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Isolation of a Purified Allergen from Kentucky Blue Grass Pollen¹

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Abstract. An allergenic fraction, designated as C-I-2-6, was isolated from among the allergenic constituents present in the dialysate of the aqueous extract of defatted Kentucky blue grass (KBG) pollen by a combination of gel filtration, ion-exchange chromatography and preparative isoelectrofocussing. This fraction was shown to consist of glycoprotein with a molecular size corresponding to 10,000 daltons and of two components with closely related pI values (4.5-4.9); its protein moiety contained all the amino acids except cysteine. As demonstrated by crossed immunoelectrophoresis with a sheep anti-R serum, fraction C-I-2-6 shared the antigenic determinants of 2 of the 15 antigenic components of the non-dialysable components, i.e. retentate (R) of the aqueous extract of KBG pollen. Remarkably, in spite of the markedly lower molecular weight than R, fraction C-I-2-6 possessed all the allergenic determinants of R responsible for inducing murine IgE antibodies.

Introduction

Conventional hyposensitization treatment of allergic patients suffering from hay-fever to a given pollen consists of a series of injections of the whole aqueous extract of the pollen. In view of the complex composition of these preparations, it may be visualized - as actually demonstrated in an earlier study [37] - that this treatment may result in the sensitization of the patients to some component(s) of the extract to which they may not have been sensitive before treat-

ment. Therefore, it would seem desirable to treat patients with a purified and well-characterized fraction(s) of the extract that is (are) devoid of components to which the patients are not sensitive. Moreover, such purified allergens would serve as reference preparations for the standardization of allergenic products obtainable from different manufacturers for use in hyposensitization treatment. In the context of this paper, the terms 'allergen' and 'antigen' will be used to designate the moieties in an allergenic preparation which combine with IgE and IgG antibodies, respectively.

There is overwhelming evidence that immediate hypersensitivity reactions are triggered by the cross-linking of IgE antibodies,

fixed to mast cells or basophils, by the appropriate polyvalent allergens [5, 11, 20]. A rational therapeutic approach would, therefore, involve inhibiting the cross-linking of cell-fixed IgE antibodies by multivalent allergens by the administration of haptens of the appropriate allergenic molecules or of fragments of these molecules containing only single determinants. These univalent compounds would then be expected to bind to the Fab regions of the IgE antibodies and thus inhibit the combination of IgE antibodies with the offending multivalent allergen(s). The feasibility of this approach gains support from the successful regimen developed by *de Weck* [10] for the treatment of penicillin allergy by the injection of large doses of the univalent penicillin derivative, benzylpenicilloyl-formyl lysine, prior to administration of penicillin to sensitive patients.

In another approach, *Malley et al.* [29] showed that suppression of formation of human IgE antibodies to timothy grass pollen allergens, which was accompanied by clinical improvement, was achieved by treatment of the patients over a period of only 2 years with a low-molecular-weight hapten-like fraction of the pollen extract. More recently, abrogation of the ongoing reaginic responses to haptens, such as the benzylpenicilloyl and 2,4-dinitrophenyl groups in experimental animals (mice, rats and dogs), was also achieved by the injection of conjugates of the appropriate hapten with nonimmunogenic carriers, such as isologous γ -globulins [24], polyvinyl alcohol [26] or the synthetic copolymer of *D*-glutamic acid and *D*-lysine [21, 41]. Clearly, this therapeutic modality could be extended to a wide spectrum of allergies on condition that the determinants of the appropriate allergens were available in the form of compounds

which could be incorporated into tolerogenic conjugates. In essence, this approach would involve the establishment of the chemical structure of the relevant allergenic determinants and the subsequent synthesis of the appropriate compounds, as has been achieved, for example, for the determinants of sperm whale myoglobin [2, 9]. In this connection, it is to be noted that low-molecular-weight hapten-like constituents were shown to be present in the dialysates of the aqueous extracts of ragweed pollen [1] and Kentucky blue grass (KBG) pollen [8].

