

Characterization of the Main IgE-Binding Components of Cat Dander

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Abstract. Cat dander extract (CDE) was fractionated by preparative isoelectric focusing (pIEF). All the fractions showed allergenic activity, but the most acidic ones (pH range 3.3-4.3) had the highest specific activity. These fractions also had the highest cat allergen 1 content and a partial antigenic identity as determined by fused rocket immunoelectrophoresis. Immunoblotting of the pIEF fractions and the crude CDE after analytical IEF showed the presence of native proteins with IgE-binding ability all along the pH range 3.3-6.2. However, when the IgE-immunoprecipitated components of the most acidic pIEF fractions were focused in the presence of urea, a neat band was found at pH 3.9. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting of CDE proved the presence of, at least, 9 polypeptides with affinity to IgE, those with molecular weights of 18,000 and 22,000 daltons being the most active ones. These polypeptides presumably belong to cat allergen 1 since they were found in the most acidic pIEF fractions as well as in the crossed immunoelectrophoresis arc corresponding to this allergen. Furthermore, we found a group of active proteins with molecular weights of 10,000, 22,000, 25,000, 50,000 and 65,000 daltons (cat albumin) and with isoelectric points between 3.3 and 6.2.

Introduction

Several studies have demonstrated that allergens derived from the domestic cat give rise to severe allergic reactions in humans [1], and that an important allergen identified in cat saliva, cat pelts, sebaceous glands of cat skin, and designated cat allergen 1, is associated with these reactions [2]. Using skin-prick test and quantitative radioallergosorbent test (RAST) inhibition, the allergenic activity of extracts obtained from the above materials correlated very highly with the allergen 1 content [3].

The isolation of this allergen from extracts of cat pelt by Ohman et al. [4, 5] resulted in the identification of an acidic protein with a molecular weight between 30,000 and 60,000 daltons. Recently, Leiterman and Ohman [6] isolated the cat allergen 1 by affinity chromatography and demonstrated that it had an apparent molecular weight of 35,000 and 18,000 daltons under native and dissociating conditions, respectively.

On the other hand, by means of crossed immunoelectrophoresis/crossed radioimmunoelectrophoresis

(CIE/CRIE) studies, the presence of minor allergens in cat epithelial extracts was also proved, but the importance of such allergens and their physicochemical and biochemical properties has not been fully established [3].

The present study describes the isolation of 10 fractions from cat dander extract (CDE) by preparative isoelectric focusing (pIEF). The identification and immunochemical characterization of the main allergenic polypeptides, especially cat allergen 1, have been established by several methods.

Materials and Methods

Source Material

Cat dander was purchased from Allergon AB (Engelhom, Sweden).

Extract Preparation

CDE was prepared by mixing the source material with phosphate-buffered saline (PBS) at a 2.5% (w/v) ratio and maintaining the mixture for 1 h at room temperature while stirring. After centrifugation at 27,000 g for 20 min to precipitate insoluble materials, the

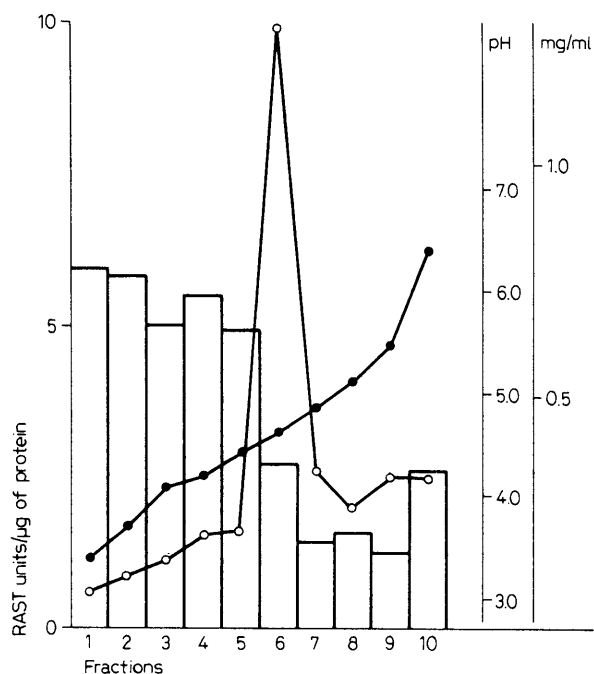


Fig. 1. pIEF. pH gradient from 3.5 to 5.5 (●) and protein content (○). The histogram indicates the allergenic specific activity measured in RAST units/microgram of protein.

supernatant was dialyzed, filtered through a 0.2-μm pore membrane and freeze-dried.

