

Isolation and Characterization of an Allergen-Rich Fraction Derived from Cultures of *Dermatophagoides pteronyssinus*

Preliminary Purification of a Protein

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Abstract. An allergen-rich fraction derived from mature cultures of *Dermatophagoides pteronyssinus* was isolated. Results of radio-allergosorbent tests and the disclosure of IgE-binding proteins after their electrophoretic transfer from sodium dodecyl sulphate-polyacrylamide gel to nitrocellulose sheets revealed that both important groups of allergens, Der p I and Der p II, seem to be contained in the above-mentioned fraction. Analysis using crossed immuno-electrophoresis (CIE) disclosed 5-7 antigens and a corresponding enzymatic assay for the specificity of patient's IgE in CIE revealed that 4 of the antigens also are allergens. Moreover, an allergen was detected which apparently was not discovered when using CIE. The fraction in question was fractionated further and subsequent analysis by CIE indicated the purification of 1 antigen.

Introduction

Allergens derived from house dust mites (family: Pyroglyphidae) are an important cause of allergic diseases such as asthma and perennial rhinitis [1, 2].

In recent years, different mite allergens from mites of the genus *Dermatophagoides* have been identified and the two most important groups have been purified [3-9]. Group I allergens are 24,000 molecular weight (MW) glycoproteins and group II allergens are 15,000 MW proteins. However, the accurate value of these figures should be applied with some caution [3-8]. The allergens in each group seem to be structurally homologous, but there is no indication, however, of a structural similarity or any cross-reactivity between the allergens from the two groups examined [6, 10].

Although the 2 dominant mite allergens have been purified, there is still an urgent need for a number of the minor allergens to be purified individually in an adequate quantity and to establish the significance of these allergens. Moreover, in order to achieve better standardization of allergen extracts for immunotherapy purposes, it is important to gain an insight into the frequency and concentration of the individual components of both major and minor allergens during the growth period of the mite population. This

study concerns the isolation and characterization of a fraction containing 5-7 antigens, of which the major part is allergens from *Dermatophagoides pteronyssinus* cultures, and the subsequent preliminary purification of such a protein.

Materials and Methods

Extracts

Extracts from cultures of the house dust mite *D. pteronyssinus* and from the culturing medium with no mites (yeast and human skin scales) were processed as described in a previous paper [11]. House dust mite cultures used for allergen isolation were grown for 16-22 weeks. In respect of extracts used for allergen isolation, dialysis was omitted because Lind and Løwenstein [12] found that allergen molecules leaked through the ordinary dialysis membranes with an MW cut-off of 8,000-15,000. Coinciding herewith, the ionic strength of the ammonium hydrogen carbonate buffer, pH 7.6, was lowered from 0.125 to 0.05 M. The protein content of the extracts obtained was estimated by the Folin-Ciocalteu method [13] using tyrosine as the standard.

Extract Purification

Rabbit antibodies against yeast (*Saccharomyces cerevisiae*) were coupled to a protein A-Sepharose CL-4B column. A crude extract was applied to the column in a 0.01 M phosphate buffer (PBS), pH 7.4, and the unabsorbed peak was collected and freeze-dried. 10 mg of this partially purified mite extract were redissolved in PBS and filtrated on a Sephadex G-75 column (40 cm, diameter 2.8 cm; flow

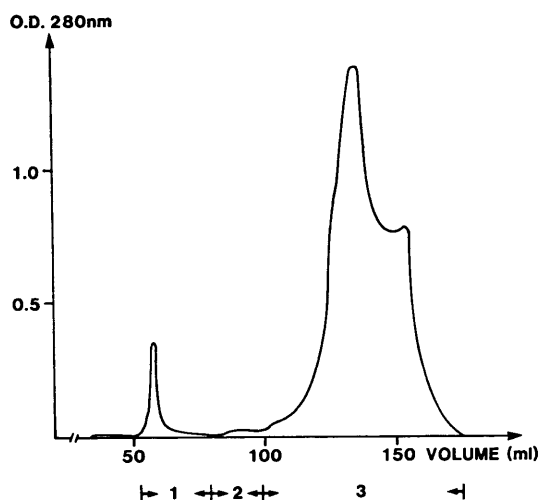


Fig. 1. Fractionation of the partially purified extract [D.pt(m)] on Sephadex G-75. The obtained fractions were pooled as indicated.