In relation to the regulation of antibody formation, it is now generally accepted that (i) collaboration [27] between T helper (T_H) and B cells is a prerequisite for the induction of the IgG and IgE antibody responses to most protein antigens (i.e. to T-dependent antigens) in mice [22, 31] and rats [39]; (ii) B lymphocytes differentiate to become antibody-producing cells; (iii) the population of T cells contains not only T_H cells, but also T suppressor (T_S) cells [33, 40] and amplifier cells which may enhance the effects of T_H and T_S cells [34, 35], and (iv) the homeostatic balance among these different subpopulations of T cells is responsible for controlling the magnitude and duration of antibody responses [23, 30, 33-35]. Obviously, from a therapeutic standpoint, it would be desirable to establish the nature of the determinant(s) of an allergenic molecule, which is(are) capable of stimulating T_S cells and which in turn would lead to the suppression of IgE antibodies to the allergen in question. In support of this concept, *Sercarz et al.* [38] have recently demonstrated the presence of a single suppressive determinant on the lysozyme molecule which promoted the generation of T_S cells capable of abrogating the formation of antibodies directed against the anti-

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genic determinants of the lysozyme molecule as a whole.

On the basis of these considerations, it is obvious that for the design of a rational basis for immunotherapy, it is essential to establish the chemical nature of the diverse determinants of an allergenic molecule. Many pollen allergens have been shown to be glycoproteins with molecular weights in the range of 10,000–14,000 daltons. Owing to the chemical diversity and heterogeneity of substances responsible for common allergies, so far the elucidation of the chemical structure has been achieved only for the codfish allergen M [15, 16] and the low-molecular-weight constituents of the ragweed allergen Ra5 [32], which have molecular weights of the order of 12,300 and 5,000 daltons, respectively.

In view of the high preponderance of respiratory allergies and hayfever to KBG, an attempt was made in this study to isolate the low-molecular-weight allergens of this pollen, since it is obvious that allergens of low molecular weight are more readily amenable to chemical studies. In an earlier study [12], it was shown that the allergenic activity of the dialyzable components of the aqueous extract of KBG pollen was associated with a fraction designated as D-I. As described in the present article, a purified allergenic fraction was isolated from D-I by a combination of gel filtration, ion-exchange chromatography and preparative isoelectrofocussing. Remarkably, this allergenic fraction, designated as C-I-2-6, was found to share all the allergenic determinants of the components present in the higher-molecular-weight, nondialysable and multicomponent fraction of the aqueous extract of KBG pollen, as demonstrated with the aid of IgE antibodies produced in mice by immunization with the latter fraction.

Materials and Methods

Preparation of Dialysable and Nondialysable Fractions of Pollen

An aqueous extract of KBG pollen (Hollister-Stier Laboratory, Mississauga, Ontario, Canada) was prepared according to the procedure previously established in this laboratory [14]. The aqueous extract was dialyzed through Visking tubing, No. 20 dialysis (Union Carbide Canada Ltd., Lindsay, Ontario, Canada), against distilled water for 24 h at 4 °C; the dialysable components are referred to as the dialysate (D). To ensure a more thorough removal of dialysable components from the nondialysable fraction, dialysis was continued for an additional 24 h; the nondialysable components are referred to as the retentate (R).

Chromatography

Since a previous study [12] had revealed that, on filtration of D through a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden), the skin-active components were confined essentially to the first fraction, referred to as D-I, this fraction was used as the starting material for the isolation of the individual dialysable allergens. Thus, fraction D-I was subjected to cation-exchange chromatography on CM Sephadex C-25 (particle size 40–120 μ m, column size 1.6 $\times$ 40 cm) which had been previously equilibrated with a sodium phosphate buffer, 0.01 M, pH 6.8. After passage of the components which had not been retained in the gel, the ionic concentration of the eluting buffer was increased linearly up to a molarity of 0.15; a final attempt to elute the material from the column was made with the initial phosphate buffer (i.e. 0.01 M) in the presence of sodium chloride at a concentration of 1 M.

For gel filtration, Sephadex G-50 (particle size 20–80 μ m) was equilibrated with phosphate-buffered saline (7.75 g NaCl, 0.54 g KH_2PO_4 , 2.14 g Na_2HPO_4 , in 1 liter of H_2O ; pH 7.2; PBS), and packed into a column (2.5 $\times$ 90 cm) at 4 °C.

All chromatographic eluates were desalted by gel filtration on Sephadex G-15, which had been swollen in distilled water (particle size 40–120 μ m) using double-distilled water as the eluant. In some cases, desalting was accomplished by ultrafiltration through UM-2 membrane, with a cutoff for molecules larger than 1,000 daltons (Amicon, Lexington, Mass., USA).

