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Hydrophobic interaction chromatography for isolation and purification of *Equ.c1*, the horse major allergen

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ABSTRACT

Equ.c1, the horse (*Equus caballus*) major allergen, was identified in a partially purified extract obtained from a crude aqueous horse dander extract, by acetonic precipitation and a salting-out process. It was isolated and purified by size-exclusion chromatography followed by hydrophobic interaction chromatography. *Equ.c1* appeared as an almost pure protein in a fraction eluted at 1.2 *M* ammonium sulphate from a phenyl Superose column. It is a single peptide with a relative molecular mass of 20 000 and a p*I* of *ca.* 3.9.

INTRODUCTION

Allergens are immunogenic and antigenic molecules that provoke a hypersensitivity response mediated by immunoglobulins E, in genetically predisposed animal or human individuals. These molecules have a various origins. Most of the allergens originating from animal danders are generally proteins or glycoproteins. The relative molecular masses of these molecules are generally rather low, between 10 000 and 50 000. It is common that the same raw material obtained from animal danders contains several different allergens. The major allergen is considered to be the one that provokes an anaphylactic reaction in a majority of individuals presenting an immediate hypersensitivity reaction against the basic raw material.

Isolated and purified allergens are essential for a fundamental study of the molecular interactions involved in the hypersensitivity reaction, as well as for clinical diagnosis and therapy.

Stanworth [1] used zone electrophoresis and clinical puncture tests to demonstrate the presence of several allergenic molecules in horse hair and dandruff. Early studies concerning the allergens originating from horse hair and danders were performed by Ponterius *et al.* [2]. Löwenstein *et al.* [3] confirmed the presence of different allergens in the same material and partially isolated some of them, using quantitative immunoelectrophoresis and ion-exchange chromatography. A more recent study was done by Franke *et al.* [4] on different commercially available raw materials currently used for clinical testing of "horse" allergic individuals. They clearly showed that the amount of allergens present depends on the origin of the raw material. The majority of the allergens identified had relative molecular masses between 20 000 and 30 000, and p*I* values of *ca.* 3.9.

In our study, we used the most natural way to obtain the raw material, namely by brushing healthy animals. Using a relatively simple physicochemical and biochemical method, based on a salting-out process and hydrophobic interaction chromatography (HIC), we isolated, purified and

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characterized the horse major allergen. It was named *Equ.c1*, according to the new allergen nomenclature. The procedure described here allows a high yield of chemically and immunologucally pure *Equ.c1* to be obtained.

EXPERIMENTAL

Partially purified horse danders extract (HHD75)

This extract was prepared from the crude horse danders extract obtained according to the method described previously for house dust [5]. $(NH_4)_2SO_4$ was the salt used in the salting-out process performed here. All the allergenic proteins present in the crude extract were precipitated at 75% saturation. The precipitate was dissolved and exhaustively dialysed against water; the resulting brownish solution, named HHD75, was lyophilized and stored.

Size-exclusion chromatography

A Superose 12 HR 16/50 column, 500 × 16 mm I.D. (Pharmacia, Uppsala, Sweden), controlled by a fast liquid chromatographic system (FPLC, Pharmacia) was equilibrated with 0.02 *M* phosphate buffer (pH 8.00) containing 1 *M* NaCl. The mean particle size was 10 μm. For analytical purposes, a 10-mg sample of HHD75 was dissolved in the same buffer (100 μl) and loaded onto the column. An isocratic run was performed with the equilibration buffer at a flow-rate of 1 ml/min. For the preparative process, a 200-mg sample dissolved in the equilibration buffer was applied to the same column and eluted in the same manner. In both cases the absorbance was monitored at 280 nm.

Hydrophobic interaction chromatography

A phenyl Superose HR 5/5 column, 50 × 5 mm I.D. (Pharmacia) was used after being equilibrated with 0.02 *M* phosphate buffer (pH 8.00) containing 2 *M* $(NH_4)_2SO_4$. The mean particle size was 10 μm. The protein fraction of interest was dissolved in the same buffer and loaded onto the column.

