

Purification and partial characterization of two major allergens from the house dust mite *Dermatophagoides pteronyssinus*

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Two major allergens of the house dust mite, *Dermatophagoides pteronyssinus* (Dp), were purified, and their molecular weight and isoelectric points (pIs) were determined. Dp 42 was purified from an acetone-precipitated mite-excrement extract by a combination of hydrophobic interaction chromatography on phenyl Sepharose and copper-chelate chromatography. The molecular weight was determined to be 18,000 and 25,000 to 30,000 by gel filtration (G-75) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis, respectively, and pI values of 4.6, 5.6, and 6.6 were obtained by sucrose gradient isoelectric focusing (IEF). These values correspond well with those described for the identical allergen, P₁. The pI 6.6 variant was considerably enriched in the purified material. Dp 42 constituted 6.4% of the dry weight of a reference whole mite-culture extract. Dp X was obtained partially purified by gel filtration (G-75), ammonium sulphate precipitation, and hydrophobic interaction chromatography. The molecular weight was 18,000 to 20,000 by gel filtration and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Multiple pIs in the range 5 to 7 were found by sucrose gradient IEF and crossed IEF. The two purified allergens carried clearly distinct activities toward human IgE and appeared as potent allergens in crossed radioimmuno-electrophoresis, RAST, and RAST inhibition. (J ALLERGY CLIN IMMUNOL 76:753-61, 1985.)

Two major allergens of the house dust mite, *D. pteronyssinus*, have recently been identified in CRIE besides several less important allergens.^{1,2} The present study deals with a further characterization of these two major allergens, Dp 42 and Dp X.

Several important properties of individual antigens may be obtained without full purification. Molecular weight, pIs, and affinities for various molecular structures may be determined by a combination of small scale fractionation and immunoelectrophoresis, based on the availability of high-quality antisera.³

Other characteristics may only be obtained by studies on the components in a reasonably pure state. This includes data on the allergenic activity of single allergens in diagnostic tests like RAST, histamine release, and skin prick test.

The present study presents data obtained through both these approaches and a verification of the allergenic importance of both identified allergens.

Abbreviations used

AML:	Acetone-precipitated mite-excrement extract
MF:	Mite-excrement extract
WMC:	Whole mite-culture extract
Dp:	<i>Dermatophagoides pteronyssinus</i>
SDS-PAGE:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
CIE:	Crossed immunoelectrophoresis
CLIE:	Crossed-line immunoelectrophoresis
CRIE:	Crossed radioimmuno-electrophoresis
FRIE:	Fused rocket immunoelectrophoresis
IEF:	Isoelectric focusing
pI:	Isoelectric point
HSA:	Human serum albumin
BSA:	Bovine serum albumin

MATERIAL AND METHODS

Allergen preparations

AML, an acetone-purified allergen preparation of Dp, was produced as described previously² by acetone/water extraction and precipitation. This preparation contained nine antigens by CIE, including the major allergens Dp 42 and Dp X,² the former five times enriched, relative to a standard WMC.

MF and WMC were produced by extraction 1:10 w/v in

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FIG. 1. Sucrose gradient IEF pattern of 20 mg AML as demonstrated by FRIE on collected fractions and pH gradient (superimposed). Fractions of 15 μ l applied; concentrations of a-Dp WMC antibodies; 4.4 μ l/cm². Pooling of fractions I, II, and III (for CIE/CRIE analysis) indicated. Arrows point to Dp 42 precipitate top points.

ammonium hydrogen carbonate buffer, dialysis, and freeze-drying.²

Rabbit antibodies

Rabbit antibodies to Dp WMC were produced as described previously.¹ Antibodies to Dp 42 and Dp X were produced by immunization of rabbits with purified Dp 42 (25 μ g per rabbit per injection) and Dp X (50 μ g per rabbit per injection) in incomplete Freund's adjuvant. The first bleeding was obtained 1 week after two injections with a 2-week interval. Booster injections and subsequent bleedings occurred, each with 2-week intervals. Immunoglobulin fractions were produced as previously described.⁴

Patient sera

Sera, rich in specific IgE to Dp (class 3 to 4 in Al [OH]₃ RAST),⁵ and serum pool for RAST inhibition were obtained from Diagnoselaboratoriet, Copenhagen, Denmark.

RAST and RAST inhibition were performed as previously described.¹

CIE, FRIE, and modifications of these techniques were performed as described previously.³ CRIE was performed as previously described.⁶

Column IEF in sucrose gradient was performed in an 8101 column (LKB Instruments, Bromma, Sweden) (140 ml). The sample was dissolved in 50 ml of 1% v/v of Ampholine, pH 3 to 10 (LKB). A linear sucrose gradient was produced by mixing with 50% w/v of sucrose containing 1% v/v of Ampholine. Focusing took place at 200 V, 2.2 mA, for 48 hours at 15° C. Fractions of 2.5 ml were collected from the column starting at the anode.