Isoelectrofocussing (ISO-EF) on Polyacrylamide Gel

For the preparation of a concentrated gel, a volume of 30 ml of a solution containing acrylamide (28%; Bio-Rad Laboratories, Richmond, Calif., USA) and bisacrylamide (0.735%; Bio-Rad) was mixed with 8 ml of a 1% solution of N,N,N',N'-tetramethylethylenediamine; Bio-Rad) and 3 ml of 40% Ampholyte buffer, pH range 3.5–10 (LKB-Product AB, Stockholm-Bromal, Sweden). For each gel, a volume of 0.67 ml of the mixture of monomers was mixed with 0.1 ml of the sample to be analyzed (100–500 μ g) and then a volume of 1.22 ml of a 0.1% solution of ammonium persulfate (Fisher Scientific Company, Fair Lawn, N.J., USA) was added. This mixture was then immediately pipetted into a glass tube (5 $\times$ 125 mm) and allowed to gel at room temperature for 30 min.

The ISO-EF was carried out in a Bio-Rad electrophoresis cell (model 300A). A solution of 0.1 N H_2SO_4 was used as the anode electrolyte and a solution of 0.1 N NaOH was used as the cathode electrolyte. To minimize undesirable effects due to the heat liberated during electrophoresis, a pulsed constant power supply (Ortec 4100, Ortec Inc., Oak Ridge, Tenn., USA) was used and the tubes containing the gel were cooled by a surrounding flow of cold tap water. The ISO-EF was performed at a potential of 350 V, with a discharge capacitance of 0.5 μ F at an initial frequency of 50 pulses/sec for 1 h, then raised to 100 pulses/sec for 1 h and continued at 200 pulses/sec for 22 h.

At the completion of the ISO-EF, the gels were removed from the tubes and fixed in 12% trichloroacetic acid (TCA) for 1 h. Following a brief washing with distilled water to remove the major portion of TCA, staining was accomplished by immersing the fixed gels for 2–3 h in a solution of Coomassie blue R250 (Ames Company, Division of Miles Laboratories, Inc., Elkhart, Ind., USA) at a concentration of 0.2% (w/v) in a solution composed of a mixture of ethanol:water:glacial acetic acid in ratios of 45:50:5 (v/v). The gels were destained in a Bio-Rad Model 172 diffusion destainer with 7% acetic acid in the presence of the ion-exchange resin AG501-XD (D; Bio-Rad Laboratories) until a clear background was obtained.

Preparative ISO-EF

The procedure of ISO-EF described by Radola [36] was adapted with slight modifications. Thus,

11.5 g of Sephadex G-100 superfine (Pharmacia) was swollen in a volume of 200 ml of distilled water. A volume of 7.5 ml of the stock solution of the Ampholyte buffer was added to the gel suspension, which was then deaerated under vacuum. The gel was poured onto a horizontal glass trough (20 $\times$ 40 $\times$ 1 cm), spread uniformly and dried (equivalent to a weight loss of 25–30%) so that the gel did not slide when the glass trough was tilted at an angle of 45°. This drying procedure was important for achieving uniform and reproducible separations.

The appropriate amounts of the allergenic preparations (e.g. 110 mg of fraction C-I-2) to be separated were dissolved in 1.5 ml of distilled water and applied over an area of 3 $\times$ 18 cm² in the central part of the gel. The gel was then allowed to dry for an additional 5 min. The glass trough was placed on the precooled metal block of the electrophoresis chamber (Desaga, Heidelberg, FRG), through which cold tap water was circulated. A strip of cellulose acetate membrane presoaked with a solution of 0.2 M H_2SO_4 for the anode, and another strip presoaked with a solution of ethylenediamine (0.4 M) for the cathode were then laid on top of the gel at the far ends, and the platinum electrodes were placed on top of the membrane. The ISO-EF was carried out for a period of 96 h at a constant voltage, starting at 50 V/cm for the 1st day, increasing it to 100 V/cm for the 2nd day and 125 V/cm for the 3rd and 4th days (Brinkman, Voltage and Current Power Supply, Brinkman, Rexdale, Ontario, Canada).

After ISO-EF, the gel was divided into 15 equal sections. A volume of 5 ml of distilled water was added to each fraction and the pH of the suspension was measured. Each gel suspension was then filtered, followed by resuspension of the gel in another volume of 10–15 ml of distilled water and the gel was again filtered. The combined filtrate was then dialysed through Spectrapor membrane tubing (molecular weight cutoff 3,500, Spectrum Medical Industries, Inc., Los Angeles, Calif., USA) against distilled water. All fractions were finally lyophilized and dissolved in 2 ml of distilled water and stored in the frozen state.

