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Immobilized metal ion affinity chromatography for the purification of *Fel d* I, a cat major allergen, from a house-dust extract

J.-P. DANDEU*, J. RABILLON, M.-J. BELTRAND, M. LUX, R. DUVAL and B. DAVID
Unité d'Immuno-Allergie, Institut Pasteur, Paris (France)

ABSTRACT

Although the efficient isolation and purification of the major feline allergen have previously been achieved using polyclonal or monoclonal antibody affinity chromatography, these methods lead to a relatively low yield of pure allergen. Therefore, attempts were made to establish a process involving ion-exchange chromatography followed by immobilized metal ion affinity chromatography. Although slightly more time consuming than the immunological methods, it gives a higher yield. It consists of four steps. From a crude house-dust extract, precipitation of a *Fel d* I enriched fraction is obtained by addition of acetone. After dissolution in water, exhaustive dialysis takes place against a citric acid solution to eliminate divalent metal ions. This gives the whole house-dust extract. Then partial isolation and purification are obtained by ammonium sulfate precipitation. The resulting precipitate is dissolved in and dialysed against water to remove ammonium salts. This partially purified house-dust extract can be submitted to chromatography on an anion exchanger and then on a copper ion charged chelating gel. All steps of the isolation and purification were controlled by immunochemical analyses using a crossed immuno-electrophoretic method and enzyme-linked immunoadsorbent assays. The HD75 Q2 Cu2 fraction so obtained can be considered to be a pure cat major allergen *Fel d* I preparation. Its homogeneity was demonstrated using physico-chemical and immunochemical methods.

INTRODUCTION

Allergens are immunogenic and antigenic molecules, generally proteins or glycoproteins, which give rise to an immunoglobulin E (IgE) antibody response in genetically predisposed humans. Most of these allergens have a very high biological potency and an apparent molecular weight (MW) between 10 000 and 50 000 daltons.

The isolation and purification of allergens, whatever their origin, house-dust mites, pollens, moulds or foods, are currently performed in many laboratories. The availability of a pure allergen from any source and a knowledge of its amino acid composition and sequencing of the protein part can be useful for the study of hyper- and hyposensitization processes in animal models and in man.

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Some allergens derived from the domestic cat (*Felis domesticus*) frequently cause severe allergic reactions. One of these allergens is found in the saliva and can be transferred to the cat pelt by licking. It can be considered as the major cause of these reactions, designated Cat 1 allergen, and more recently *Fel d* I, first described by Leitermann and Ohman[1]. It has a native MW of *ca.* 35 000 daltons[1,2] and is composed of two chains which can be easily dissociated as they are not covalently linked. *Fel d* I has been defined as a homodimer, each monomer (17 000 daltons) having an equivalent allergenic and antigenic potency[1,2].

The presence of cat allergens in house dust has been shown by Ohman and co-workers[1,3]. To extract all soluble and allergenic components from the house dust, harvested during house vacuum cleaning, we followed the method originally described by Guibert and Causse-Combes[4].

Fel d I was first isolated by biochemical processes with some success but immunochemical methods of purification with immunosorbents, prepared with either polyclonal or monoclonal antibodies, were more efficient, although they gave a poor yield[1,2]. The biochemical method described here leads not only to a higher yield but could easily be scaled up for various purposes.

EXPERIMENTAL

Whole house-dust extract (WHDE)

The whole extract from a pool of house dusts harvested with a vacuum cleaner was essentially prepared according to Guibert and Causse-Combes[4]. Briefly, it consists of two steps, an aqueous extraction followed by two acetone precipitations at 25% and 75%, the last precipitate being termed the WHDE (Scheme 1).