Analytical procedure. After an isocratic step with solution A, *i.e.* the equilibration buffer, a linear decreasing concentration gradient of $(NH_4)_2SO_4$ was applied. This was followed by a plateau at 100% B, namely a 0.02 *M* phosphate buffer (pH 8.00).

Preparative procedure. Stepwise elution was performed: an isocratic run was applied with the equilibration buffer, followed by a second step with a 1.2 *M* $(NH_4)_2SO_4$ solution in the same phosphate buffer. The final two elution steps were carried out with a 0.8 *M* $(NH_4)_2SO_4$ solution and with the phosphate buffer alone, respectively.

For both the analytical and preparative procedures, elution was performed at a flow-rate of 0.5 ml and the absorbance was monitored at 280 nm.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Analyses were performed using homogenous 12% gels on a Novex apparatus (San Diego, CA, USA). Before electrophoresis, samples were treated either with SDS or with SDS and 50 m*M* dithiothreitol (DTT) as reducer and then alkylated. After electrophoresis, Coomassie brilliant blue and silver nitrate stains were applied.

Isoelectric focusing

Analyses were performed on Novex gels (pH 3–7) with a Novex apparatus.

Crossed immunoelectrophoretic methods

To follow the purification of the antigens/allergens, crossed immunoelectrophoresis (CIE) methods were used as previously described [6]. Rabbit IgG antibodies raised against HHD75 were used, and each chromatographic fraction was tested by crossed-line rocket immunoelectrophoresis (CLRIE). The intermediate gel of a crossed-line immunoelectrophoresis (CLIE) is lengthened on the right-hand side, allowing a rocket-line experiment to be performed. All these methods lead to an easy identification of each antigen.

IDALI test [7]

To study individual sensitization to the different allergenic constituents of an allergenic extract, the IDALI test (immuno-detection of allergenic compounds for individual patients: in French, Immuno-Detection des ALlergènes Individuels) was performed as follows. Crossed immunoelectrophoresis was carried out with HHD75 rabbit IgG and with the partially purified extract HHD75 previously submitted to electrophoresis in the first dimension. A CLIE, the right-hand side of which is lengthened by line immunoelectrophoresis (LIE), was performed under the conditions described for the CIE. The intermediate gel contained HHD75. After electrophoresis, narrow strips were cut off and each of them was incubated overnight with 50 μl of a horse-sensitized human patient serum, at room temperature. After incubation, the strips were washed three times in saline. The strips were incubated overnight with 50 μl of ^{125}I-labelled rabbit IgG anti-IgE (Pharmacia). After being washed three times in saline with 1% BSA and dried, they were submitted to autoradiography for 48 h at −70°C in a cassette. An intensifier screen was used as described previously for crossed radioimmunoelectrophoresis [6]. Results could be quantified by using a densitometer.

RESULTS

Immunochemical study

Before attempting to isolate and purify any major allergen from the available partially purified extract HHD75, we performed an immunological study of this extract. CIE and CRIE were carried out, and results are shown on Fig. 1. Four main antigens may be identified (Fig. 1a) and defined as being allergenic (Fig. 1b), because they bind specific IgE from a human horse-sensitive patients sera pool, as revealed by autoradiography.