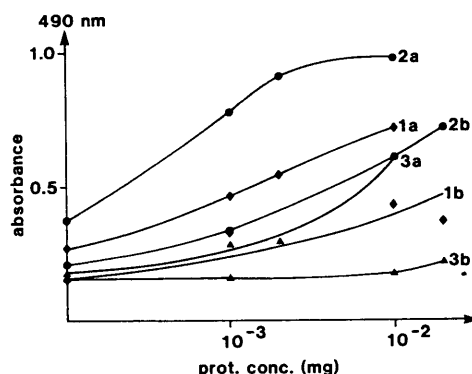


Fig. 2. Direct RAST determinations of the obtained fractions (1, 2 and 3) using either the tracer serum (a) or the serum pool (b) to evaluate the allergenicity.

rate: 3 ml/h). The fraction to be examined (fig. 1: pool No. 2) was further separated according to its charge using a cation-exchanger SP-Sephadex C50. The exchanger was activated with 50 mM sodium phosphate, pH 7.0, before equilibration using 50 mM sodium phosphate, pH 5.0. After applying the sample to the column and performing a thorough washing with the equilibrium buffer, a continuous salt gradient to 0.5 M NaCl was applied. The gel materials were pretreated and used in accordance with the manufacturer's instructions (Pharmacia Fine Chemicals, Uppsala, Sweden) and, moreover, the column effluent was monitored at 280 nm.

Rabbit Antisera

Laboratory rabbits were immunized using Freund's complete adjuvant and 0.9% NaCl (1:1) at the first immunization. Then for every 14 days during the next 2 months, the rabbits received a boost-

ter immunization containing the immunogen in a saline solution. Once a month, i.e. 8 days after the animals had been injected with the immunogen, the rabbits were bled from the ear vein and the sera obtained.

Human Sera

The tracer serum used in this study originates from a patient previously described [14] with both clinical and laboratory evidence of hypersensitivity to house dust mites, i.e. based on skin prick test and a radio-allergosorbent test (RAST) score of 4. A reference standard was provided by a serum pool made by pooling equal volumes of serum from 10 allergic patients showing allergy towards house dust mites, i.e. based on skin prick tests and RAST scores at 3-4. Finally, a patient (No. 2) who had never received immunotherapy but also had a documented history of house dust mite allergy (RAST score of 4 and positive skin prick test) was included. Human sera for control purposes were obtained from non-atopic individuals chosen among healthy laboratory staff members.

Crossed Immuno-Electrophoresis

Crossed immuno-electrophoresis (CIE) was performed in 1% agarose in 0.05 M di-ethylbarbitur-sodium buffer, pH 8.5 (Indobiose, EEO = -0.10; Pharmindustrial, Clichy, France), basically as described by Löwenstein [15].

Assay for the Specificity of Patient's IgE in CIE

The assay as described by Skjød et al. [16] was followed with few adjustments. In brief, following the second-dimension electrophoresis of CIE, the gel was pressed and washed (3×15 min) with 0.01 M PBS, pH 7.4, with Tween 20 (1:10,000). Afterwards, the gel was incubated with the tracer serum adequately diluted in incubation buffer [PBS with 1% bovine serum albumin (BSA)] overnight at room temperature. After 3 subsequent washings, the gel was incubated with peroxidase-conjugated rabbit anti-IgE (1:200) in incubation buffer for 3 h at room temperature followed by yet 3 washings of which the last one was 0.02 M ammonium acetate buffer, pH 5.0. The gel was then incubated in staining solution prepared as follows: 10 mg 3,3'-diaminobenzidine dissolved in 100 ml of 0.02 M ammonium acetate buffer, pH 5.0. Immediately prior to use, this solution was mixed with 80 μ l of 30% H_2O_2 . The reaction was stopped by washing the gel in redistilled water.