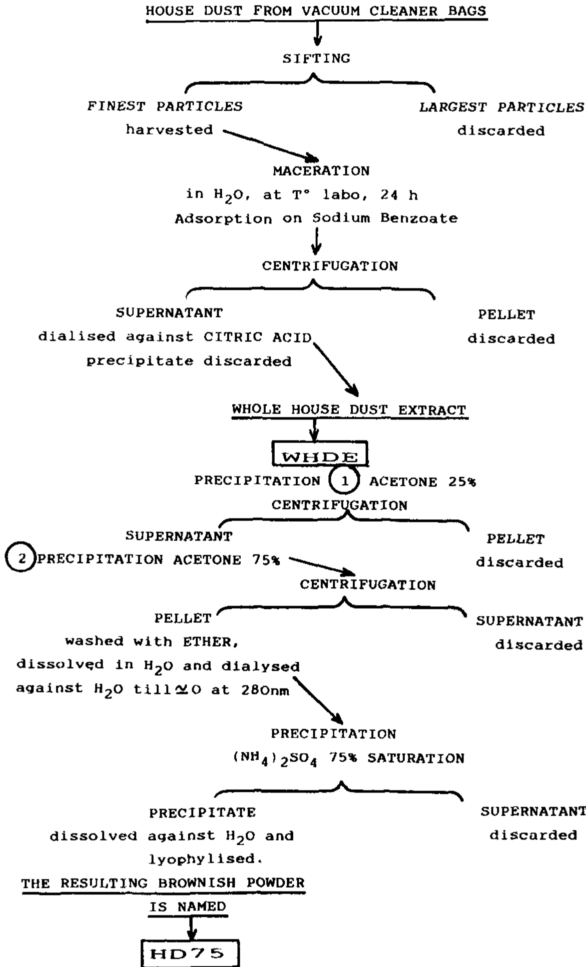
Partially purified extract from house dust

From the whole house-dust extract, a partially purified extract was obtained by precipitation at 75% ammonium sulphate saturation in order to concentrate medium- and high-molecular-weight proteins and leave small molecules in the supernatant. After solubilization of the resulting precipitate, a dialysis against distilled water was performed to eliminate ammonium sulphate and other coprecipitated and dialysable molecules, using a dialysis bag with a 6000–8000-dalton MW cut-off. The resulting brownish solution was named HD75 and lyophilized (Scheme 1).

Fractionation methods

Ion-exchange chromatography. As preliminary experiments showed that the majority of HD75 antigenic components were negatively charged and *Fel d* I was shown to have a p*I* 3.8, an anion exchanger was used. A Mono Q HR 10/10 column (Pharmacia, Uppsala, Sweden) controlled by a fast protein liquid chromatographic (FPLC) system (Pharmacia) was loaded after equilibration with 0.02 *M* Tris–HCl buffer (pH 8.6)–0.125 *M* sodium chloride with a batch dissolved in the same buffer. A stepwise elution was performed; an isocratic run with the equilibration buffer was carried out followed by a second and a third step with the same buffer containing 1 and 2 *M* sodium chloride, respectively.

Immobilized metal ion affinity chromatography. Chelating Sepharose Fast Flow (Pharmacia) was used to pack an empty HR 10/10 column after being washed



Scheme 1.

according to the manufacturer. It was charged, at saturation, with Cu^{2+} ions from aqueous copper(II) chloride solution (5 mg/ml). Unbound copper ions were washed out by a run with water. Before use, a blank run at a flow-rate of 3 ml/min was performed. The process was as follows: we began with a 15-min run with the equilibration buffer (solution A, 1 *M* sodium chloride in 0.02 *M* sodium phosphate buffer, pH 7.0), then a short 5-min linear gradient of molarity was applied to reach 100% B (solution B, 1 *M* ammonium chloride in 1 *M* sodium chloride–0.02 *M* sodium phosphate buffer, pH 7.0). A 15-min plateau at this concentration of B was followed by a decreasing linear gradient (5 min) to 0% B, then after a further run of 15-min with A the column was ready for use. The fractionation process was controlled by an FPLC

system, UV detection was done at 280 nm and events were recorded on a three-way recorder. A pre- and a post-column packed with the chelating gel without metal ions were inserted in the system to remove any free metal ions that might interfer with the chelating process; the columns used were of the HR 5/5 type.

Immunoelectrophoresis

To follow the purification steps of the cat major allergen *Fel d* I, crossed immunoelectrophoresis (CIE) methods were used as described by Axelsen *et al.*[5] and a rocket line experiment (RLIE) as described by Rabillon and co-workers[6,7] using a hyperimmunized rabbit sera pool against the partially purified house-dust extract HD75. The immunization process was as described by Le Mao *et al.*[8]. Each fraction obtained by chromatographic techniques was tested in a crossed-line rocket immunoelectrophoresis (CLIE) where the intermediate gel is lengthened on the right-hand part of the CLIE, allowing us to perform an RLIE leading to the easy identification of each antigen.