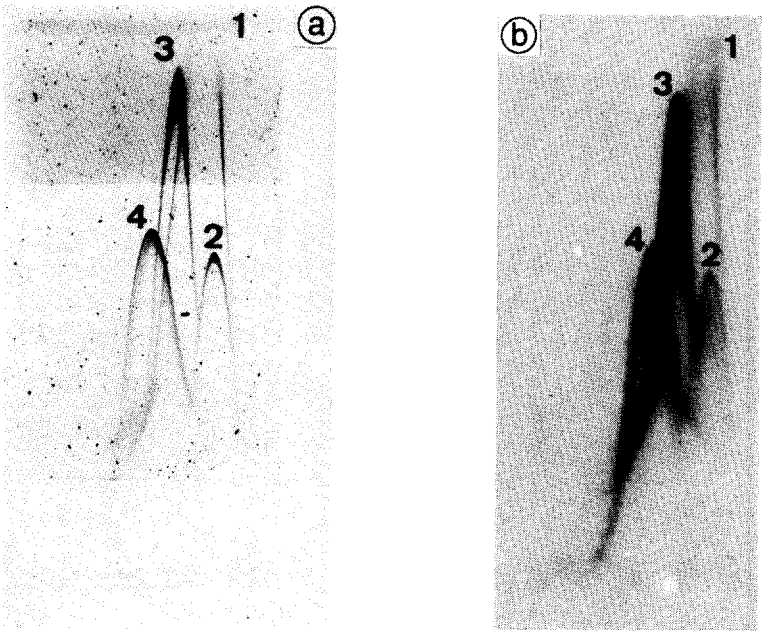

Fig. 1. Immunological analysis of HHD75 by CIE (a) and CRIE (b), after reaction of HHD75 with homologues rabbit antibodies. In CRIE the allergens are revealed by human IgE from a pool of horse-sensitized human sera. In CIE, the immunoprecipitates are revealed by Coomassie blue staining. In CRIE, the immunoprecipitates are revealed in two steps: first, they react with human IgE from a pool of allergic patients' sera (sensitive to horse allergens), and secondly, human IgE bound to these immunoprecipitates are revealed by rabbit ^{125}IgG anti-human IgE (autoradiography).

Using IDALI, one of these antigens has been defined as the horse major allergen. As shown in Fig. 2b, there is no reaction from either a control serum from a non-allergic patient, (strip 22) or a patient sensitive to a different allergen, pollen from *Dactylis glomerata* (strip 23). Strip 24 corresponds to the saline used for washing. In the group of 21 horse-sensitive patients, radiolabelling greatly differs for each of the four previously defined allergenic constituents. In almost every