RAST

Activation of paper discs with BrCN and coupling of proteins under increased incubation time were done as described by Ceska et al. [17] and Tovey and Vandenberg [18], however, with the exception that peroxidase-conjugated anti-IgE was chosen instead of isotope-marked anti-IgE [14].

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in accordance with Laemmli's method [19] using a 15% separating gel and a 5% stacking gel. The proteins were solubilized by heating with 1% SDS (w/v) without a reducing agent in the loading buffer and electrophoresed at 45 V for 18 h. Proteins in the gel were eventually stained with Coomassie brilliant blue R-250 and the MW of the separated proteins was calculated using the calibration kit from Pharmacia.

Immunoblotting

Protein bands were transferred from the polyacrylamide gel to nitrocellulose (pore size: 0.45 μ m, Schleicher & Schull) by using the method of Towbin et al. [20], modified with the rapid semidry electroblotting system (constant current of 80 mA for 1 h) of Kyhse-Andersen [21]. The buffer (Tris-glycine, methanol, SDS) for the SDS gels was used as proposed by Sutton et al. [22]. To visualize the transferred proteins, strips of nitrocellulose were stained with the colloidal gold staining method as basically described by Moere-mans et al. [23].

To detect the IgE-binding zones, the methods of Sutton et al. [22] and Tovey and Baldo [24] were followed with a few modifications. In brief, the unstained membrane was washed for 5 min in 0.01 M PBS, pH 7.4, followed by 2×30 min washing in PBS with 10% methanol to remove the detergent used. The remaining absorptive places on the membrane were blocked through an immersion of the membrane in 3% BSA in PBS for 2.5 h. After washing for 3×10 min with the washing buffer (Tween-PBS 1:10,000), the nitrocellulose sheet was incubated for 18–24 h with serum diluted 1:5 in incubation buffer (1% BSA in PBS). After additional 3 washings, the membrane was incubated with rabbit anti-IgE (1:150) for 2.5–3 h. This was again followed by washing for 3×10 min and incubation with swine anti-rabbit (1:150) for 1.5 h, and another washing (3×10 min) before the final incubation with 1:250 complex of peroxidase-rabbit anti-peroxidase (Dakopatt, Denmark) for 1.5 h. After 3 further washings of which the last one was in 20 mM ammonium acetate buffer, pH 5.0, the complex was detected by adding the chromogen. This last step was performed exactly as described in the section dealing with the specificity of the patient's IgE in CIE. Through the entire washing and incubation process, which took place at room temperature, the mixture was subjected to occasional shaking in order to ensure mixing.

Results

The non-relevant antigens originating from the yeast were removed from the crude *D. pteronyssinus* extract by affinity chromatography on protein A-Sepharose. The resulting partially purified extract [= D.pt.(m)] was fractionated on Sephadex G-75. The fractions were pooled as indicated (fig. 1) and the allergenicity of the three fractions obtained was assessed by direct RAST binding studies.

Previous experiments [14] including dose-response studies revealed that the optimal protein concentrations bound to paper discs were in the range of 0.5–1,000 μ g when using crude extracts prepared from old cultures of *D. pteronyssinus*. In this study, it was preferred to bind less amounts of protein to the discs (range: 0.1–20 μ g) since it was the partially purified extract [= D.pt.(m)] which was used for the fractionation. In addition, by using diluted protein concentrations for the couplings materials were saved for further analyses.