Allergenicity of these antigens can be previously challenged, before Coomassie blue staining, in applying the crossed radioimmunoelectrophoresis process described by Weeke and Lowenstein[9], using a cat-sensitized patient sera pool.

Enzyme-linked immunosorbent assay (ELISA)

To specifically detect the presence of the major cat allergen *Fel d* I in the different fractions obtained, ELISAs were performed using monoclonal antibodies (anti *Fel d* I), Mab 6F9 and biotinylated Mab 3E4 from Charlottesville University (VA, U.S.A.), Martin Chapman Laboratory and the two-site immunoassay described by Chapman *et al.*[2].

A CEB Immunoplate (Centre Européen de Biotechnologie, France) was coated with 1 μg per well of Mab 6F9 (anti *Fel d* I) in 0.1 *M* hydrogen carbonate buffer (pH 9.6) overnight at 4°C. The plate was then washed twice with phosphate-buffered saline (PBS) (pH 7.4), containing 1% Tween 20 and treated for 1 h with 100 μl per well of a bovine serum albumin (BSA) solution at 1% in the same buffer.

The wells were then incubated for 1 h at room temperature with 100 μl of diluted fractions (from 10^2 to 10^7), using double dilutions of a reference *Fel d* I fraction (Q2 Cu2, 10–0.04 μg/ml of PBS) to obtain a calibration graph.

After washing five times, the wells were incubated for 1 h at room temperature with 100 μl of a 10 μg/ml solution of biotinylated Mab 3E4 (anti *Fel d* I). The plate was then washed a further five times and the wells were incubated for 30 min with 1/1000 streptavidin–peroxidase (Sigma S-5512, 0.25 mg of protein reconstituted in 1 ml of distilled water).

Finally, the assays were developed by adding 100 μl per well of 1 m*M* 2,2′-azino-di(3-ethylbenzthiazoline sulphonate) (ABTS)–5% hydrogen peroxide in 70 m*M* citrate–phosphate buffer (pH 4.2) (ABTS Sigma A-1888). The reaction was stopped after 10 min by adding 100 μl per well of 2 m*M* sodium azide solution. The absorbance was read at 414 nm in an ELISA microplate reader.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a PHAST apparatus (Pharmacia) according to the procedure described by the manufacturer; after electrophoretis silver staining was used.

Gel filtration

A 50 cm × 1.6 cm I.D. column was packed with Superose 12 by the Chromatography Department of Pharmacia. The column was used after connection to an FPLC system with two pumps providing precise and accurate flow at relatively low back-pressure. It was equilibrated with 0.02 *M* Tris–HCl buffer (pH 8.6) containing 1 *M* sodium chloride to avoid ionic interactions. Buffer and protein samples were filtered through 0.45-μm membranes in order to eliminate foreign particles and dissolved gas.

Calibration of the column using protein molecules of known molecular weight was performed. To control the exclusion limit, Blue Dextran 2000 (MW $2 \cdot 10^6$ daltons) was added to the following mixture: ferritin, MW = $4.4 \cdot 10^5$, bovine γ-globulin, MW = $1.6 \cdot 10^5$, bovine serum albumin, MW = $6.9 \cdot 10^4$, α-chymotrypsin, MW = $2.35 \cdot 10^4$, egg lysozyme, MW = $1.75 \cdot 10^4$, bovine approtinin, MW = $6.5 \cdot 10^3$, and mellitin from honey bee venom, MW = $2.5 \cdot 10^3$ daltons. A 500-μl volume of the mixture was loaded onto the column.

RESULTS

Immunochemical analysis of HD75 by CIE

Homologous reactions between HD75 and the anti HD75 rabbit sera pool revealed at least eight antigens, the allergenicity of wich was demonstrated by CRIE using a cat-sensitized human patient sera pool. The results of these experiments are shown in Fig. 1a and b. In these experiments (Fig. 1a and b) a very marked uptake of radioactivity on the precipitin line corresponding to the antigens numbered 1, 2, 5 and 8 was obtained with cat-specific human IgE.

