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Isolation and Characterization of the Allergen Dpt 12 from *Dermatophagoides pteronyssinus* by Chromatofocusing

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Abstract. The allergen Dpt 12, from the house dust mite *Dermatophagoides pteronyssinus*, was isolated from spent growth medium by chromatofocusing. The allergen was isolated in the pI range 5.86-6.95, indicating heterogeneity with regard to pI. However, on sodium dodecylsulphate - polyacrylamide gel electrophoresis the allergen appeared homogeneous with an apparent molecular weight of 27,000 daltons. Radioallergosorbent scores utilizing paper discs coupled with Dpt 12 correlated significantly ($p < 0.0005$) with those obtained using discs coupled with whole mite extract, although the corresponding titres were higher with Dpt 12 ($p < 0.0005$).

Introduction

Several groups of workers have described the isolation of allergens from the house dust mites *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* [10, 14, 15]. Whilst earlier studies described the allergens in broad terms, only recently have the physicochemical characteristics of individual allergens been determined [5, 19].

The use of isoelectric focusing, either in a preparative or analytical mode, has been a feature of these studies which have shown, especially for the low-molecular weight allergens such as P₁ and Dpt 12, heterogeneity suggestive of the presence of isoallergens. For example, Chapman and Platts-Mills [5] demonstrated that the allergen P₁ focused into two main peaks with pI of 4.7-5.4 and 6.6-7.1, respectively, with a minor peak in between. Data from this laboratory, using crossed radioimmunoelectrophoresis in combination with analytical isoelectric focusing, also indicated that the low-molecular weight allergen Dpt 12, analogous to P₁, was heterogeneous [19]. These studies, however, showed that the majority of the allergen focused in the pI range 6-7, although minor amounts were detected in the pI range 4-5.

The recently developed technique termed chromatofocusing is analogous to isoelectric focusing in that proteins are separated on the basis of their isoelectric

point. However, the pH gradient required to achieve separation is generated using a combination of an ion exchange resin and a buffer mixture rather than an electric field [17, 18]. Potentially, this technique offers several advantages over isoelectric focusing.

The following study describes the use of chromatofocusing in the isolation of the low-molecular weight allergen Dpt 12 from spent mite growth medium devoid of whole mites, but rich in allergens [12, 20, 21]. The results obtained using this technique have been compared with those obtained using conventional isoelectric focusing.

Materials and Methods

Preparation of Mite Extracts

Whole mite (HDM), cuticle, spent growth medium (SMM) and unused growth medium (UMM) were kindly provided by Commonwealth Serum Laboratories (Parkville, Victoria). However, for commercial reasons, the manufacturer did not provide details of the composition of the mite growth medium. Extracts of these materials were prepared in a manner similar to that described by Kabasawa and Ishii [10]. Briefly, the material to be extracted was homogenized (X3) with 0.1 M Tris-HCl buffer, pH 7.4, (5% w/v) for 20 min at 0°C. The combined supernatants were clarified by centrifugation at 15,000 g for 15 min, dialysed against four changes (each of 5 litres) of distilled water and freeze-dried. Extracts were stored desiccated at -20°C. The allergenicity and composition of these extracts will be described elsewhere [20].

Chromatofocusing of Mite Allergens

Antisera

2 mg of HDM extract were dissolved in 1 ml of 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl (phosphate-buffered saline), emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into sheep. A secondary injection of 2 mg of HDM in Freund's incomplete adjuvant was given 28 days later and the sheep bled 10 days after this and subsequent booster injections. The specificity of this antiserum has been described [20]. An anti-Dpt 12 antiserum was raised in a rabbit against a purified allergen fraction isolated by gel filtration and isoelectric focusing (pI 6.25-6.55) from 50% saturated ammonium sulphate-precipitated SMM [20].

Immuno-electrophoretic Analysis

Immuno-electrophoresis was performed in 1.2% (w/v) agar (Difco Noble) in barbitione buffer, pH 8.6 (I = 0.05), according to the method of Grabar and Williams [8] and developed overnight at 4°C. After washing, the plates were pressed and dried according to the method of Laurell [11] and stained with Biebrich scarlet [6] purchased from Searle Diagnostic.