Cat albumin was purified from cat serum by a modification of Cohn et al.'s method [7].

Throughout the whole process, protein concentrations were measured by two different methods: Lowry et al.'s method [8] and Bradford's technique [9] using bovine serum albumin (BSA) and ovalbumin as standard proteins, respectively.

RAST Inhibition

RAST inhibition was performed according to the procedure reported by Yman et al. [10]. Paper discs were activated with cyanogen bromide and coupled with CDE, basically as described by Ceska et al. [11]. In order to compare allergenic activities, specific activity was defined in RAST units/microgram of protein. A RAST unit is the amount of protein that produces a 50% inhibition.

Human Sera

A pool of human sera (positive pool) from 30 patients who contained specific IgE to CDE (RAST class 3 or 4; Pharmacia) was used throughout the experiments. These patients had a positive skin-prick test to CDE and clinical history of hypersensitivity to cat. A pool of human sera from healthy individuals without specific IgE to CDE was also used as a control (negative pool).

Rabbit Antisera

Rabbits were injected intramuscularly every 2 weeks with 1 mg of CDE emulsified in Freund's complete adjuvant. After the second injection the same immunization schedule was followed using Freund's incomplete adjuvant. From the 2nd month on, rabbits

were bled periodically and antisera were assayed by immunodiffusion against CDE. Those antisera with a suitable titre were pooled, aliquoted and stored at -80°C.

Isoelectric Focusing

CDE (120 mg) was fractionated by pIEF in a pH gradient from 3.5 to 5.5 using LKB Ultradex granulated gel. The proteins were eluted from the gel with 0.5 N NaOH, dialyzed against distilled water and freeze-dried. The protein content of the fractions was determined by Bradford's technique [9] because the ampholyte interference was smaller than with Lowry et al.'s method [8].

Analytical IEF (aIEF) with and without urea was carried out in a 5% polyacrylamide gel using a pH gradient from 3.5 to 9.5.

CDE proteins separated by aIEF were transferred onto nitrocellulose membranes according to Towbin et al. [12]. After blocking the remaining adsorptive sites with 1% BSA in PBS, immunodetection of human IgE-binding proteins was achieved by incubating the blots with a 1:5 dilution of the human serum pool and then with 200,000 cpm/ml of ¹²⁵I-labeled mouse anti-human IgE monoclonal antibody [13]. Finally, the nitrocellulose membranes were dried and autoradiographed.

¹²⁵I-Labeling

The mouse anti-human IgE monoclonal antibody was ¹²⁵I-labeled to a specific activity of about 10 μCi/μg according to Fraker and Speck [14]. Similarly, 20 μg of protein of CDE pIEF fractions were labeled with 1 mCi of ¹²⁵I using the same method.

Immunoelectrophoretic Methods

Fused rocket immunoelectrophoresis (FRIE) was done basically according to Svendsen [15]. Immunodetection of human IgE-binding proteins (FRRIE) was performed by incubating the FRIE plate with the human serum pool and ¹²⁵I-anti-human IgE as described for aIEF.

CIE was done as reported by Weeke and Lowenstein [16]. In order to correlate CIE and immunoblotting results for Cat-1, we applied in the first dimension of CIE a pool of pIEF fractions 1:5 mixed with 5 × 10⁵ cpm of the same fractions labeled with ¹²⁵I. When the second dimension against the antibody-containing gel was completed, the precipitation arc corresponding to Cat-1 was cut, dissolved in Laemmli's buffer [17] and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) under reducing conditions. Then, the gel was dried and autoradiographed.

Quantification of Cat Allergen 1

Radial immunodiffusion (RID) for quantification of cat allergen 1 was carried out using Cat-1 and anti-Cat-1 antibody references generously supplied by Dr. Baer (FDA, Bethesda, Md.).

Polyacrylamide Gel Electrophoresis

PAGE was performed in the presence of sodium dodecyl sulfate (SDS-PAGE) under reducing conditions as described by Laemmli [17]. Further immunodetection of IgE-binding polypeptides was achieved as described previously.