Identification of *Fel d* I among the HD75 antigens and allergens and discrimination from cat albumin, a potent allergen, were performed using a monoclonal immunosorbent-purified *Fel d* I and cat albumin purified by gel filtration from a whole cat serum, in one CIE and two CLIE, Fig. 1c, d and e.

Fractionation of HD75

Anion-exchange chromatography. This was carried out on a Mono Q HR 10/10 column equilibrated with 0.02 *M* Tris–HCl buffer (pH 8.6)–0.125 *M* sodium chloride. A sample of lyophilized HD75 (100 mg) previously dissolved in the same buffer was loaded onto the column. A stepwise elution was performed, beginning with an isocratic run during which one peak was eluted (Q1), and followed by elution with 1 *M* sodium chloride of almost all the remaining components of the extract (Q2). Finally, the column was eluted with 2 *M* sodium chloride, giving a third peak (Q3), essentially composed of pigmented material (Fig. 2). Immunochemical analysis using the rabbit sera pool against HD75 in CLIE and RLIE showed that only fraction Q2 contained *Fel d* I in a partially purified form (Fig. 4).

Application of a linear gradient of molarity between 0 and 1 *M* sodium chloride did not give a better purification but it allowed us to define the correct concentration, *i.e.*, 0.125 *M*, in the first run leading to the elution of the house-dust component of interest.

Copper chelate Sepharose chromatography. A 50-mg amount of the HD75 Q2 fraction from the Mono Q column dialysed against water and then lyophilized was