Hydrophobic interaction chromatography

A Pharmacia K 16/40 column was packed with either 20 ml or 50 ml of phenyl Sepharose Cl-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Unless otherwise indicated, the column was equilibrated with 0.05 mol/L of acetate

buffer, pH 5.0, containing 1.0 mol/L of ammonium sulphate. Samples were applied and dissolved in the same buffer, after which elution took place.

Metal-chelate affinity chromatography⁷

Iminodiacetic acid Sepharose (Pierce, Rotterdam, The Netherlands) was packed into plastic syringes, and the gel was washed in two bed volumes 0.05 mol/L of EDTA and equilibrated with 0.1 mol/L of copper sulphate in 0.1 mol/L of sodium phosphate buffer, pH 7.5. Stepwise elution of applied samples took place with buffers of decreasing pH and with EDTA.

Gel filtration chromatography

Sephadex G-75 (Pharmacia Fine Chemicals) was equilibrated with 0.125 mol/L of ammonium hydrogen carbonate buffer, pH 7.6, containing 15 mmol/L of sodium azide. A K 26/100 column (Pharmacia) was used for preparative fractionation, whereas an econocolumn (57 ml) (Bio-Rad Laboratories, Richmond, Calif.) was used for molecular weight determination on purified Dp 42. The samples were applied, dissolved in equilibration buffer, and eluted with flow rates of 11.2 ml/hr and 6.6 ml/hr, respectively.

Purification procedure for Dp 42

One gram of AML (undialyzed) was dissolved in equilibration buffer and applied to a 50 ml phenyl Sepharose CL-4B column. The column was eluted with a flow rate of 50 ml/hr, and 8.3 ml fractions were collected: (1) two bed volumes of equilibration buffer, (2) followed by 20 volumes of a linear gradient of phosphate/carbonate buffers (500 ml of 0.05 mol/L of sodium phosphate buffer, pH 5.3/500 ml of 0.05 mol/L of sodium carbonate buffer, pH 10.2), (3) one volume of carbonate buffer/ethylene glycol, 80%/20% v/v, and (4) two volumes of carbonate buffer/ethylene glycol, 50%/50% v/v. Fractions, eluted with carbonate buffer/ethylene glycol, which contained the bulk of Dp 42

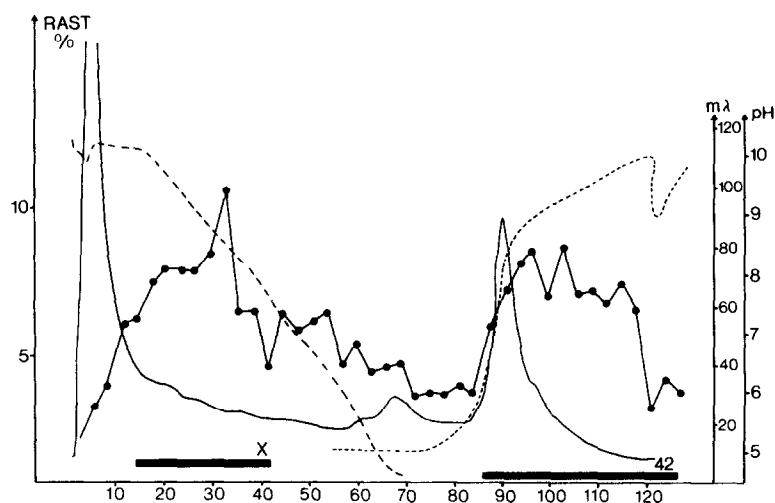


FIG. 2. Preparative fractionation of 100 mg of AML on a 20 ml phenyl Sepharose CL-4B column; application and elution (See Material and Methods). RAST activity of the fractions (—●—), obtained after coupling of two paper discs with 1 ml of the fractions, diluted 1:30. Horizontal bars indicate fractions containing Dp X and Dp 42, respectively, as demonstrated by FRIE. Ultraviolet absorption (280 nm) (—); conductivity (---); pH (---).

as demonstrated by FRIE, were pooled and dialyzed against 0.05 mol/L of acetate buffer, pH 5.0, for 4 times $\frac{1}{2}$ day, and ammonium sulphate was added at 1 mol/L. Rechromatography took place by use of the same eluents, and fractions eluted with carbonate buffer/ethylene glycol were pooled and applied to a 10 ml iminodiacetic acid Sepharose column equilibrated with 0.1 mol/L of copper sulphate and 0.1 mol/L of phosphate buffer, pH 7.5. Elution took place with (1) six bed volumes of the phosphate buffer, (2) 12 volumes of 0.1 mol/L of acetate buffer, pH 4.65, and (3) six volumes 0.4 mol/L NaCl, 0.05 mol/L of EDTA. Fractions eluted with EDTA, demonstrated by FRIE to contain Dp 42, were pooled, dialyzed, and freeze-dried.