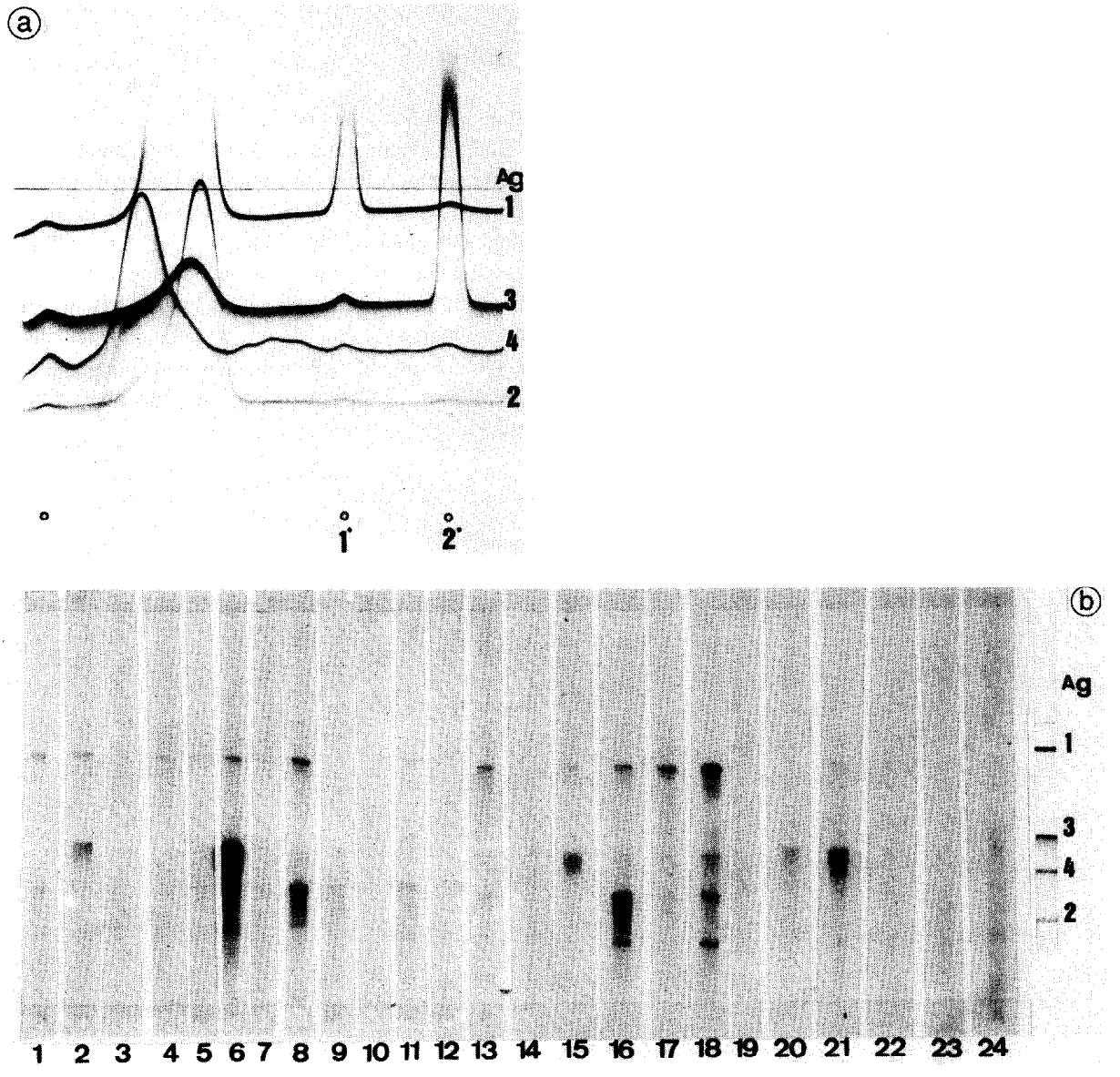

Fig. 2. (a) CLRIE: HHD75 reacts with homologous rabbit antibodies; it is also present in the intermediate gel. The first rocket corresponds to the purified Ag1 (*Equ.c1*), and the second to horse albumin. (b) IDALI: Autoradiography of 24 strips treated as described in Experimental. The first 21 strips correspond to the serum samples from different human patients referred in the text. The last three strips correspond to a non-allergic patient serum, a pollen-sensitive patient serum, and the saline used for washing. On the right-hand side of the figure, a strip stained with Coomassie blue is shown for a clear identification of each line. Antigens are numbered as in on Fig. 1a.

case, the highest intensity is observed for Ag1 (16/21 = 76%). This result indicates that Ag1 may be the horse major allergen. Note that only 5/21 (23.8%) react with Ag2, 7/21 (33.3%) with Ag3 (horse albumin) and 7/21 (33.3%) with Ag4.

Size-exclusion chromatography

The partially purified extract HHD75 was analysed by SEC on a Superose 12 HR 16/50 column on which a 10-mg sample was loaded. Isocratic elution with the equilibration buffer gave the results shown in Fig. 3. The first peak was eluted with the dead volume of the column and is composed of high-molecular-mass substances, among which none of the antigens of interest could be identified. A second major peak was eluted surrounded by three shoulders insufficiently separated from each other to lead to a useful fractionation. Moreover, the antigens/allergens desired were not separated, as shown by CLRIE (results not shown). Nevertheless, this experiment was scaled up to eliminate all the high-molecular-mass components present in HHD75 and representing 40% of the whole material. The resulting fraction was named HHD75 SII.

Hydrophobic interaction chromatography

After several unsuccessful attempts to isolate and purify the horse major allergen using classical chromatographic techniques on anion-exchange suppports or by immobilized metal-affinity chromatography (IMAC), we tried HIC. HHD75 SII is only partially soluble in a 2 *M* $(NH_4)_2SO_4$ solution (corresponding to a 50% ammonium sulphate saturation). The solution is cleared by centrifugation. The supernatant SII-IS50 and the pellet SIIP50, after being dissolved, were exhaustively dialysed against water till no reaction took place with the Nessler reagent (potassium tetraiodomercurate(II)). Both of these fractions were analysed using immunochemical methods. SIIS50 contains the horse major allergen.