Polyacrylamide Gel Electrophoresis (PAGE)

Conventional PAGE was performed at pH 8.3 in the GE-4 gel electrophoresis apparatus (Pharmacia South Seas Pty. Ltd.) using 12% polyacrylamide slab gels [7]. Sodium dodecylsulphate (SDS)-PAGE for the determination of apparent molecular weights [22] was performed using commercially prepared gradient gels (PAA 4/30, Pharmacia South Seas Pty. Ltd.) according to the manufacturer's instructions. Both types of gel were stained for protein using Coomassie blue G-250 [2]. Apparent molecular weights were calculated by linear regression analysis of curves constructed with low-molecular weight protein standards obtained from Pharmacia.

Radioallergosorbent Test (RAST)

RAST assays were performed essentially as described by Wide et al. [24] using ¹²⁵I anti-human IgE (Pharmacia South Seas Pty. Ltd.). Allergens were coupled to cyanogen bromide activated paper discs as described by Ceska et al. [3] and the concentration of allergen coupled expressed in micrograms of protein per millilitre. Individual sera from atopic patients with both clinical and laboratory evidence of hypersensitivity to *D. pteronyssinus* were obtained from the Princess Margaret Hospital. Sera from non-atopic individuals were obtained from healthy laboratory personnel. Pools of sera from atopic and non-atopic subjects were prepared from sera obtained as described above.

Fractionation of Mite Allergens

Initial fractionation of the low-molecular weight mite allergens from SMM was achieved by precipitation with 50% saturated ammonium sulphate [20]. The precipitated material (800 mg) was redissolved in 0.05 M ammonium bicarbonate buffer, pH 8.0, and, after dialysis, applied to two columns (2.5 × 80 cm) of Sephacryl S-300 connected in tandem and equilibrated with the same buffer. Fractionation was achieved at 4°C by upward elution at a flow rate of 20 ml/h and 6-ml fractions were collected using an LKB Ultrarac fraction collector. Column effluents were monitored at 280 nm for protein and by fused rocket immuno-electrophoresis for specific antigens [23] using a sheep anti-HDM antiserum. The low-molecular weight fraction (approximate molecular weight 10,000-30,000 daltons) from the S-300 run was further fractionated on Sephadex

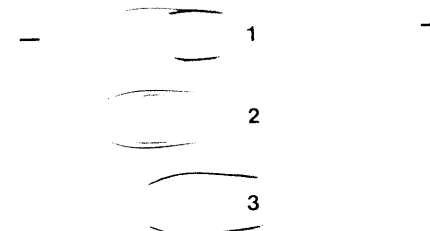


Fig. 1. Immuno-electrophoretic analysis of spent mite medium (1) and the 50% saturated ammonium sulphate supernatant (2) and precipitate (3) each at 20 mg/ml against anti-whole mite antiserum.

G-50 equilibrated with 0.05 M ammonium bicarbonate buffer, pH 8.0.

Chromatofocusing of Mite Allergens

Chromatofocusing was performed at room temperature according to the manufacturer's instructions (Pharmacia South Seas Pty. Ltd.). Briefly, 19 mg of a freeze-dried, crude allergen fraction isolated by consecutive gel filtration on Sephacryl S-300 and Sephadex G-50 were dissolved in 0.025 M imidazole buffer, pH 7.4. This solution was then applied to a 0.9 × 43 cm column of PBE 94 ion exchange resin equilibrated in the same buffer. Proteins were eluted over the pH range 5-7 using Polybuffer 74 adjusted to pH 5 with HCl. The buffer was pumped at a flow rate of 20 ml/h using a Pharmacia P-3 peristaltic pump and 3-ml fractions were collected. Protein elution was monitored by measuring the absorption of each fraction at 280 nm and the pH profile was constructed by determining the pH of each fraction. The elution with Polybuffer was terminated when the pH reached 5, after which the column was treated with 1 M NaCl to elute any strongly bound material. Appropriate fractions were pooled and the proteins precipitated with 60% saturated ammonium sulphate to remove the Polybuffer. The precipitated proteins were redissolved in phosphate-buffered saline and re-precipitated a further two times. After dialysis against phosphate-buffered saline, protein concentrations of these and other fractions were measured using the Coomassie blue G-250 assay described by Sedmak and Grossberg [16]. Fractions were stored frozen until required.