Purification procedure for Dp X

Five hundred milligrams of MF II was dissolved in 5 ml 0.125 mol/L ammonium hydrogen carbonate and applied to a 480 ml Sephadex G-75 column. Elution took place at 10 ml/hr with collection of 5 ml fractions. Fractions of apparent molecular weight <20,000, containing predominantly Dp X and Dp 42 according to FRIE (see Fig. 3), were pooled. The supernatant after 2.5 mol/L of ammonium sulphate precipitation at pH 5 was applied to a 50 ml phenyl Sepharose column equilibrated in 0.05 mol/L of acetate buffer, pH 5.0, containing 2.5 mol/L of ammonium sulphate. Elution took place with (1) two volumes of equilibration buffer, (2) eight volumes of 0.05 mol/L of acetate buffer, pH 5.0, containing a linear gradient of ammonium sulphate from 2.5 mol/L to 0.6 mol/L, and (3) two bed volumes of 0.05 mol/L of acetate buffer plus 50% ethylene glycol (6 by 8 ml fractions per hour). Fractions 38 to 66, demonstrated by CLIE to contain Dp X, were pooled, dialyzed, and freeze-dried.

SDS-PAGE

A modification of the Laemli procedure was carried out as described.⁸ Unless otherwise stated, the total concentrations of acrylamide and relative concentration of the cross-link were as follows: stacking gel: 5% T (w/v) and 5% C (w/w); gradient: 12.5% T, 0.5% C to 20% T, and 0.8% C.

Agarose IEF and crossed IEF

Focusing occurred in 0.8% HSIF (Litex, Glostrup, Denmark) agarose and 2% Ampholine (pH 3 to 10) on a Pharmacia flat bed apparatus (FBE-3000) with 100 V for 20 minutes, 200 V for 40 minutes, and 300 V for 10 minutes. Maximum current obtained was 10 mA. Transferred gel strips for crossed IEF were covered with a 2 cm broad gel of HSA agarose (total thickness, 2 mm), after which an antibody-containing gel of 1.5 mm thickness was cast on the anodic side. Electrophoresis, pressing, washing, and staining were carried out as for CIE. Neighboring gel strips were cut in sections for pH measurements of 0.5 cm length and eluted in a small volume of distilled water.

RESULTS

Preliminary fractionation experiments and partial characterization of the major allergenic components were carried out by use of the AML and MF preparations in combination with polyspecific antibodies.

Column IEF of 20 mg of AML revealed a marked heterogeneity in pI for several of the antigens (Fig. 1). Dp 42 focused as at least three variants with pI values 4.6, 5.6, and 6.6. Three pools of eluted frac-

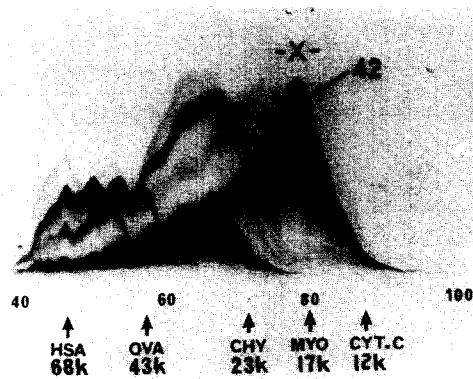


FIG. 3. Gel filtration (Sephadex G-75) pattern of 500 mg of MF demonstrated by FRIE. Column, K 26/100 (Pharmacia); flow rate, 11.2 ml/hr; fractions of 3.7 ml collected. Conditions for FRIE, 2 μ l of fractions of 37 to 112 applied after 1-hour diffusion; electrophoresis at 2 V per centimeter for 18 hours in antibody-containing gel with 6 μ l of a-Dp WMC/cm². Elution of molecular weight markers in a parallel run are indicated. Fractions 76 to 89 pooled for purification of Dp X. Ova, ovalbumin; chy, chymotrypsinogen; myo, myoglobin; cyt.C, cytochrome c.

TABLE I. Ammonium sulphate precipitation pattern for Dp 42 and Dp X

(NH ₄) ₂ SO ₄ - molarity	% Recovered in precipitate*	
	Dp 42 (%)	Dp X (%)
0.48	<5	<5
0.93	18	<5
1.36	50	10
1.75	76	22
2.13	88	39

*Determined by addition of (NH₄)₂SO₄ to AML (10 mg/ml) at pH 5.0, 5° C incubation overnight and centrifugation at 1000 $\times$ g. Quantitation of allergens in redissolved precipitate occurred by single radial immunodiffusion²⁰ by use of rabbit antibodies to Dp 42 and Dp X.

tions (I, pH 3.7 to 5.2; II, pH 5.2 to 6.3; and III, pH 6.3 to 8.1) were further investigated by CRIE by use of a serum pool from mite-allergic patients. Radiostaining after 1 day of exposure was obtained from the Dp 42 precipitate on the CIE plates with all three fractions, indicating significant allergenic activity (IgE binding) of the three variants. Furthermore, CRIE demonstrated radiostaining of Dp X in all three fractions.