IgE Immunoprecipitation

Polyvinyl chloride (PVC) wells were coated with 100 μl of purified mouse anti-human IgE monoclonal antibody (20 μg/ml) and, after saturation with BSA, they were incubated overnight with

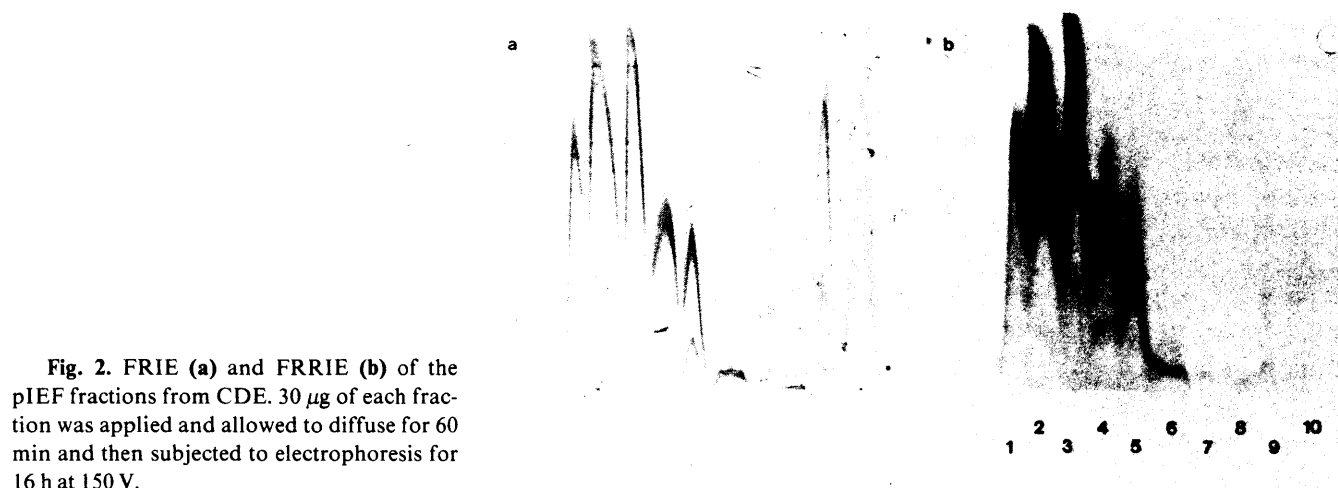


Fig. 2. FRIE (a) and FRRIE (b) of the pIEF fractions from CDE. 30 μ g of each fraction was applied and allowed to diffuse for 60 min and then subjected to electrophoresis for 16 h at 150 V.

100 μ l of human serum pool. After washing, the wells were incubated with 2.5×10^5 cpm of ^{125} I-labeled pIEF fractions for 18 h at room temperature. They were then again washed and the radioactivity bound to them was dissolved in the IEF sample buffer and subjected to aIEF (pH range 3.5–9.5) in the presence of 8 M urea. Finally the gel was dried and autoradiographed.

Results

CDE, prepared as indicated in Materials and Methods, gave a final protein concentration of 3.5 mg/ml. 120 mg of this protein was subjected to pIEF in a pH gradient from 3.5 to 5.5 for 18 h at 8 W. The gel was then divided into 10 fractions and the pH, protein content and allergenic activity of each fraction were measured (fig. 1). The highest protein concentration was found in fraction 6 (pI 4.6) which corresponded to cat albumin. By RAST inhibition, all the fractions showed IgE-binding ability; however, fractions 1 (pI 3.3), 2 (pI 3.8), 3 (pI 3.9), 4 (pI 4.1) and 5 (pI 4.3) seemed to possess the highest specific allergenic activity.

Figure 2 shows a FRIE/FRRIE experiment of the pIEF fractions. A great deal of antigenic precipitation arcs was found in all fractions (fig. 2a). Following incubation with human serum and ^{125} I-anti-human IgE, 5 main arcs showed IgE-binding capacity (fig. 2b). These arcs fused indicating a possible relationship of identity among them. By means of RID using Cat-1 reference and anti-Cat-1 rabbit antibodies, it was possible to confirm the presence and the quantity of this allergen mainly throughout fractions 1–5 (table I), in agreement with the FRRIE results.

Table I. Quantification of cat allergen 1 by RID

pIEF fractions	FDA units/ml ¹
1–5	81.0
6	3.0
10	1.5

¹ Arbitrary units for quantification of Cat-1 proposed by Anderson and Baer [3] of the FDA.