Radioimmunoassay of Mite Allergen

20 µg of mite allergen obtained by chromatofocusing (fraction 2, pI 6.82-6.95) were labelled with 1 mCi of ¹²⁵I (Radiochemical Centre, Amersham) by the chloramine-T method [9]. Free iodine was removed by gel filtration on Sephadex G-25. The radiolabelled allergen was 96% precipitable by 10% (w/v) trichloroacetic acid, 77% immunoprecipitable by an antiserum raised against the mite allergen Dpt 12, and 82% immunoprecipitable using an allergic serum pool.

The radioimmunoassay used to quantitate the amount of allergen in preparations of HDM, cuticle, SMM and UMM was essen-

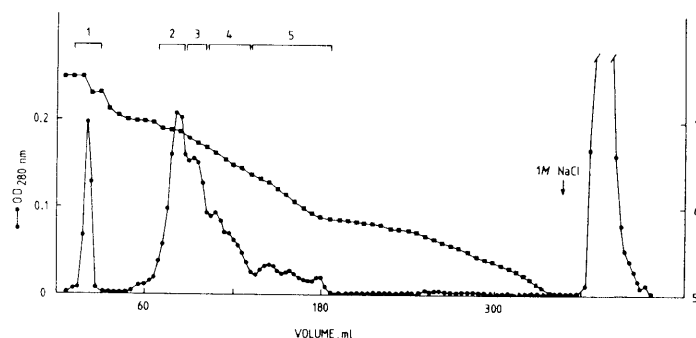


Fig. 2. Chromatofocusing fractionation of the Dpt 12-containing material (19 mg) from the Sephadex G-50 and Sephacryl S-300 fractionations of the 50% saturated ammonium sulphate precipitate of spent mite medium over the pH range 5-7. Fractions were pooled as indicated.

tially similar to that described by Chapman and Platts-Mills [5]. ^{125} I-labelled allergen (5.5 ng) was added to 100 μ l of a 1/40 dilution of normal rabbit serum containing either sample or standard. To this was then added 100 μ l of a 1/5,000 dilution of rabbit anti-Dpt 12 antiserum described above and the mixture incubated for 4 h at 4°C. Bound antigen was precipitated by incubating the mixture with 100 μ l of undiluted sheep anti-rabbit IgG antiserum at 4°C overnight. The precipitate was washed three times with borate-buffered saline and the ^{125} I present counted in a gamma counter (Autogamma, Packard Instruments). Inhibition curves were constructed using doubling dilutions of chromatofocusing fraction 2 over the range 0.1-1 μ g/ml. Each dilution of standard and unknown sample was assayed in triplicate and, in the case of the unknown samples, at two different concentrations. All dilutions were performed in phosphate-buffered saline containing 0.3% (w/v) bovine serum albumin and 0.0125 M EDTA.

Results

Isolation of Mite Allergen

On the basis of preliminary experiments [20] an initial fractionation of the low-molecular weight allergen, Dpt 12, was achieved by 50% saturated ammonium sulphate precipitation of SMM. The allergen was shown to be present in the precipitate (fig. 1) with little contamination from other mite antigens although media components were shown to be present [unpublished observations].

The precipitate was initially fractionated on Sephacryl S-300 and those fractions containing the allergen Dpt 12 were identified by fused rocket immunoelectrophoresis using a sheep anti-whole mite antiserum (data not shown) as described earlier [19]. These fractions were pooled, concentrated by freeze-drying and fractionated further on Sephadex G-50. Those fractions containing the allergen Dpt 12 were identified

by fused rocket immunoelectrophoresis (data not shown) and pooled. This material was then dialysed against 0.025 M imidazole buffer and subjected to chromatofocusing over the pH range 5-7 to yield the elution profile shown in figure 2. Fractions were pooled as indicated and processed to remove Polybuffer. Fraction 1, representing material which did not bind to the column, and the strongly bound fraction eluted with 1 M NaCl were not examined in detail since they were shown to be unrelated to Dpt 12 [unpublished observations]. Fractions 2, 3, 4 and 5 (pI range 5.86-6.95) represented 45% of the UV-adsorbing material recovered from the column and contained 1.03, 0.76, 0.51 and 0.14 mg of protein, respectively. Not all the applied material was recovered from the chromatofocusing column since a brown-coloured band, similar to the colour of the original material, remained at the top of the column.