Hydrophobic interaction chromatography on phenyl Sepharose CL-4B was carried out on 100 mg of ALM (Fig. 2). Direct RAST on paper-disc coupled fractions

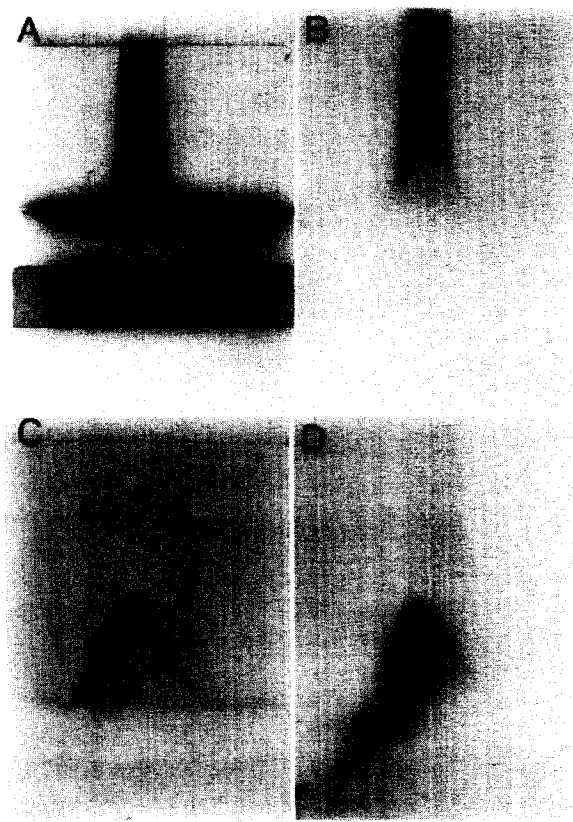


FIG. 4. A-B. CIE and corresponding CRIE of 10 μ g of purified Dp 42 and C-D, 10 μ g of purified Dp X. Concentration of a-Dp WMC antibodies: A-B, 8.6 μ l/cm²; C-D, 6 μ l/cm². Electrophoresis occurred in 1% agarose (HSA, Litex) in TRIS-Veronal buffer, pH 8.6; I = 0.02. First dimension, 10 V/cm for 25 minutes; second dimension, 2 V/cm for 18 hours. A and C stained with Coomassie brilliant blue. B and D incubated with serum pool from house dust mite-allergic patients and radiolabeled anti-IgE. Autoradiography for 3 (B) and 6 (D) days, respectively.

(diluted 1:30) revealed two major peaks of allergenic activity. FRIE demonstrated the presence of Dp 42 in the second peak. The pooled dialyzed and freeze-dried fractions of the first peak were investigated by CIE. The material contained Dp X and contaminants. No Dp 42 could be detected by incorporating large amounts of this Dp X fraction (1 mg) in the intermediate gel of the AML CIE system.

The Sephadex G-75 elution profile in FRIE for 500 mg of MF is demonstrated in Fig. 3. Identification of allergens Dp 42 and Dp X on the FRIE plate was performed by CRIE and CLIE.

The apparent molecular weight for Dp 42 and Dp X as determined from the precipitate top points were 18,000 and 18,000, respectively. Most other antigens eluted apparent molecular weights >30,000. Smaller

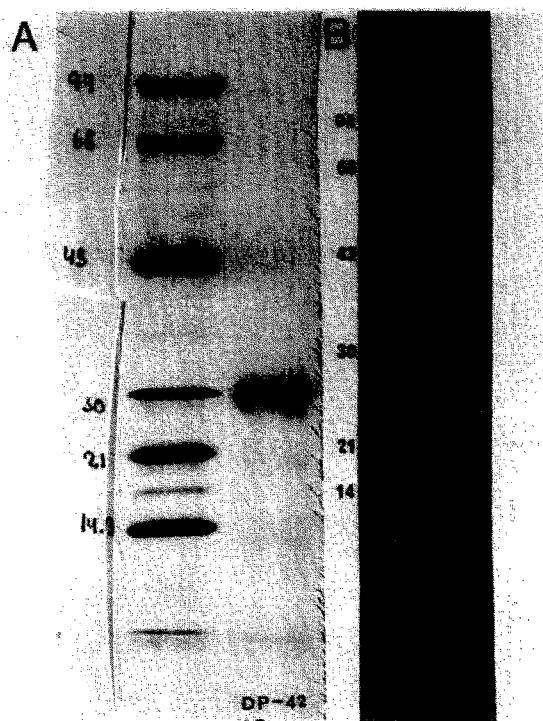


FIG. 5. SDS-PAGE on purified Dp 42 (A) and Dp X (B). The latter was radioiodinated according to standard chloramine-T method.¹⁹ Samples (10 to 20 μ g of protein) were precipitated with trichloroacetic acid, washed in acetone, and boiled for 2 minutes in TRIS-HCl buffer containing 1% w/v SDS, 5% v/v mercaptoethanol, and 5% v/v glycerol. Gel gradients: A, 12 T, 0.6 C to 16 T, and 0.8 C. B, 15 T, 0.75 C to 20 T, and 1.0 C. Molecular weight markers were phosphorylase B (94,000), BSA (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soy bean trypsin inhibitor (21,000), and lysozyme (14,000). A, Gel was stained in Coomassie brilliant blue. B, The 3-day autoradiography of the gel strip is illustrated.