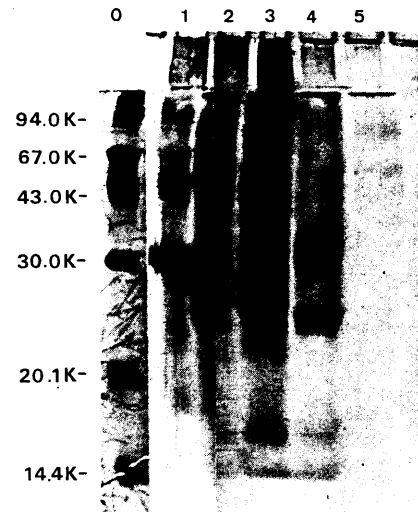
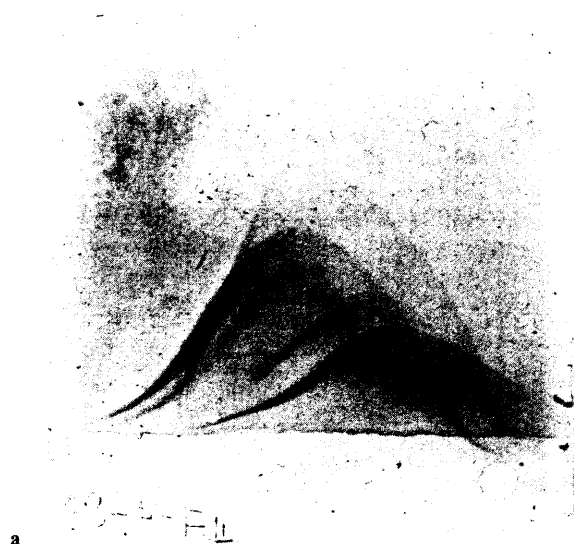


Fig. 3. Detection of the IgE-binding proteins on a nitrocellulose sheet after electrophoretic transfer from an SDS-polyacrylamide gel (tracks 2–5). Lane 0 = MW markers on the SDS-polyacrylamide gel after the transfer. Track 1 = MW markers on the nitrocellulose sheet (stained with gold); track 2 = a part of the affinity-fractionated extract [D.pt.(m)] was stained with gold, the other part was like the rest of the sheet incubated with serum. The IgE-binding proteins were detected with a sandwich antibody staining technique; track 3 = fraction 1; track 4 = fraction 2; track 5 = fraction 3.

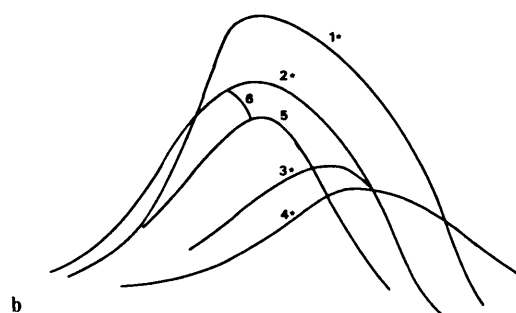
On both occasions the results of RAST (fig. 2) using either the tracer serum or the serum pool revealed that fraction 2 clearly exceeded the other two fractions despite its low optical density at 280 nm (fig. 1). The allergenic potency was judged by the shape of the curve and by the concentration of the material required to bind 50% of the maximum obtainable. RAST analysis of the mite medium without mites showed that the allergenicity was not related to these components.

Samples taken from the partially purified extract [D.pt.(m)] and from the above-mentioned fractions were separated according to size using SDS-PAGE. Non-reduced samples were applied to avoid destruction of the allergenicity even if an unfolding of the proteins might not be completely achieved. Therefore, information pertains to the molecule's native conformation which is not necessarily the same as its absolute size.

Immediately after the transfer, a strip of the nitrocellulose containing the transferred MW markers and a part of the affinity-fractionated extract [D.pt.(m)] was cut off and stained with gold. A comparison of the stained SDS-PAGE after electrophoretically hav-



a

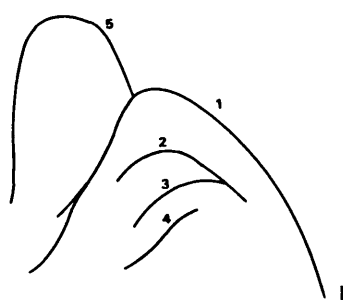


b

Fig. 4. a CIE analysis of fraction 2 using polyspecific rabbit antibodies. **b** Drawing illustrating the individually detected arcs.



a



b

Fig. 5. a Enzymatic assay for detection of the specific serum IgE in a corresponding CIE. **b** Drawing illustrating the individually detected lines.

ing transferred proteins from the gel to the membrane and the gold-stained membrane made it possible to predict the accurate locations of the MW markers on the nitrocellulose.