Analytical HIC. A 10-mg amount of SII was

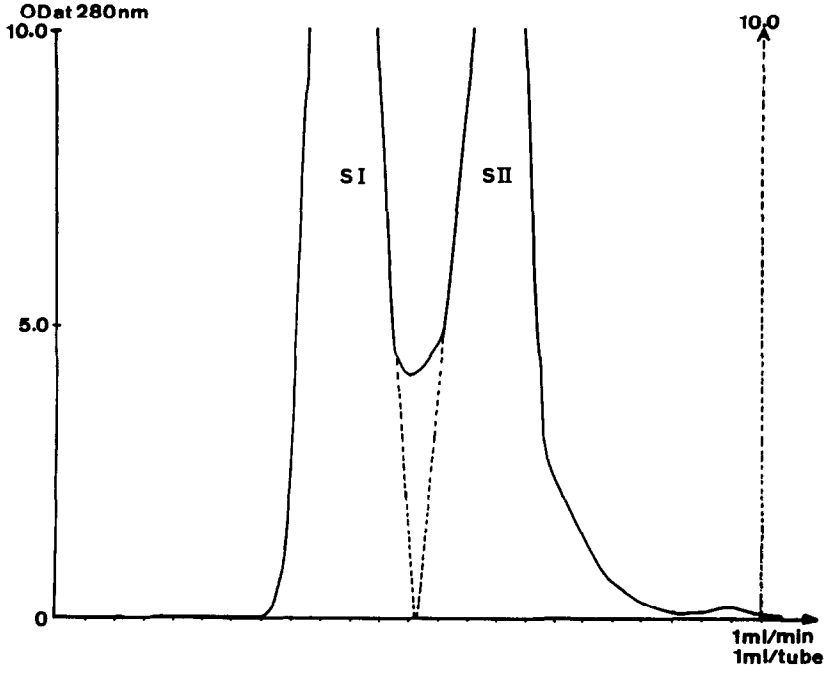

Fig. 3. Preparative SEC performed on a Superose column HR 16/50. Equilibration and elution were performed with the same buffered salt solution, 1 *M* NaCl and 0.02 *M* phosphate buffer (pH 8.00), and 200 mg of HHD75 were loaded onto the column.