Figure 3 shows the immunoblotting of all the pIEF fractions after aIEF in a pH gradient of 3.5–9.5. As expected, the highest binding capacity to human IgE was exhibited by native proteins which focused, basically, along fractions 1–5 (pH range 3.3–4.3). Fraction 6 with pH 4.6 and fraction 10 with pH 6.2 also showed a relatively high allergenic activity. All these proteins with other less active proteins in fractions 7–9 were responsible for the total allergenic activity of CDE (fig. 3, E). Cat albumin was found in fractions 6 and 7, as demonstrated with a marker of purified cat albumin (fig. 3, A). Its capacity to bind specific human IgE was weak in relation to the high protein concentration it had.

Immunoblotting of the main active fractions and crude CDE, previously separated by SDS-PAGE under reducing conditions, is shown in figure 4. As many as 9 different polypeptides from crude CDE were able to bind specific human IgE (fig. 4, E). Two of them, found in fractions 1–5 and with apparent molecular weights of 22,000 and 18,000 daltons,



Fig. 3. Immunodetection of IgE-binding molecules of the pIEF fractions (1-10), purified cat albumin (A) and whole CDE (E), after aIEF. 60 μ g of protein was applied in all cases. As control, a nitrocellulose strip with the blotted CDE proteins was also incubated with the negative human serum pool and no band was detected.

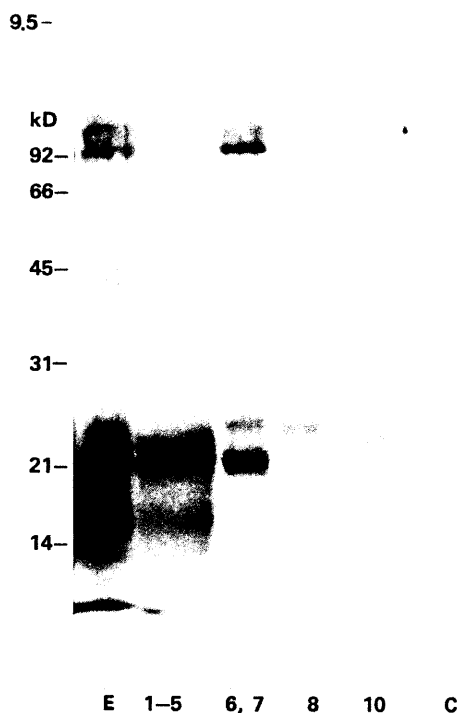


Fig. 4. Immunodetection of IgE-binding polypeptides of the whole extract (E) and pIEF main fractions after SDS-PAGE under reducing conditions. 125 μ g of protein was applied per sample. C is a negative control with the negative serum pool. KD = Kilodaltons.

seemed to belong to cat allergen 1. Moreover, in these fractions, it was possible to detect the presence of another active protein of about 10,000 daltons. Analysis of the polypeptide pattern of fractions 6 and 7 also showed the presence of a 22,000-dalton protein be-

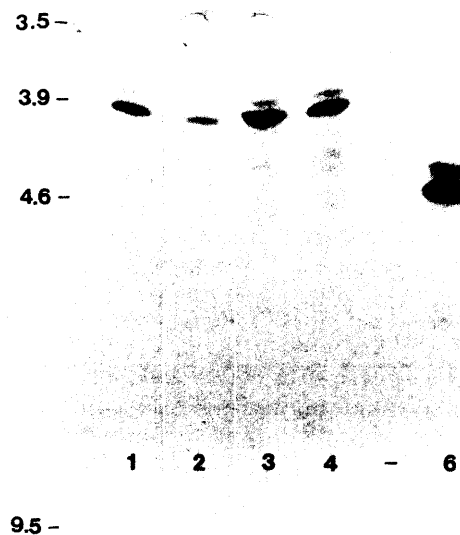


Fig. 5. Autoradiography of aIEF in the presence of urea of the immunoprecipitates obtained by coating PVC wells with anti-human IgE, then incubating with the positive serum pool and finally with ^{125} I-labeled fractions 1-4. 6 = Cat albumin; - = negative control obtained by incubation with the negative serum pool.

sides cat albumin (65,000 daltons) and another minor band with an apparent molecular weight of 25,000 daltons, which exhibited a weaker capacity of binding human IgE. Likewise, in fraction 8, as well as in fraction 10, we found other active polypeptides with mo-

