongly absorb at 254 nm do not contains any specific IgE binding activity. When fraction 9, the central part of the Gaussian distribution, is run again under the same conditions, the chromatogram depicted in Fig. 2A was obtained. Integration with the Waters data module showed 96% purity.

Fig. 2B depicts the chromatogram of the *D. pteronyssinus* purified allergenic extract Pharmalgen. Both allergens show similar degrees of purity and have a com-

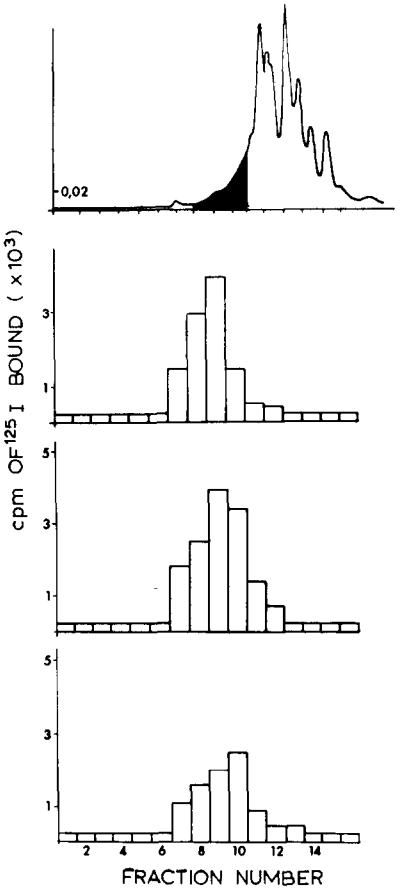

Fig. 1. HPLC of crude extract of *D. pteronyssinus*. Conditions as in ref. 8. Three sera (class 4 RAST to *D. pteronyssinus*) were used in the SPRIA analysis of the different fractions.

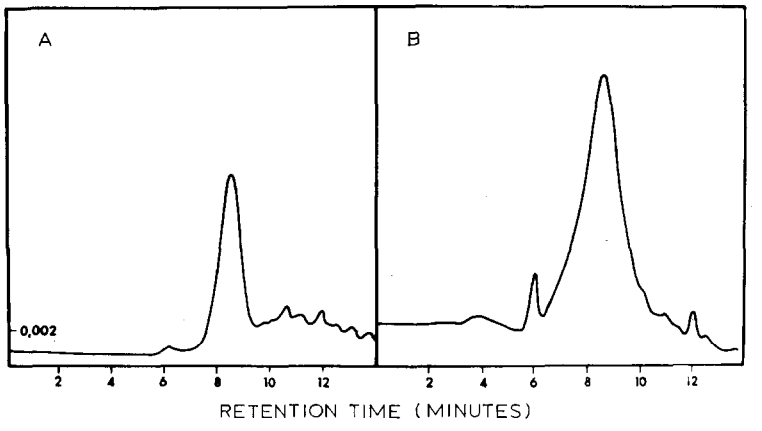

Fig. 2. Re-chromatograms of HPLC-purified fraction 9 (A) and *D. pteronyssinus* allergenic extract Pharmalgen (B). Both allergens were run under the same conditions.

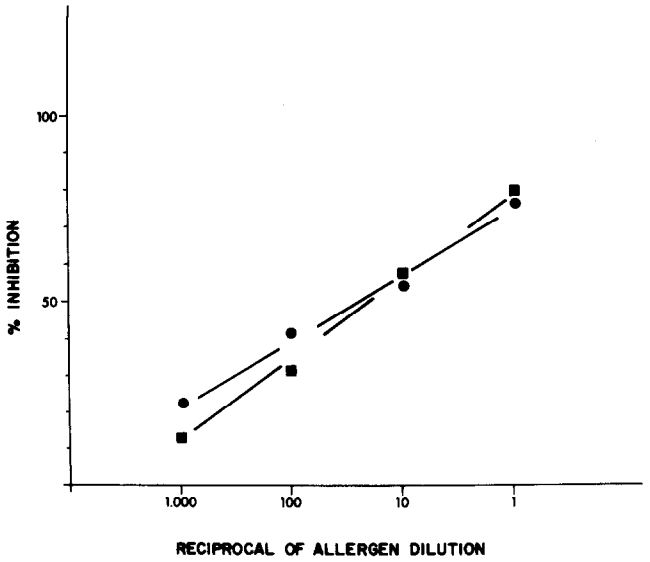

Fig. 3. RAST inhibition assay. The results are expressed as percentage inhibition of binding of specific IgE achieved by addition of serial dilutions of allergen. The undiluted Pharmalgen extract corresponds to a 100 000 BU/ml concentration. ■, HPLC-purified allergen; ●, Pharmalgen extract.

mon retention time of 8.6 min. This position is intermediate between bovine pancreas DNase (MW = 31 000) and soybean trypsin inhibitor (MW = 20 100), in very good accord with a molecular weight of 24 000 reported by Chapman and Platts-Mills[2] for its purified allergen, termed P_1.

A very good recovery of around 80% was calculated from the integration data provided by the data module.

In vitro immunological analysis

The *in vitro* allergenic activity of the purified molecule was compared with the Pharmalgen product by RAST inhibition. A pool of sera from patients sensitive to mites was used as a source of specific IgE. Both products were almost identical in their allergenic potencies (Fig. 3).