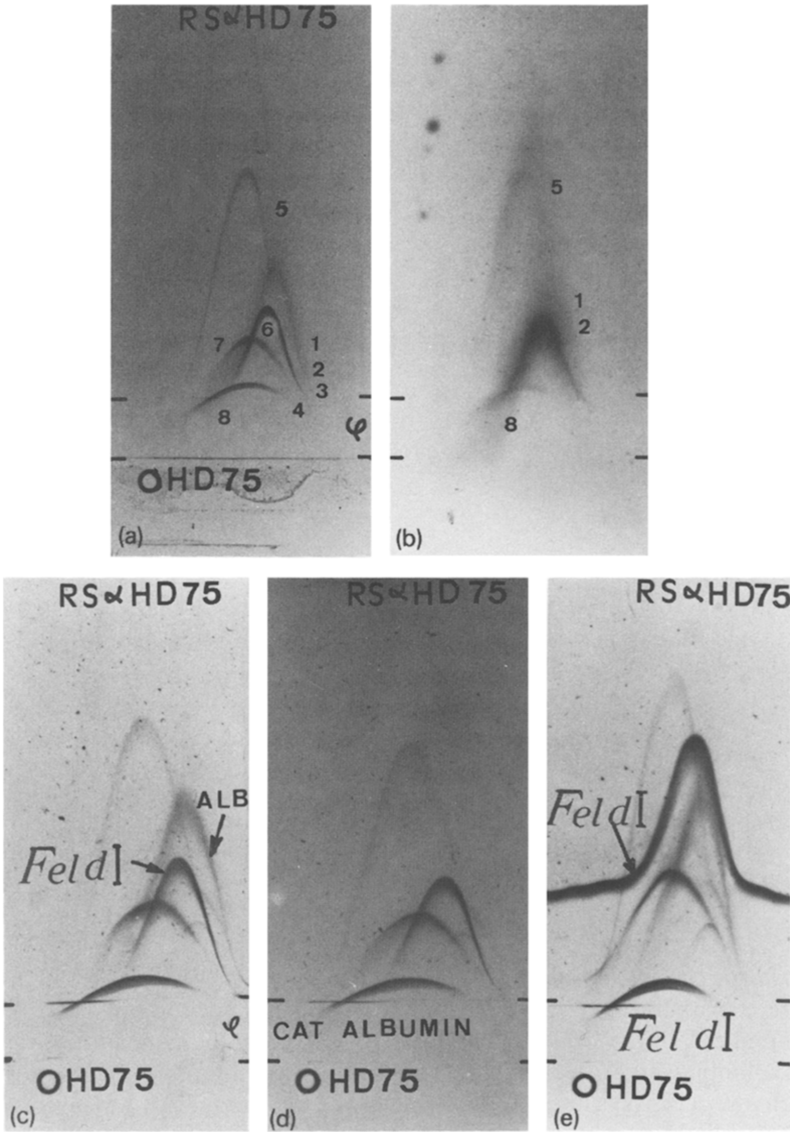


Fig. 1. Immunochemical analysis of HD75 by CIE, CRIE and CLIE. (a and b) CIE and CRIE where HD75 reacts with homologous rabbit antibodies, allergens are revealed by human IgE from a pool of cat-sensitized human sera. Autoradiography. (c, d and e) CIE and CLIE where cat albumin and *Fel d* I are identified by using their respective standards.

dissolved in 500 μl of the equilibration buffer and loaded onto the copper column prepared as described under Experimental.

The following elution programme was controlled by the FPLC system. The first step was a 60-min isocratic run with solution A in order to remove unbound material and to desorb proteins weakly bound to the immobilized copper ions. The second step

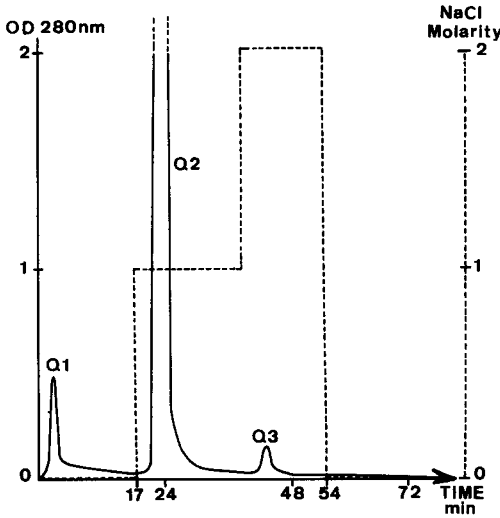


Fig. 2. Chromatogram of HD75 on a Mono Q HR 10/10 column equilibrated with 0.125 *M* NaCl in 0.02 *M* Tris–HCl buffer (pH 8.6) (solution A). Elution is carried out with 1 *M* NaCl in 0.02 *M* Tris–HCl buffer (pH 8.6) (solution B). Flow-rate, 1 ml/min. UV detection at 280 nm.

was a 30-min run using 50% solution B and the third a 30-min run at 100% solution B to desorb strongly bound protein molecules. All these runs were performed at a flow-rate of 0.5 ml/min. Finally, a 30-min run at a flow-rate of 3 ml/min with an injection of 20 ml of a 50 m*M* EDTA solution in solution A was carried out to elute all the material for which all other attempts had failed. The chelating Sepharose elution profile is shown in Fig. 3. Five peaks were obtained, and the last one, Cu5, was essentially composed of brown pigments. The other four fractions, Cu1, Cu2, Cu3 and Cu4, were immunochemically tested in the same system as used above and the results

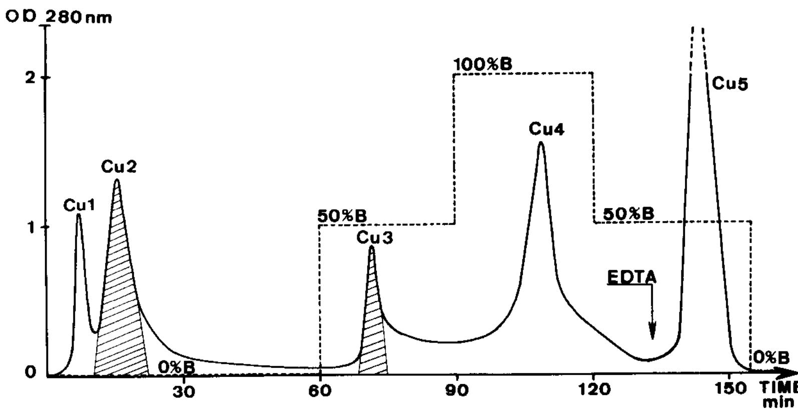


Fig. 3. Chromatogram of HD75 Q2 on a chelating Sepharose fast flow HR 10/10 column, charged with Cu^{2+} ions equilibrated with 1 *M* NaCl in 0.02 *M* sodium phosphate buffer (pH 7.0) (solution A). Elution is carried out with 1 *M* ammonium chloride in solution A (solution B). Flow-rate, 0.5 ml/min. UV detection at 280 nm.