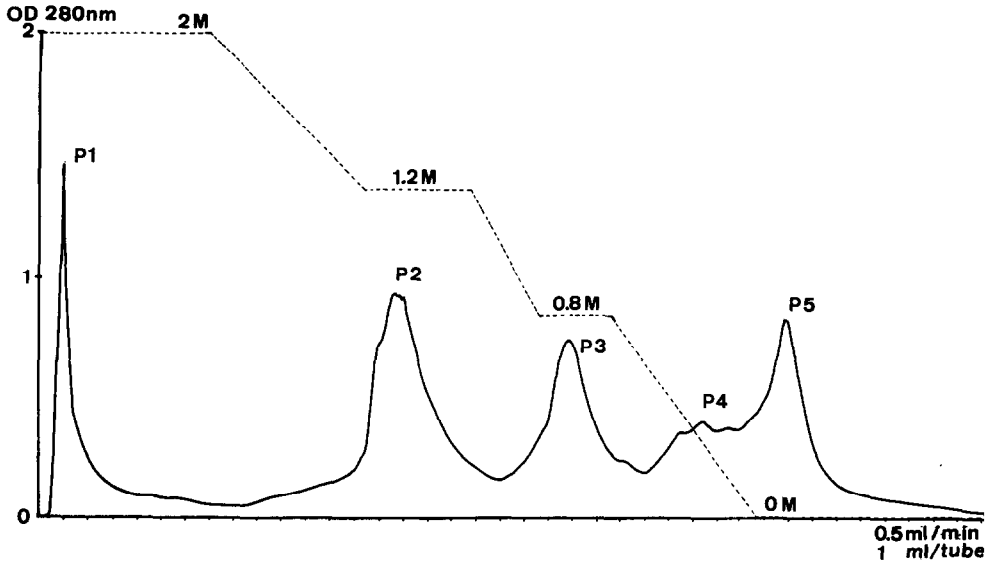

Fig. 4. Chromatogram obtained by analytical hydrophobic interaction chromatography of SIIS50 on a phenyl Superose column HR 5/5 equilibrated with 2 *M* $(NH_4)_2SO_4$ in 0.02 *M* phosphate buffer (pH 8.00) (solution A). A 10-mg amount of SIIS50 was loaded onto the column. Elution was carried out in four steps. A first isocratic run with 100% solution A was followed by a linear decreasing gradient. Two plateaus were introduced at 40% and 60% of solution B. The last step was performed with 0.02 *M* phosphate buffer (pH 8.00) (solution B). Flow-rate, 0.5 ml/min; UV detection wavelength, 280 nm.

dissolved in 500 μl of solution A, 2 *M* $(NH_4)_2SO_4$ in 0.02 *M* phosphate buffer (pH 8), and clarified by centrifugation. It was then loaded onto the phenyl Superose HR 5/5 column previously equilibrated with the same buffered salt solution. After an isocratic run, during which one peak was eluted, a linear decreasing concentration gradient of $(NH_4)_2SO_4$ was applied, which gave two major peaks and two minor ones. The last elution step consisted in a plateau at 100% B to elute the more strongly adsorbed brownish material (Fig. 4). Immunochemical analyses were performed by fused rocket experiments (results not shown). Peak 1 contained no antigenic material, and peaks 2, 3, 4 and 5 contained *Equ.c1*. The last peak contains a mixture of Ag1 (*Equ.c1*), Ag2, Ag3 and Ag4. The majority of the *Equ.c1* allergen is eluted in peak 2, between 0 and 40% of solution B (that is equivalent to 1.2 *M* $(NH_4)_2SO_4$). The process was simplified and scaled up as described below.

Preparative HIC. An 80-mg amount of SII, dissolved in 2 ml of solution A after being clarified by centrifugation, was loaded onto the phenyl Superose HR 5/5 column. The column was previously equilibrated with solution A. An isocratic run was carried out during which only one peak, P1, was eluted. A second elution was performed at 40% of solution B as previously defined, a third one at 60% and a last one at 100% of solution B. Each one gave only one peak (results not shown). The three peaks were named P2, P3 and P4, respectively. The material eluted at 40% of solution B (*i.e.* 1.2 *M* $(NH_4)_2SO_4$), P2, can be considered as a pure horse major allergen *Equ.c1* preparation, as shown by CLRIE (Fig. 5).

SDS-PAGE analysis

The results of this analysis are shown in Fig. 6. *Equ.c1* appears as a homogeneous protein fraction, consisting of only one peptide with relative molecular mass of 20 000. Under reducing conditions no cleavage occurs. A reversible change of apparent molecular mass is observed: it increases from 20 000 to 25 000 after reduction of the disulphide bonds by DTT.