When a calculation in Biological Units (BU)* was performed with the Pharmalgen product, 6000 BU were required to achieve 50% inhibition in RAST, which corresponds to 8.4 μg of HPLC-purified allergen. Hence, in this system, 1 Biological Unit is equivalent to 1.4 ng of pure protein.

The IgG binding properties of the molecule are not impaired by the purification method, as demonstrated in Fig. 4. A pool of sera from patients subjected to a complete treatment of immunotherapy, which contained detectable levels of *D. pteronyssinus* specific blocking IgG antibodies[10], was used to precipitate the ^{125}I-labelled

* An allergen solution is assigned 1000 Biological Units when it gives a mean wheal reaction of the same size as 0.1% histamine hydrochloride.

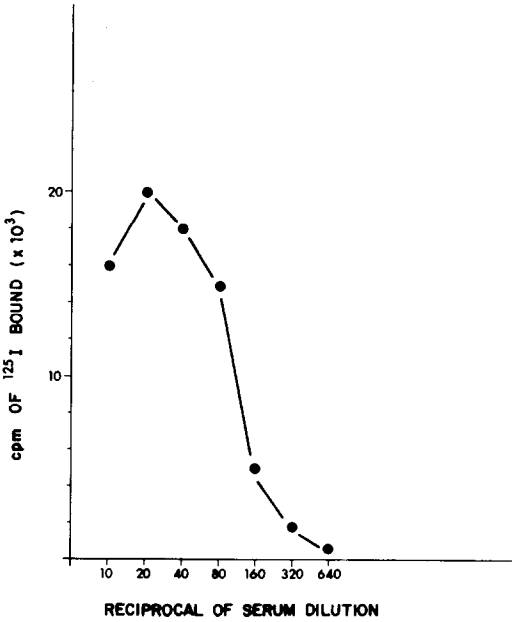

Fig. 4. Precipitation of antigen IgG antibody complexes by *S. aureus* (Cowan strain). 100000 cpm of HPLC-purified labelled allergen were precipitated with two-fold dilutions of a serum containing IgG blocking antibodies.

pure allergen. *S. aureus* Cowan strain was employed as immunoadsorbent and a dose–response titration curve was obtained (Fig. 4).

Protein A from the cell wall of *S. aureus* binds to the Fc portion of human IgG but not the IgE Fc region[11,12], ensuring the isotopic specificity of the test.

The low percentage of maximal binding ($20 \cdot 10^3$ out of $100 \cdot 10^3$ cpm) of the label could be explained by the competition of IgE and IgG for the antigen molecule.

In vivo allergenic tests

Skin reactions to purified allergen in five patients suspected of *D. pteronyssinus* allergy are summarized in Table I.

TABLE I

SKIN PRICK TEST REACTIONS (WHEAL DIAMETERS IN mm) TO HPLC-PURIFIED *DERMATOPHAGOIDES PTERONYSSINUS* MAIN ALLERGEN

Patient No.	*Positive control (0.1% histamine · HCl)*	*Negative control (PBS)*	*D. pteronyssinus purified allergen*		
			45 μg/ml	*4.5 μg/ml*	*0.45 μg/ml*
1	9	3	5	4.5	3.5
2	7.5	0	8	2.5	0
3	7	0	1.5	1.5	1.5
4	5.5	2.5	5	5	3.5
5	7	0	4	4	3

All patients but No. 3 showed positive reactions (2 mm wheal diameter), indicating that the HPLC-purified molecule is still active *in vivo*. The highest concentration of allergen used (45 μg/ml), equivalent to 32 142 BU/ml, seems low if we take into consideration that 100 000 BU/ml are recommended in the prick test to avoid false negatives[13].

A decrease in the diameter of the wheal was observed in all positive patients in proportion to the dilution of the allergen employed.

Patients 4 and 5 showed a plateau at doses of 45 and 4.5 μg/ml.

DISCUSSION

Whole cultures of mites were approximately three times more allergenic that purified mite bodies[14]. This is due to the specific major sensitization to mite faecal particles[3]. Therefore, whole culture extracts are the best source of allergenic material.

Nevertheless, such cultures contains in addition to mite products and bodies, cuticles and eggs, the culture media and some enzymes such as esterases, phosphatases, aminopeptidases and glycosidases[14]. We performed the extraction under mild conditions imitating those prevailing in the human mucosa. To reduce the degradation caused by enzymes, the temperature was kept at 0°C and PMSF added to prevent protease activity[8] (except in purifications used for human *in vivo* tests).

The time of extraction was reduced to 30 min, because it has previously been shown that the major allergens are released within 3 min[3]. With such starting material we set up the conditions for purification that can be achieved in a 15-min run using HPLC. This period is extremely short compared with purification schemes such as Sephadex G-100 chromatography, Pevikon block electrophoresis and preparative isoelectrofocusing.

A recovery of 80% and a 96% purity of the allergen could be achieved. The biochemical quality of the pure product is as good as that of commercially available materials for allergy immunotherapy.

The allergen fully retains its binding capacity for specific IgE and IgG and it is active also *in vivo* as measured in the prick test on allergic patients.

In this second paper on the purification of allergens, we have demonstrate that HPLC is even more useful with mites that in the purification of graminiae pollen main allergen, repported previously[8].

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