amounts of DP 42 and Dp X were also recovered in fractions $>40,000$. The asymmetric elution pattern for the allergen may be caused by adherence to one or more proteins of larger molecular weight.

A distinct difference was observed in the solubility of the two major allergens in ammonium sulphate at pH 5 (Table I). At 1.75 mol/L (half saturation) 76% of Dp 42 was precipitated from AML, whereas only 22% of Dp X appeared in the precipitate.

Characterization of purified allergens

Purified Dp 42 demonstrated one major precipitate when it was analyzed in CIE against polyspecific a-Dp WMC antibodies (Fig. 4). Two very weak precipitates near the base line represent contaminants. When the Dp 42 was subjected to CRIE by use of pooled patient serum, the Dp 42 precipitate exhibited intense radiostaining (clearly visible after 1 day of

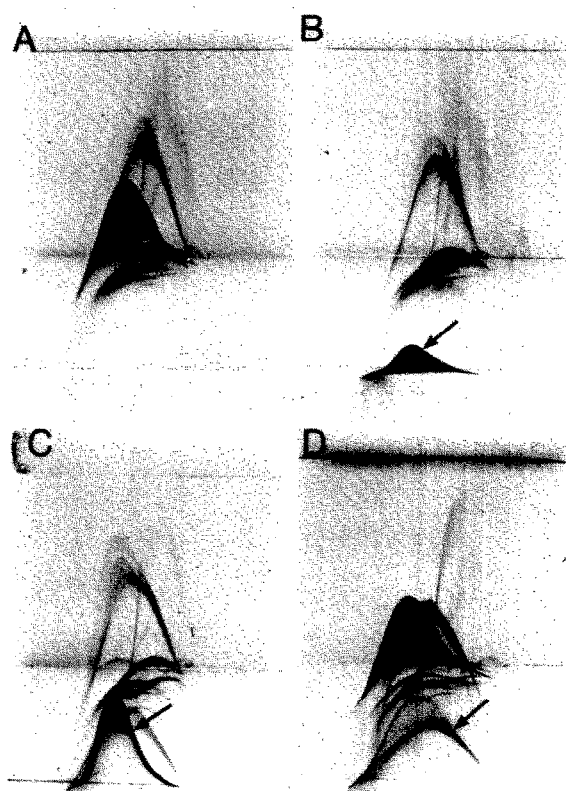


FIG. 6. CIE of WMC against anti-Dp WMC. Rabbit antibodies raised against purified components in the intermediate gel. A, Normal rabbit serum. B-C, Antiserum to Dp 42 from rabbits 1 and 2, respectively. D, Antibodies to Dp X, pooled from two rabbits. Arrows point to Dp 42 and Dp X precipitates.

exposure). IEF in HSIF agarose demonstrated several blurred bands in the pH region 5 to 7. In crossed IEF the precipitate peak occurred at pI 6.9 (not shown).

In SDS-PAGE one major broad band was observed close to the chymotrypsinogen marker at 25,000 MW (Fig. 5). Reduction of the sample with mercaptoethanol had no effect on the position. A few weak bands demonstrated the presence of small amounts of contaminants. When Dp 42 was subjected to SDS-PAGE in a different gel (gradient: 12% T, 0.6% C to 16% T, and 0.8% C), the band took a position at MW = 30,000 (equal to the carbonic anhydrase marker of this system). Sephadex G-75 gel filtration performed on 90 μ g of purified Dp 42 elicited an elution volume corresponding to the myoglobin marker (MW = 17,000).