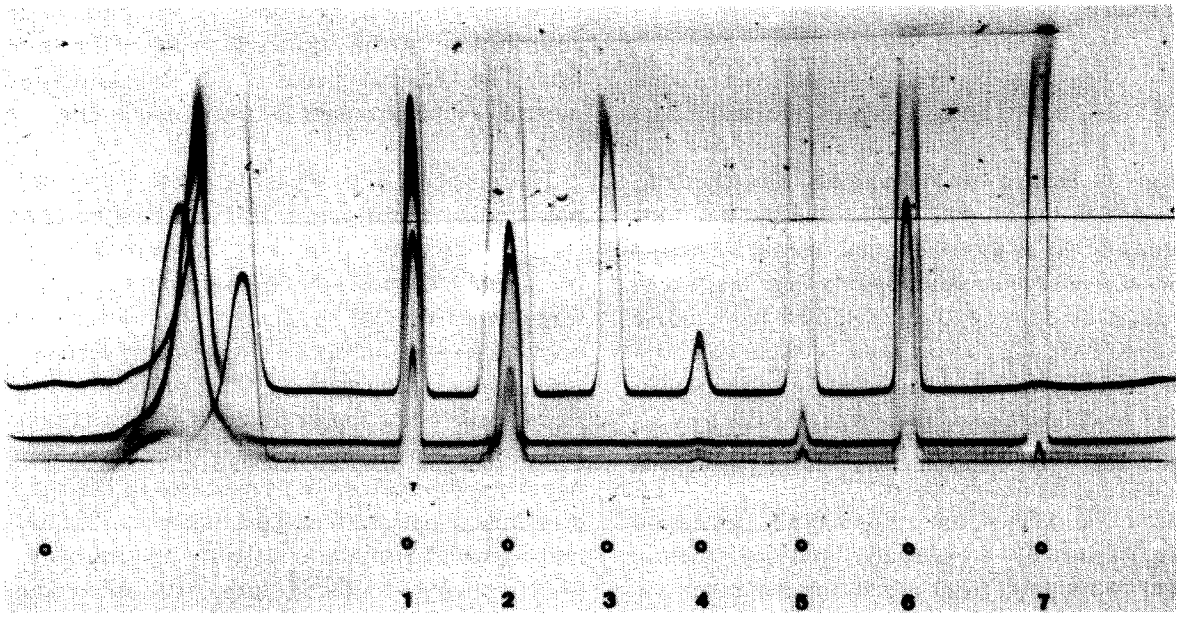

Fig. 5. In CLRIE HHD75 reacted with homologous rabbit antibodies. It was also present in the intermediate gel. Each fraction obtained by HIC was deposited in a well and submitted to electrophoresis in the second dimension: 1 = HHD75; 2 = SII; 3 = P2; Ag1; 4 = P1; 5 = P3; 6 = P4; 7 = horse albumin.

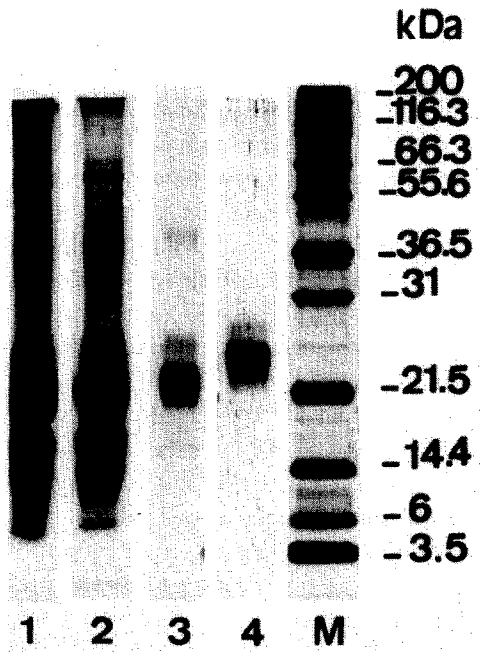

Fig. 6. SDS-PAGE performed in an homogeneous 12% gel. A 10-μl sample of either dithiothreitol-treated or untreated *Equ.c1* were deposited and submitted to electrophoresis. The gel was stained with silver nitrate. Lanes: 1 = native HHD75 (100 μ); 2 = SII (100 μg); 3 = native *Equ.c1* (100 μg); 4 = reduced *Equ.c1* (100 μg); M = protein markers.

Isoelectric focusing

Between pH 3 and pH 7, this experiment indicated a p*I ca.* 3.9 for the *Equ.c1* allergen (Fig. 7).

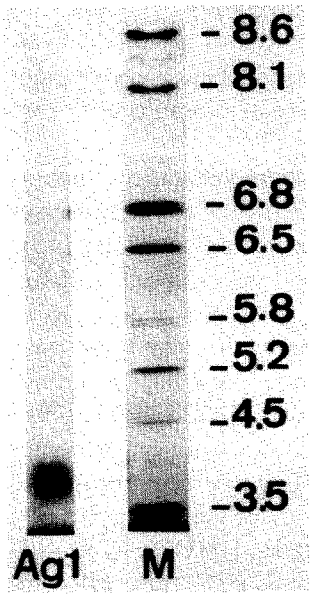

Fig. 7. Isoelectric focussing performed in a Novex gel (pH 3–7): Ag1 = pure *Equ-c1* (100 μg); M = protein markers of known p*I*.