Physicochemical Analysis

Chromatofocusing fractions were analysed by immunoelectrophoresis and, apart from fraction 2 where an extremely faint precipitin band inside the major band was detected, contained only 1 precipitin band using both anti-HDM and anti-Dpt 12 antisera (fig. 3). These bands were immunochemically identical and corresponded to the densely staining precipitate seen in the immunoelectrophoretic analysis of the SMM and its ammonium sulphate precipitate (fig. 1).

Analytical PAGE analysis (figure 4) showed that some of the chromatofocusing fractions differed in electrophoretic mobility. Fractions 2 and 3 were electrophoretically similar but fractions 4 and 5 comprised electrophoretically faster materials at pH 8.3.

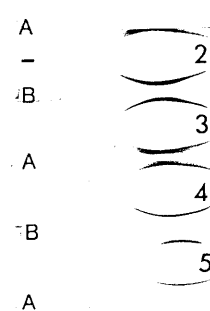


Fig. 3. Immunoelectrophoretic analysis of the chromatofocusing fractions 2-5 against anti-whole mite (A) and anti-Dpt 12 (B) antisera.

SDS-PAGE showed that, despite the difference seen in electrophoretic mobility, each fraction contained material with similar apparent molecular weights, namely 27,000 daltons (fig. 5).

Quantitative Studies

The amount of Dpt 12 present in various mite extracts was determined using a radioimmunoassay. The allergen was present in all extracts apart from UMM. While similar amounts were present in extracts of HDM and SMM, namely 3.5 and 3.4 μ g/mg dry extract weight, approximately half this amount (1.9 μ g/mg) was found in extracts of cuticle.

RAST Studies

The allergenicity of the chromatofocusing fractions 2-5 was determined using a direct RAST binding assay and the results compared with those obtained using HDM and SMM (fig. 6). All fractions showed similar binding curves and comparable allergenicity as judged by the concentration of material required to bind 50% of the ^{125} I-labelled anti-IgE, namely 2.5 μ g/ml. The slopes of the curves were similar to that of SMM but slightly shallower than that of HDM. The chromatofocusing fractions were approximately 4- and 8-fold more allergenic than HDM and SMM which required 10.5 and 20 μ g/ml, respectively, to bind 50% of the maximum bindable ^{125} I-labelled anti-IgE. Extracts of UMM were non-allergenic and sera from non-atopic individuals did not bind to discs coated with fractions 2-5 at a concentration of 200 μ g/ml.

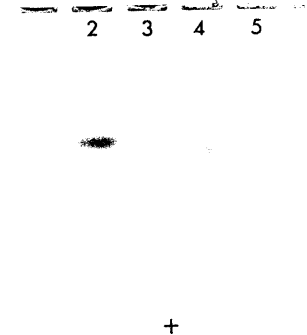


Fig. 4. 12% slab PAGE analysis of the chromatofocusing fractions 2 (10.3 μ g), 3 (7.6 μ g), 4 (10.2 μ g) and 5 (4.2 μ g). Gels were stained with Coomassie blue G-250.

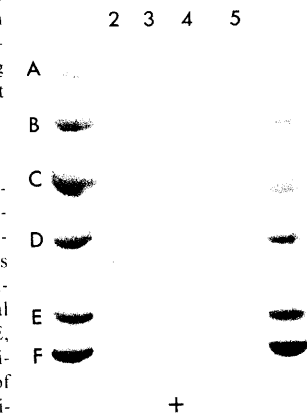


Fig. 5. SDS-gradient PAGE analysis of the chromatofocusing fractions 2-5. The molecular weight markers used were A = phosphorylase b (molecular weight 94,000); B = albumin (molecular weight 67,000); C = ovalbumin (molecular weight 43,000); D = carbonic anhydrase (molecular weight 30,000); E = trypsin inhibitor (molecular weight 20,100); F = α -lactalbumin (molecular weight 14,400).