The Dp X preparation demonstrated two precipitates in CIE (Fig. 4). The one with the lowest elec-

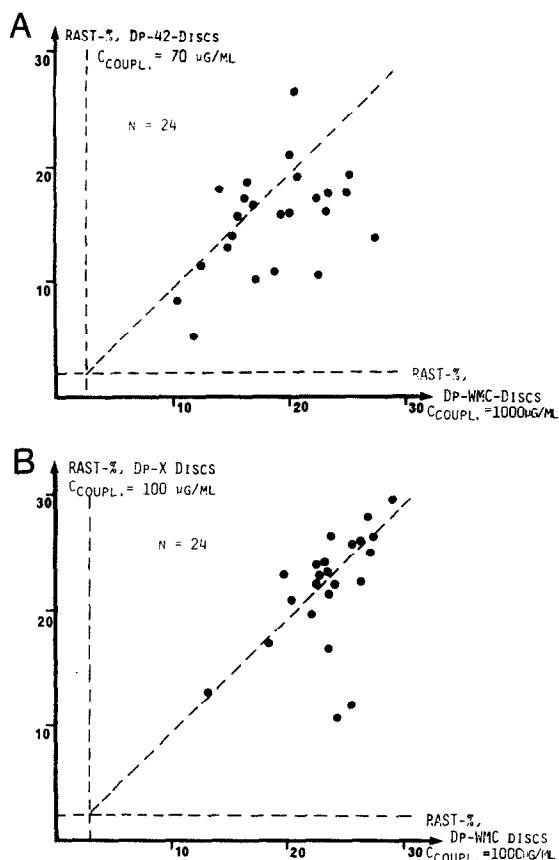


FIG. 7. Comparison of RAST reactions from 24 mite-allergic patients. **A**, Reaction to Dp WMC and Dp 42. **B**, Reaction to Dp WMC and Dp X. Figures on axes denote the retained radioactivity as percent of total added counts.

trophoretic mobility was identified as antigen Dp X by CLIE and tandem CIE. The Dp X precipitate exhibited intense radiostaining in CRIE (Fig. 4). No distinct bands were observed after fixating and Coomassie staining when purified Dp X was investigated in agarose IEF. Two precipitate peaks at pH 5.0 and 6.4 were observed in crossed IEF.

In SDS-PAGE a weak, blurred band was observed in the region of 12,000 to 17,000. Radioiodinated Dp X elicited one band of radiostaining on the autoradiography at 18,000 to 20,000 (Fig. 5).

Immunization with purified allergens

The specificity of rabbit antisera raised against purified Dp 42 and Dp X was studied by incorporating these antibodies in the intermediate gel of the reference CIE system (Fig. 6). Of two rabbits immunized with Dp 42, one produced monospecific antibodies; the second rabbit reacted to other antigens as well. Both rabbits immunized with purified Dp X reacted to other components besides Dp X.

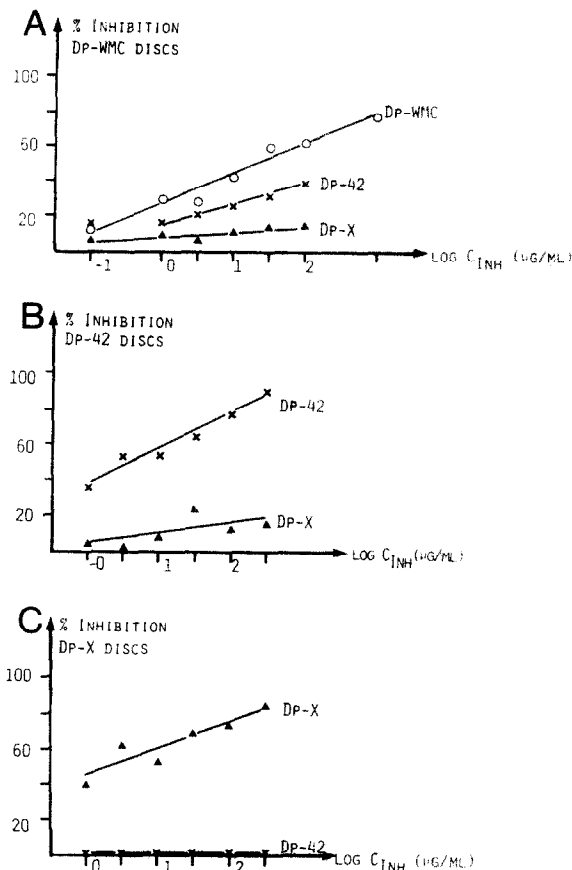


FIG. 8. RAST inhibition obtained with WMC, Dp 42, and Dp X on WMC discs (**A**), Dp 42 discs (**B**), and Dp X discs (**C**), by use of pooled serum from mite-allergic patients.

Allergenic activity of purified allergens

The direct RAST activity of Dp 42 and Dp X was compared to WMC by use of 24 sera from house dust mite-allergic patients.

The coupling concentration was 70, 100, and 1000 µg/ml for Dp 42, Dp X, and WMC, respectively. The coupling solution of WMC contained 64 µg of Dp 42 per milliliter and 150 µg of Dp X per milliliter. Both purified allergens exhibited significant specific IgE binding as compared to WMC (Fig. 7). The mean relative activities (RAST [Dp 42 or X]/RAST [WMC]) were 80% (range 43% to 129%) for Dp 42 and 90% (range 44% to 117%) for Dp X.

Both purified allergens inhibited significantly the RAST reaction to WMC (Fig. 8, A). Within the range of inhibitor concentrations used, maximum inhibitions obtained were 77%, 39%, and 15% for WMC, Dp 42, and Dp X, respectively.