DISCUSSION

Forty years ago [8], animal danders were stated to be the source of horse allergens. Horse danders are only present nearby the animal, and so horse allergy is limited to individuals directly concerned with the animal. Horse allergy may thus be considered as a professional disease.

Several allergenic components were defined in an aqueous crude extract obtained from horse danders [1–3]. Up to now none of them has been isolated and purified. Therefore, we tried to purify the so-called *Equ.c1* (according to the new allergen nomenclature [9]), the horse major allergen and we have succeeded.

First, we defined the antigenic and allergenic composition of a partially purified extract HHD75 obtained from crude horse danders extract by acetonic precipitation and a salting-out process. Both these techniques are founded on hydrophobic interactions.

Separation of low-molecular-mass from high-molecular-mass components was achieved using size-exclusion chromatography.

Hydrophobic interaction chromatography (HIC) was used after several unsuccessful attempts to isolate and purify each of these allergens by ion exchange chromatography and immobilized metal affinity chromatography. Nevertheless, these techniques had been very efficient for the purification of similar biomolecules from a cat dander extract [6].

HIC is a technique where substances such as proteins are separated according to their interaction with the hydrophobic groups, such as phenyl groups, bonded to the bed material of a column. Moreover, hydrophobic molecules in an aqueous solvent will self-associate due to these hydrophobic interactions. Antichaotropic salts, *e.g.* ammonium sulphate, increase the hydrophobic effect [10]. Ammonium sulphate in solution promotes protein aggregation, leading to precipitation, and this property was used to prepare the partially purified horse dander extract HHD75. In a solid–liquid phase system, such as HIC, these salts emphasize the interaction with the immobilized hydrophobic groups [11,12]. They have also been shown to promote hydrophobic interactions in contributing to the surface tension of the solution. The salt that increases the surface tension most gives the strongest hydrophobic interaction [13].

The first gels of practicable use for HIC were prepared by Porath *et al.* [14] and Hjerten *et al.* [15]. More recently, HIC was shown to be useful to purify enzymes [16,17], hormones [18] and fragments from IgM [19]. HIC has never been used to isolate and purify allergenic molecules.

We have achieved a 99% purification of *Equ.c1*, the horse major allergen, after only one run through a column packed with phenyl Superose. Phenyl Superose is a derivative of the rigid, crosslinked agarose-based gel Superose 12 (Pharmacia), which contains covalently bonded hydrophobic phenyl groups. Elution of adsorbed components in order of increasing hydrophobicity was achieved by decreasing the salt concentration as described previously [20]. This was done in a linear gradient for analytical purposes and stepwise for a preparative goal. Vorauer *et al.* [21] showed that scale up of HIC is possible while maintaining constant the ratio of column height to column diameter. We thus believe that we will be able to prepare larger amounts of *Equ.c1*.

Our results indicate a relative heterogeneity of this allergenic material. We must keep in mind that no particular precautions were taken during the collection and extraction of the horse danders to avoid chemical and enzymic denaturation. This mimics the hazardous life of natural allergenic substances.

It is interesting to note that a brownish pigmented material was strongly retained on the phenyl Superose and was desorbed only by the buffer alone without antichaotropic salts.

Physicochemical, biochemical and immunochemical analyses lead us to define *Equ.c1* so prepared as a pure protein consisting of only one peptide, with a relative molecular mass of 20 000 and a p*I* *ca.* 3.9. After reducing treatment, it seems to have a greater molecular mass than in its native state. It may be assumed that breakage of disulphide bonds leads to an unfolded state, which explains the retardation of the electropho-

retic migration observed and the increase of its apparent molecular mass to 25 000. Though unfolding can be reversible under oxidative conditions, the molecular mass of *Equ.c1* does not return to its original value.

Accumulation of information on the structure of allergenic molecules could help to answer to the most intriguing question: what makes an allergen an allergen?

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