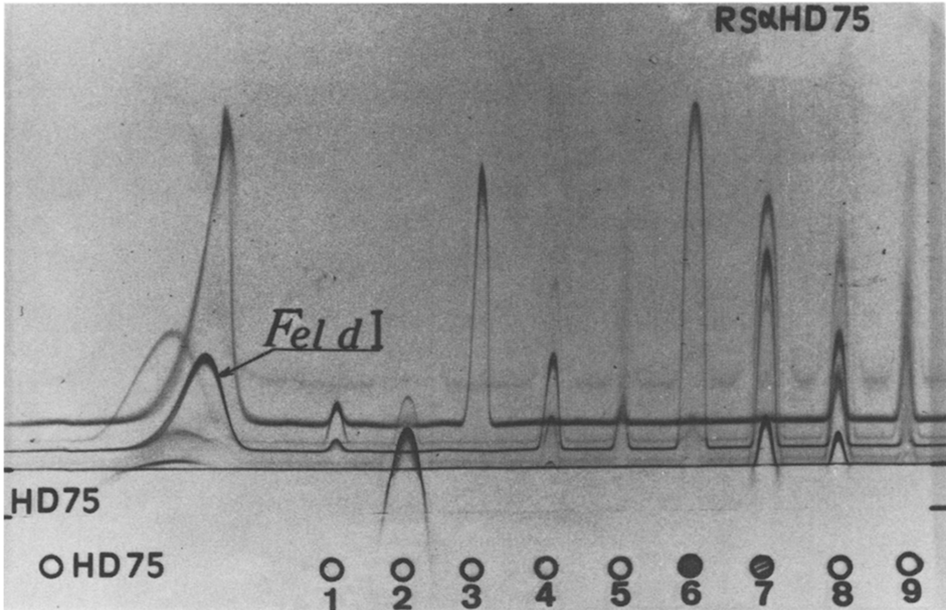


Fig. 4. Immunochemical analysis of chromatographic fractions by CLIE and RLIE using rabbit antibodies against HD75. Only HD75 is submitted to the first-zone electrophoresis. HD75 is present in the whole intermediate gel. Each fraction is tested in a contiguous RLIE. 1 = Cat saliva; 2 = HD75 Q1; 3 = cat albumin; 4 = HD75 Q2; 5 = Q2 Cu1; 6 = Q2 Cu2; 7 = Q2 Cu3; 8 = Q2 Cu4; 9 = Q2 Cu5.

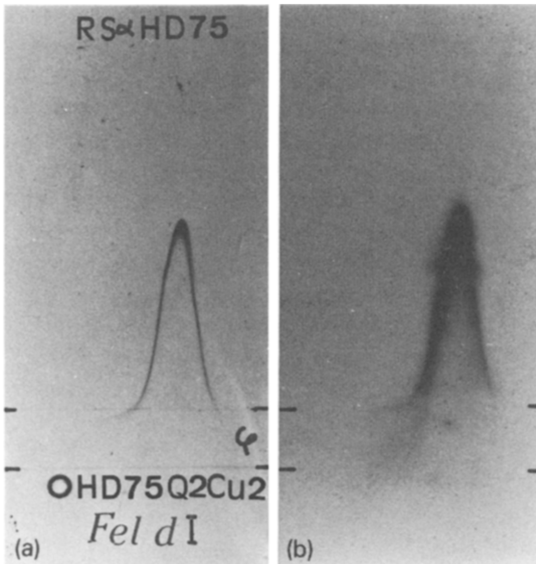


Fig. 5. CIE and CRIE where pure *Fel d* I (Q2 Cu2) reacts with rabbit antibodies against HD75. Allergenic activity is revealed by specific human IgE. Autoradiography.

are shown in Fig. 4. Fraction Q2 Cu2 appears as an immunologically pure allergen *Fel d* I and more particularly when tested in CIE, its allergenic activity being challenged by CRIE using a pool of cat-sensitized patient sera (Fig. 5a and b). Q2 Cu3 does contain *Fel d* I but a lower concentration and rather contaminated.