The allergenic proteins were visualized by transferring the proteins to the nitrocellulose membrane and performing subsequent incubations of the membrane with serum from allergic individuals. Identification was carried out by the enzyme-conjugated antibody staining technique.

The potential for detecting IgE-binding proteins is demonstrated using the tracer serum as probe (fig. 3). A duplicate experiment was performed using patient No. 2 who had never had immunotherapy. The binding capacity of this patient's IgE showed the same basic pattern as the tracer's serum, however, the response was weaker. The results demonstrated by the three fractions revealed an intense staining of fraction 1, followed by fraction 2, and as third fraction 3

showing nearly no response at all (fig. 3). This latter result corresponds with the results of RAST which also showed the lowest allergenic activity in association with fraction 3 (fig. 2).

Judged by the staining intensity appearing on the nitrocellulose, the IgE-binding capacity of fraction 2 appears to be particularly two groups of proteins lying in the area corresponding to proteins with MW of about 22.0–25.0 and 29.0–32.0 kilodaltons K, respectively. These two groups are also present in fraction 1, however, their appearance is blurred by the presence of other proteins. Some of the intermediate staining, especially seen in fraction 1, could be the result of allergen adherence to proteins of larger MW which might retard and blur the separation.

The results of RAST and the corresponding determination of the MW of fraction 2 point to the fact that the major group I allergen Der p I [3–5] with an MW of about 24.0 K is contained in fraction 2. More-

over, this might explain the apparent discrepancy between the results of RAST and the total staining intensity on the nitrocellulose of fractions 1 and 2. Loss of protein might occur if loading exceeds the capacity of the nitrocellulose [20]. So if the concentration of the important allergens is high, the probability of loosing these proteins is also particularly high. The staining intensity of the nitrocellulose might, therefore, not be directly related to the absolute concentration of the protein in question.

The allergens of group II, Der p II, with an MW of about 15.0 K [5, 25] might be represented by one of the two other groups of stained proteins seen in both fraction 1 and 2 in the area corresponding to proteins with MW of 13.5–14.5 and 15.0–16.0 K, respectively (fig. 3).

Since fraction 2 seems to contain both important groups of allergens as judged by the results of RAST and immunoprint, it was decided to continue the analysis using this particular fraction.

CIE analysis of fraction 2 and the corresponding assay for the specificity of the tracer's IgE in CIE are shown in figures 4 and 5 together with drawings illustrating the individually detected arcs. In the drawings differences in the concentrations applied have been taken into account.

A total of 6 precipitating lines are seen in CIE (fig. 4a, b) and the tracer's IgE revealed (fig. 5a, b) that 4 of these antigens also are allergens (symbolized with asterisks). Moreover, an allergen (No. 5, fig. 5a, b) was detected which apparently was not identified by the corresponding CIE.

Using cation exchange, fraction 2 was fractionated further and an unbound protein component was eluted with the starting buffer. The remaining parts of the sample were very strongly bound and elution was first achieved by varying both the buffer pH and the ionic strength. Attempts are now in progress with the elution and separation of these components.

A subsequent analysis of the eluted unbound protein component by CIE revealed the appearance of 1 antigen (fig. 6).