Maximum inhibition of the RAST reaction to Dp 42 discs was 83% and 25% for Dp 42 and Dp X, respectively, whereas the corresponding figures for Dp

TABLE II. Dry weight and protein weight of reserved antigens Dp 42 and Dp X

Separation procedure		Yield	Recovery (%)	Purification ratio*	Protein content of dry weight† (%)
Dp 42	Mite excreta → acetone extraction and precipitation → phenyl Sepharose → copper chelate chromatography → Dp 42	2.5 mg	4.9	15.6	53
Dp X	Mite excreta → aqueous buffer extraction → gel filtration (G-75) → ammonium sulphate precipitation → phenyl Sepharose → Dp X	2.0 mg	9.8	6.7	35

*Calculated as $\frac{\text{dry weight of Std (WMC)}}{\text{dry weight of purified allergen}}$ at equal precipitate area in CIE.

†Estimated by the Lowry method with BSA as a standard.

X discs were 83% (Dp X as inhibitor) and 1% (Dp 42 as inhibitor) (Fig. 8, B and C).

DISCUSSION

Purification of the two allergens studied took place from two different extracts (Table II). However, this is not an essential point, since Dp 42 may be purified from MF by use of gel filtration and hydrophobic chromatography followed by, e.g., copper-chelate chromatography, and Dp X may be obtained in reasonable purity by hydrophobic chromatography of AML (results not shown). The described procedures were the first to lead to purified material in sufficient amounts to allow a further characterization. The rather low recoveries obtained (5% to 10%) are probably due to repeated dialysis of dilute solutions. We have also met considerable difficulty in desorbing all applied Dp 42 from the phenyl Sepharose column. The purification ratios obtained (relative to a well characterized reference material²) indicate that the two major allergens together may constitute up to 20% w/w of a typical house dust mite-allergen extract. The estimate of protein weight per dry matter weight obtained by the Lowry method (with BSA as standard) may indicate the presence of residual salt and water after dialysis and freeze-drying. Pure proteins may, however, exhibit a several fold difference in extinction coefficients with the folin reagent as compared to BSA.⁹

The purified allergens were not completely free from antigenic contaminants as demonstrated by CIE/CRIE and SDS-PAGE. Some caution is therefore necessary when interpreting, e.g., RAST or histamine release reactions to the purified components.

The presence of contaminants was also demonstrated by the specificity of the rabbit antibodies obtained by immunization with Dp 42 and Dp X. One rabbit produced monospecific antibodies to Dp 42,

whereas the second rabbit produced antibodies to a contaminant as well. The presence of several contaminants in the Dp X preparation is deduced from the oligospecificity of the rabbit anti-Dp X antibody. The presence of contaminants in so-called "pure antigens" has been demonstrated^{10, 11} in other cases by such sensitive tests.

Cross inhibition in RAST may indicate some kind of contamination of one major allergen preparation with the other (Fig. 8). Inhibition was, however, only obtained when Dp X inhibited the RAST reaction to Dp 42 discs and not in the opposite system. A slight contamination of the Dp 42 preparation with Dp X could explain this. The lack of inhibition of Dp X RAST with Dp 42, within the range of inhibitor concentrations used, then sets an upper limit for contamination at <0.1% (Fig. 8). Alternatively, the Dp X preparation could be assumed to contain a peptide fragment of Dp 42, unable to couple to the paper but retaining the ability to partially inhibit the RAST reaction.

Some important characteristics were deduced from the fractionation results and the analysis of the purified allergens. Hydrophobic interaction between proteins, or between protein and matrices like phenyl Sepharose, is strongest at high ionic strength in the solvent and at a pH close to pI. As the salting out of a protein depends on hydrophobic interaction, there is, generally, a close relationship between the behavior of a protein in salting-out fractionation and in a hydrophobic chromatography.¹²

The salting-out properties of Dp 42 and Dp X paralleled the behavior on the hydrophobic columns. Dp 42 salted out at a lower ammonium sulphate molarity than Dp X and exhibited a much stronger binding to phenyl Sepharose (elution required low ionic strength and pH >8). Dp 42 probably contains one or more strongly hydrophobic domains. It was possible to ob-

TABLE III. MW and pI data for Dp 42 and Dp X

	Molecular weights		Isoelectric points	
	Sephadex G-75	SDS-PAGE	Column IEF	Crossed IEF
Dp 42	18,000*	25,000	4.6, 5.6, 6.6*	6.9†
	17,000‡	30,000‡		
Dp X	18,000*	18 to 20,000§		5.0, 6.4‡

*Determined by FRIE on the allergen in mixture.

†Purified allergen.

‡Different gel gradient used. See text.

§Obtained on ¹²⁵I-labeled antigen and autoradiography.

tain a close to qualitative separation of the two major allergens on phenyl Sepharose, whereas ammonium sulphate precipitation only allowed partial separation.