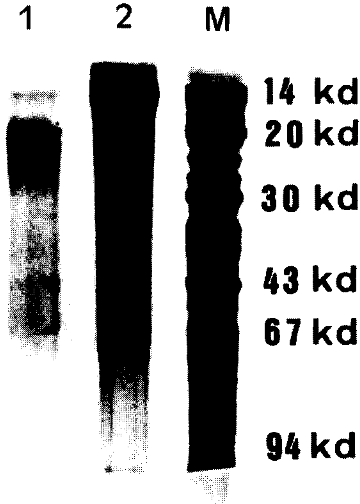


Fig. 6. Physico-chemical analysis o *Fel d* I enriched fractions. SDS-PAGE performed in a PHAST system (Pharmacia) according to the manufacturer. Phast gel gradient 8–25. A 1-μl sample of each native proteinic fraction is deposited, corresponding to 10 μg dry weight. The gel was silver stained. M = protein markers of known molecular weight; 1 = Q2 Cu2; 2 = Q2 Cu3. kd = kilodaltons.

SDS-PAGE

An SDS-PAGE experiment performed under non-dissociating conditions showed that the fraction Q2 Cu2 gives a homogeneous band but a minor one around 14 000 daltons and a second band, more diffuse, between 17 000 and 20 000 daltons. Fraction Q2 Cu3 gives a highly stained and very homogeneous band at 14 000 daltons (Fig. 6).

Gel filtration

In a gel filtration experiment performed on a Superose 12 column as described under Experimental, we showed that the Q2 Cu2 fraction appears as a peak with one shoulder, the main peak corresponds to a molecular species with MW = 14 000 daltons and the first-eluted shoulder to one with MW = 30 000 daltons. The Q2 Cu3 fraction is eluted from the column as a homogeneous peak essentially composed of three proteinic molecules with MW of 20 000, 30 000 and 50 000 daltons. All these results are shown in Fig. 7a and b.

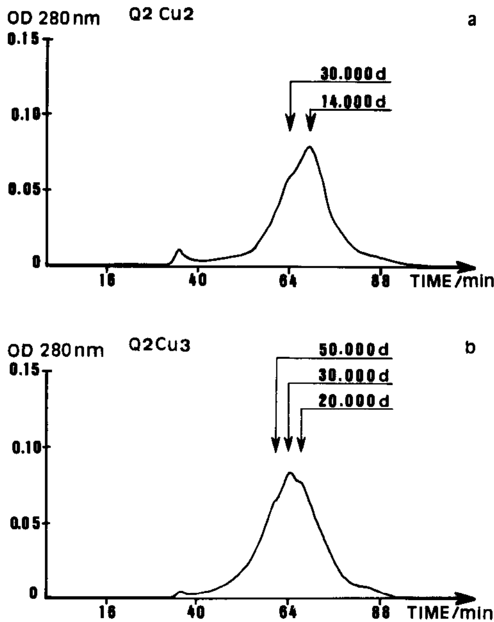


Fig. 7. Gel filtration experiments performed on a Superose 12 HR 16/50 column, equilibrated in 1 *M* NaCl–0.02 *M* Tris–HCl buffer (pH 8.6). Flow-rate, 1 ml/min. UV detection at 280 nm. (a) Q2 Cu2 analysis; (b) Q2 Cu3 analysis. d = daltons.

Increase in purity over the purification process

This was assayed using ELISA (a two-site assay) with two different specificity monoclonal antibodies against *Fel d* I: Mab 6F9 for coating of the plate wells and the biotinylated Mab 3E4. Fraction Q2 Cu2, since it was demonstrated by all other methods to be pure *Fel d* I, was used as a standard reference to challenge the presence of *Fel d* I allergen in every fraction obtained during its purification process. The results are shown in Table I.