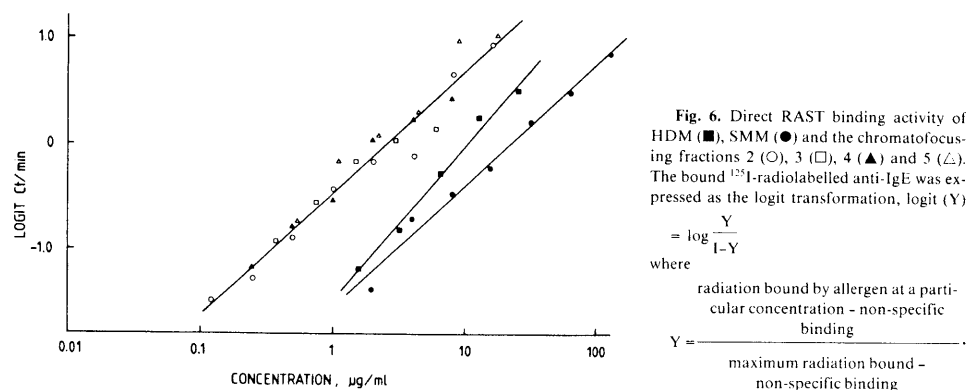


Fig. 6. Direct RAST binding activity of HDM (■), SMM (●) and the chromatofocusing fractions 2 (○), 3 (□), 4 (▲) and 5 (△). The bound ^{125} I-radiolabelled anti-IgE was expressed as the logit transformation, logit (Y)

$$Y = \log \frac{Y}{1-Y}$$

where

$$Y = \frac{\text{radiation bound by allergen at a particular concentration} - \text{non-specific binding}}{\text{maximum radiation bound} - \text{non-specific binding}}$$

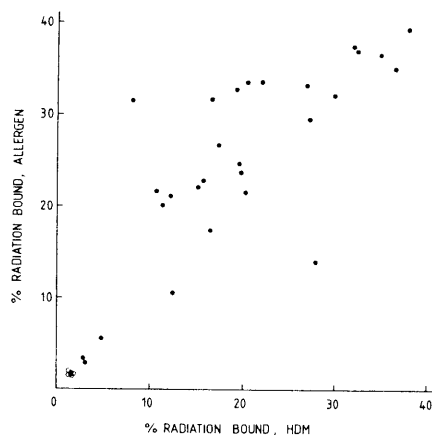


Fig. 7. Correlation of the percent radiation bound by discs coupled with whole mite (HDM) or the purified allergen, Dpt 12, when tested against a panel of sera from 28 atopic (●) and 7 non-atopic (○) patients.

The RAST binding activity of a panel of 35 sera (7 non-atopic and 28 atopic) was measured using discs coated with either HDM or chromatofocusing fraction 2. Linear regression analysis (fig. 7) indicated a direct correlation between the counts per minute (cpm) bound using the two types of discs. The curve obtained had a slope of 1.01 and a correlation coefficient of 0.87 ($p < 0.0005$). These data also indicated

that a high proportion (68%) of the atopic sera gave higher counts per minute bound with discs coated with the purified allergen than with discs coated with the whole mite using a coefficient of variation of 10%. In one instance, the percentage counts per minute bound by an atopic serum rose from 8% using discs coated with HDM to 32% with discs coated with the pure allergen. The difference between the means of the RAST scores obtained with the two kinds of discs was shown to be statistically significant using the paired *t* test ($p < 0.0005$).

Discussion

Chromatofocusing, in combination with other fractionation procedures, was found to be a useful and reproducible technique for isolating Dpt 12 from ammonium sulphate precipitates of SMM. This allergen had been previously isolated from SMM by flat bed isoelectric focusing followed by gel filtration [20]. Although the experiments described in this paper were not performed in a manner directly analogous to this, it was apparent that chromatofocusing was better than isoelectric focusing in that a brown pigment associated with SMM was poorly resolved on isoelectric focusing, contaminating all the fractions, whereas in chromatofocusing this material remained at the top of the column throughout the run.