The focusing pattern for Dp 42 obtained by sucrose gradient IEF of AML demonstrated a marked heterogeneity of pIs. The focusing pattern obtained for purified Dp 42 by crossed IEF, however, indicated one major pI value for this preparation.

This discrepancy may be attributed to selective purification of the isoelectric variant with pI approximately 6.6, e.g., during the phenyl Sepharose separation. The minor difference between the pI values for this variant obtained by sucrose gradient IEF (6.6) and crossed IEF (6.9) may be due to the less accurate cross-reference between the precipitate top point in the antibody-containing gel and the pH gradient.

Chapman and Platts-Mills^{13,14} were the first authors to describe purification of a major allergen, P₁, from Dp. P₁ is identical to our Dp 42.^{2,15} The spectrum of pI values obtained for P₁ by preparative focusing is almost identical to our results for Dp 42 (major peaks for P₁ at pH 4.7 to 5.4 and pH 6.6 to 7.1 with a small intermediate peak). They also demonstrated a total immunochemical identity between the two main variants, which were also of equal amino acid composition. Because their cultivation medium, extraction conditions, and preliminary purification steps were quite different from ours, this particular pI spectrum is likely to be a quite specific characteristic of Dp 42 alias P₁.

We have observed significant changes in average electrophoretic mobility at pH 8.6 of Dp 42 after periodate treatment at pH 5 without changes in antigenicity to rabbit antibodies (results to be published); therefore, one reason for the variation in pIs may be a different content of charged carbohydrate residues. All three fractions pooled after performing sucrose gradient IEF of AML exhibited binding of IgE in CRIE (although this happened with slightly different intensity of radiostaining).

On the basis of these data, we conclude that the main epitopes for human IgE on Dp 42 are present on the peptide backbone. Certain carbohydrate structures may, however, be imagined to form a "steric hindrance" for attachment of human IgE.

The pI values for purified Dp X, as analyzed by crossed IEF (5.0 and 6.4) are accordant with the scattered distribution of Dp X on sucrose gradient IEF where Dp X occurred in all three pooled fractions.

A distinct difference in the molecular weight of Dp 42 was observed, depending on the technique used (Table III). A gel filtration (G-75) value of 17,000 to 18,000 was obtained when both the complex mixture, MF, or the purified Dp 42 was investigated. In SDS-PAGE, molecular weights in the range 25,000 to 30,000 were obtained, depending on the actual gel gradient used, but were independent of previous reduction with mercaptoethanol to break disulphide linkages. The values obtained by SDS-PAGE are comparable to the 24,000 obtained for P₁ by the same technique.¹⁴ The specific reason for the discrepancy between gel filtration and SDS-PAGE estimates of molecular weight is unknown. An overestimation might take place in SDS-PAGE caused by carbohydrate side chains. Alternatively, the gel filtration value may be an underestimate caused by adherence to the Sephadex matrix. In this context it is interesting that Stewart and Turner¹⁶ determined the molecular weight of their antigen Dpt 12, later demonstrated to be identical to P₁,¹⁷ to be 23,000 by gel filtration on a different matrix, Sephacryl S-300.

Some difficulties were encountered by the determination of the molecular weight for Dp X because this antigen stains poorly with the protein stain. Radiolabeling was necessary to obtain a reliable molecular weight value by SDS-PAGE. The molecular weight estimates by gel filtration and SDS-PAGE agree quite well (Table III).

The allergenic importance of the two purified allergens was assessed preliminarily in the RAST sys-

tem with WMC as the reference. Direct comparison of RAST responses (Fig. 7) elicited large activities, relative to WMC, for both allergens. This is not surprising, since most mite-allergic patients respond to both allergens in CRIE,^{1,2} and the binding of IgE to individual allergens on the WMC disc may be lower than maximal caused by competition for reactive sites at the solid-phase coupling of the complex mixture.

The RAST inhibition experiment demonstrated the allergenic complexity of the reference WMC preparation. Neither of the two purified allergens inhibited the RAST reaction more than half as effectively as the reference preparation itself (Fig. 8, A). The maximum inhibitory capacity cannot, however, be assessed because the saturation levels were not reached.

We have previously defined major allergens of Dp on the basis of their IgE binding frequency in CRIE.¹ Dp 42 and Dp X both exhibited radiostaining with >50% of the patients' sera after 3 days of autoradiography and with equal intensity. The direct RAST activity of the two purified components was in accordance with the declared activity as major allergens. Dp 42 appeared, however, as a more potent RAST inhibitor than Dp X. This may possibly be caused by a relative dominance of IgE to Dp 42 in the particular patient serum pool used for RAST inhibition.

The allergenic activity of Dp 42 and Dp X has been confirmed by histamine release from basophil leukocytes. These results will be presented elsewhere.¹⁸

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