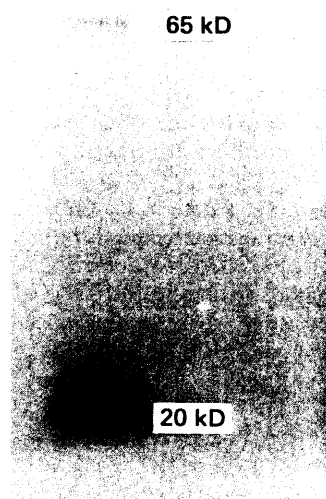


Fig. 6. Analysis by SDS-PAGE of the Cat-1 precipitation arc obtained by CIE. ^{125}I -labeled pIEF fractions 1-4 were subjected to CIE and the Cat-1 precipitation arc was detached from the CIE plate and subjected to SDS-PAGE.

lecular weights between 22,000 and 25,000 daltons. Finally, fraction 10 also showed other polypeptides of higher molecular weight with a weaker radiostaining.

To further investigate the nature of the molecules identified in fractions 1-4, an IgE immunoprecipitation of these fractions was performed as mentioned in Materials and Methods. Figure 5 shows the band pattern of the 4 fractions after aIEF in the presence of urea and autoradiography. Under these conditions, cat allergen 1, which was spread along the 4 fractions and immunoprecipitated with specific human IgE, focused mainly as a neat band at pH 3.9 instead of the pH range 3.3-4.1 obtained by aIEF in the absence of urea (fig. 3).

To determine the relationship between the arc corresponding to Cat-1 in CIE and its pattern by SDS-PAGE, a mixture of ^{125}I -labeled fractions 1-4 was cross-immunoelectrophoresed. The Cat-1 precipitation arc was detached from the CIE plate and subjected to SDS-PAGE in the presence of 2-mercapto-

ethanol. As shown in figure 6, a diffuse band was located in the region of 20,000 daltons which could correspond to the double band of molecular weights 22,000 and 18,000 daltons detected by immunoblotting (fig. 4). Another band of about 65,000 daltons (molecular weight of cat albumin) was found, indicating a possible contamination when the Cat-1 precipitation arc was cut from the CIE plate.

Discussion

In order to select the most suitable cat extract, a comparative study among different raw materials was carried out. The result of this examination proved that CDE turned out to be the extract of choice based on its high specific allergenicity, high Cat-1 level and low albumin/Cat-1 ratio (about 2/1 w/w; data not shown).

The results presented in this paper demonstrate that CDE contains, at least, 9 polypeptides with ability to bind specific human IgE when the whole extract was analyzed by SDS-PAGE under reducing conditions. Two of them, with apparent molecular weights of 22,000 and 18,000 daltons, showed the most allergenic activity (fig. 4) and corresponded to several native proteins which focused between pH 3.3 and 4.3 (fig. 3). These 2 polypeptides seem to constitute the main components of cat allergen 1, since they were found in the corresponding CIE arc and had a molecular weight in agreement with previous studies [6]. However, the same allergens focused, forming mainly a single band, at pH 3.9, after human IgE immunoprecipitation and aIEF in the presence of urea. We do not have an explanation for this finding, but it might be suggested that masked groups could be exposed due to the urea treatment, thus changing the pI of the various polypeptide chains [18, 19]. The fact that multiple native allergens, with charge heterogeneity, resolve into one or a few polypeptides, following SDS denaturation, has been substantiated in other allergenic materials like *Parietaria judaica* [20], *Dermatophagoides* [21, 22] or moulds [23]. Furthermore, we found another polypeptide of an apparent molecular weight of 10,000 daltons with IgE-binding ability. This protein might be an allergenically active subunit arising from Cat-1, however, we could not identify it by SDS-PAGE following CIE (fig. 6).

Cat albumin is the most abundant protein in CDE. However, its capacity to bind specific human IgE is

rather low, confirming that albumin is not an important cat allergen [2].

In addition to albumin, fraction 6 contains also cat allergen 1 and a third allergenic component which seems to have a slightly higher pI than Cat-1 and possibly a molecular weight of about 25,000 daltons. Its presence has been recently demonstrated by means of a mouse monoclonal antibody that recognizes an allergenic protein with pI lower than albumin and which does not recognize Cat-1 [in preparation].

Finally, it is worthy of note that there is a group of polypeptides with ability to bind specific IgE, with molecular weights around 25,000 daltons and distributed between fractions 8 and 10.

At present, we are trying to characterize monoclonal antibodies to CDE allergens which will allow their isolation and better knowledge of their molecular structures.

Acknowledgments

This work has been supported by Alergia e Inmunología Abelló Laboratories. We gratefully thank Isabel Courinha for manuscript preparation.

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Received: March 27, 1987

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