House dust (kindly provided by the Allergens Department, Institut Pasteur) contains 8 μg/g of *Fel d* I as tested in the first aqueous extract, WHDE. Thus, from

TABLE I

CONCENTRATION OF *Fel d* I AS DETERMINED BY A TWO-SITE IMMUNOASSAY (ELISA)

Fraction	*Fel d I (%)*
WHDE	0.8
HD75	8.0
HD75 Q2	75.0
Q2 Cu1	17.0
Q2 Cu2	100.0
Q2 Cu3	62.0
Q2 Cu4	21.0
Q2 Cu5	4.2

35 kg of house dust containing *ca.* 300 mg of active *Fel d* I molecules only 10% were obtained in a pure and homogeneous form as Q2 Cu2 fraction.

DISCUSSION

In order to purify the cat major allergen *Fel d* I from house dust, we developed a procedure combining two chromatographic techniques. The first is a stepwise elution process which gives three fractions from HD75, the partially purified house dust extract when it has been loaded on an anion-exchange column. Of these fractions only the second, Q2, contained *Fel d* I allergen as assayed by RLIE and ELISA (a two-site immunoassay)[2]. Thus for further purification, immobilized copper ion affinity chromatography was used.

Immobilized metal ion affinity chromatography (IMAC) is certainly one of the most powerful methods available for fractionating protein mixtures. First described about 15 years ago by Porath *et al.*[10], is is not currently used. A knowledge of the protein to be isolated is generally necessary as binding of protein(s) to divalent metal ions depends on the presence of certain amino acid residues, whereas for ion-exchange chromatography and gel filtration only charge and/or size characteristics have to be known. Amino acids form stable chelates with metal ions[11], but many workers have demonstrated that exposed imidazole and thiol groups on the protein molecules are the most important binding sites for copper-containing adsorbents[12]. Therefore, peptides containing histidine and/or cysteine residues bind more stably with the immobilized copper ions. Nevertheless, arginine residues can also bind metal ions via their guanidinium group[11].

From the amino acid composition of *Fel d* I published by other workers[2], we could only presume a relatively weak affinity of this allergenic protein for copper ions. Cysteine was not determined and histidine was totally absent. However, it could be useful in any case to challenge divalent metal ions bound to any available support in order to choose either a biochemical or an immunochemical method of purification for the protein of interest. Preliminary experiments led us to use a chelating Sepharose fast flow column, charged with copper ions. A four-step elution process was performed. All buffers contained 1 *M* sodium chloride which increases the adsorption capacity of the solid phase as shown by Porath and Olin[13]. Ammonium, considered to be a competitive ligand, was used (as ammoniom chloride) to achieve displacement of the highly adsorbed proteinic molecules.

From the *Fel d* I enriched fraction Q2, among the five fractions so obtained, only two contain *Fel d* I: Q2 Cu2, a highly pure monomeric form of *Fel d* I, with MW = 17 000 daltons and slightly contaminated by a proteinic component with MW = 14 000 daltons, and Q2 Cu3, which shows a higher affinity for copper ions and contains cat major allergen contaminated by a proteinic component with MW = 14 000 daltons.

In a gel filtration experiment, pure *Fel d* I as present in Q2 Cu2 seemed to undergo a dimerization as described previously[1,2]. In this homodimer, monomers are not engaged in covalent interactions and can be easily dissociated in the SDS-PAGE experiment without reduction. Aggregation could be evoked to explain the behaviour of Q2 Cu3 in a similar gel filtration process.

Our results are consistent with those obtained by other workers[1,2], who stated

that *Fel d* I exists as two molecular species a monomer of MW *ca.* 17 000–19 000 daltons and an homodimer of MW 35 000–39 000 daltons.

It must be emphasized that our process for purification from the crude house-dust extract is fairly drastic and could lead to some denaturation of the allergenic molecules. Nevertheless, antigenic and allergenic activities are totally preserved. Allergenic activity was challenged by CRIE and skin tests.

In conclusion, the results confirm IMAC to be a powerful tool not only for protein purification but also for the study of their native or post-synthetic molecular structure. In the case of allergen purification we can conclude that combination of anion-exchange chromatography and IMAC provides a very efficient biochemical process which may certainly benefit from subsequent scale-up.

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