The chromatofocusing elution profile obtained with the purified allergen from SMM was very similar to the profile seen with allergen Dpt 12 from HDM on

crossed radioimmuno-electrophoresis in combination with analytical PAGE-isoelectric focusing (CIEF) [19]. These and other data from this study, such as analytical PAGE, electrophoretic mobility and radioimmunoassay, showed that the allergen isolated by chromatofocusing from SMM was identical to the allergen Dpt 12 described earlier [19]. In addition, it has recently been shown that the chromatofocusing fractions SMM and HDM react strongly with an anti- P_1 antiserum kindly provided by Drs. Chapman and Platts-Mills [unpublished observations], thus indicating that allergens P_1 and Dpt 12 are the same.

However, the isoelectric focusing profile of P_1 was slightly different from that seen with Dpt 12 isolated from both SMM and HDM. In their studies they showed that P_1 focused into two major peaks with *pI* of 4.7–5.4 and 6.6–7.1 respectively [5]. Whilst the latter peak probably corresponds with those seen on CIEF [19] and chromatofocusing described here, little material was found to correspond to the former. The reasons for this difference are not clear but may reflect the fact that the mites used in both studies were grown on different media, which in turn could have influenced carbohydrate or amide content thus influencing *pI* values. It is also possible that these differences could have occurred due to small changes in chemical structure induced by proteolytic enzymes.

Although analytical PAGE studies showed that the allergen was heterogeneous, SDS-PAGE studies showed that this heterogeneity was not associated with the presence of different molecular weight species. Previous studies using PAGE in combination with crossed immunoelectrophoresis showed that the bands were immunochemically identical [19, 20]. The apparent molecular weight of 27,000 daltons for the allergen was slightly higher than that obtained for Dpt 12 by gel filtration, namely 24,000 daltons [19], and also higher than that obtained by SDS-PAGE for P_1 which was shown to have a molecular weight of 24,000 daltons [5]. Whether this difference is real or just reflects the use of different starting materials or methods is not known.

The allergen was found by radioimmunoassay to be present in extracts prepared from HDM, cuticle and SMM but not in extracts of UMM. These data are in agreement with those from crossed radioimmuno-electrophoresis studies [20]. It is probable, on the basis of previous data which showed that the allergen P_1 was associated with faecal pellets [21], that the allergen detected by radioimmunoassay in extracts of cuti-

cle was derived from faecal pellets attached non-specifically to their surface since such pellets were observed microscopically.

RAST studies showed that the allergen was potent, being 8- and 4-fold more potent than the SMM and HDM, respectively. In addition, the RAST studies showed that *pI* did not influence allergenicity, since all the chromatofocusing fractions were equally potent. The results agree with those obtained by CIEF and isoelectric focusing reported earlier [5, 19].

All sera from mite-allergic individuals reacted with discs coupled with the purified allergen. These data and subsequent data, which demonstrated a good correlation between RAST scores obtained with discs coupled with HDM and with purified allergen, indicate that the allergen is a major one based on the criteria proposed by Marsh [13]. In addition, data showed that the radiation bound by the purified allergen-coupled discs was significantly higher than with those coupled with HDM. This latter observation suggests that the use of purified allergens such as Dpt 12 may improve the already good correlation between RAST and *in vivo* assays, a suggestion proposed earlier by Aas and Lundkvist [1]. These data are in accord with those of Chapman and Platts-Mills [4, 5], who showed that IgE antibody to antigen P_1 detected using an antigen-binding assay correlated with RAST and that up to 75% of the IgE antibody to the original mite extract was directed toward P_1 .

In conclusion, data presented here show that chromatofocusing is a useful adjunct to other fractionation procedures, particularly in the isolation of complex mixtures of allergens. In the study described here, chromatofocusing was used to isolate an allergen from *D. pteronyssinus* corresponding to Dpt 12 and P_1 . Further characterization of this allergen is presently under way.

Acknowledgements

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