Discussion

Immunotherapy has shown its clinical efficacy in patients who especially suffer from asthma caused by house dust mites [26]. However, an optimal result of immunotherapy is based on the correct qualitative

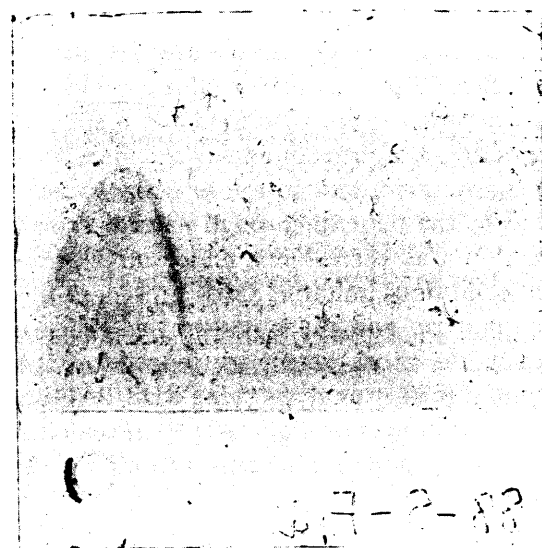


Fig. 6. CIE analysis of the purified antigen using polyspecific rabbit antibodies.

and quantitative composition of the various components and well-documented raw materials are therefore an obvious necessity.

A study on standardization of *D. pteronyssinus* extracts [27] presents the conclusion that the total allergen activity of an extract represents a spectrum of several separate allergens. In another paper concerning purification and partial characterization of 2 major allergens from *D. pteronyssinus*, Lind [5] puts forth that both major allergens together might constitute up to 20% w/w of a typical house dust mite allergen extract. However, the individual contribution of the various major as well as minor allergens to the total allergen activity has not yet been estimated, probably due to lack of a sufficient quantity and number of purified mite allergens [5, 26, 28].

The purpose of this study was to describe the isolation and characterization of an allergen-rich fraction of *D. pteronyssinus* and, furthermore, to outline the purification of one of these proteins. Now work is being carried out with the aim to produce monospecific rabbit antibodies against the purified antigen, and purification of some of the other contained components is also being performed.

For this study the source material was obtained from *D. pteronyssinus* cultures grown for a period of 16–22 weeks, at which age the mite population is at its peak and the relative frequency of the crawling stages is nearly constant [11]. At this stage in the cultivation, the amount of protein more or less reflects the popu-

lation density and the allergenic activity of the culture continues to increase, probably reflecting the accumulation of feces present in the culture medium [14]. The mature mite culture with its components might correspond to a simplified model of the mites in their natural environment and to the allergen source encountered by the patients through natural exposure.

Extraction was carried out under mild physicochemical conditions imitating those present in the respiratory mucosa and the bloodstream. Fraction 2 was clearly the most potent as judged by RAST (fig. 2), and a further analysis using SDS-PAGE and the immunoblotting technique (fig. 3) revealed that both important groups of allergens, Der p I and Der p II, seem to be contained herein.

To evaluate if the IgE-binding pattern might be affected by the immunotherapy treatment received by the tracer patient some years ago, a duplicate experiment was performed using serum from patient No. 2, who had never received immunotherapy. The results revealed that IgE from this patient showed the same binding pattern as the tracer's. Since, however, the response was weaker, it was decided to continue the investigation using the potent serum from the tracer.

The results of CIE (fig. 4a, b) and the parallel assay of the specificity of the tracer's IgE in CIE showed that 4 of the precipitated antigens were recognized by IgE. One of these allergens (No. 1, fig. 5a, b) in particular gave rise to an intense staining. As with the CRIE method, the staining intensity depends on a sufficient number of sites on the precipitated antigen available for the reaction with IgE and it moreover depends on the affinity of the various specificities of IgE [15]. Since the precipitating arc of this particular antigen almost surrounded the area containing the other antigens, the resulting staining nearly obscured the specific individual response shown by the other antigens. Precipitation lines, however, could still be detected. In the sensitive CRIE the covering effect might be even more pronounced as a result of the dark radiostaining. Furthermore, the enzyme assay appears to have other advantages when compared with CRIE. It is less time-consuming, handling of isotopes is avoided, and the lines detected might be identified more easily if the enzymatic staining is followed by an additional staining with Coomassie brilliant blue [16]. For identification of allergens in CIE, the enzymatic assay, therefore, appears to be a useful alternative to CRIE when identifying allergens in CIE.

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