Molecular Weight Estimation

For the estimation of the molecular weights of the pollen constituents, they were subjected to filtration through a Sephadex G-50 column calibrated with human serum albumin, α -chymotrypsin

and cytochrome C (molecular weights are listed below), or alternatively to electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) according to the procedure described by Weber and Osborn [42]. Prior to electrophoresis, samples were incubated at 37 °C for 2 h in 0.1 M sodium phosphate buffer, pH 7.0, containing SDS at a concentration of 1% and 2-mercaptoethanol, also at 1%. The samples were fractionated on 10% polyacrylamide gels, 120 mm in length, prepared in phosphate-SDS buffer (7.8 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 38.6 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 2 g SDS and water added to a volume of 1,000 cm^3). Electrophoresis was performed at a current equivalent to 8 mA/tube and stopped when the tracking dye, bromophenol blue, migrated close to the bottom of the gel (in approximately 3.25 h). Proteins with known molecular weights were used as standards for the calibration of gels, i.e. human serum albumin, 68,000 (Pentex, Miles Laboratories, Elkhart, Ind., USA); ovalbumin, 43,000 (ICN Pharmaceuticals, Cleveland, Ohio, USA); pepsin, 35,000 (Worthington, Freehold, N.J., USA); α -chymotrypsin, 24,000 and the two chains of α -chymotrypsin, 13,000 (chain B) and 11,000 (chain C; Worthington); cytochrome C, 11,700 (Sigma, St. Louis, Mo., USA).

Amino Acid Analysis

The samples were hydrolyzed with HCl at 110–112 °C for 24 h according to the procedure used previously in this laboratory [14]. The hydrolysates were then analyzed by Dr. F. Stevens of the Department of Biochemistry, University of Manitoba, with the Beckman automatic amino acid analyzer, Model 120–139 (Beckman, Palo Alto, Calif., USA); the tryptophan content was determined spectrophotometrically [6].

Protein Measurement

The protein content of pollen fractions was determined by the method of Lowry *et al.* [28].

Carbohydrate Analysis

Carbohydrates were detected by spot tests [17]. A pinch of the sample was placed in a microcrucible. One drop of syrupy-phosphoric acid was added and the crucible was covered with a disc of filter paper moistened with aniline acetate solution (10% aniline in 10% glacial acetic acid). A watch glass was used as a paper weight. The bottom of the crucible was continuously heated for 30 sec with a microflame. The furfural formed during

hydrolysis of the carbohydrate reacted with aniline to give violet products.

The carbohydrate analysis was performed by Dr. J. R. Clamp, Department of Medicine, University of Bristol, Bristol, UK, by gas-liquid chromatography (GLC). For this purpose, methanol (500 ml) was dried by heating under reflux for 1 h with magnesium turnings (2.5 g) and iodine (0.1 g). Hydrochloride gas was bubbled into the dried methanol until the acid content was slightly in excess of 1 N and its concentration was finally adjusted to 1.0 M by dilution with dried ethanol. The solution of methanol-HCl was then transferred to 10-ml ampoules which were purged with nitrogen gas, sealed and stored at –10 °C until required.

The samples were placed into 2-ml glass ampoules. To each ampoule was added an amount of 0.1 mol of each of the standards arabinol, mannitol and peritol. The contents of the ampoules were thoroughly dried and then 0.5 ml of 0.1 M HCl-methanol was added. The ampoules were purged with nitrogen for 10 sec, sealed and placed in an oven at 85 °C for 24 h. The contents were then neutralized with solid silver carbonate and 0.05 ml of acetic anhydride was added. The ampoules were covered with aluminum foil and left for 6 h at room temperature. After thorough trituration, the ampoules were centrifuged and the supernatants transferred to 5-ml pear-shaped flasks. The trituration and centrifugation steps were repeated by three further additions of 0.5 ml of methanol to each sample. The combined supernatant fractions from each sample were then dried by rotary evaporation at 35 °C under reduced pressure, and the flask was stored in a vacuum desiccator over phosphorus pentoxide until required. To the dried material was added 0.05–0.10 ml of silylating agent [trimethyl chlorosilane, hexamethyldisilazone and pyridine, in the proportions (1:1:5)]. The mixture was thoroughly triturated and the stoppered flask allowed to stand for 30 min at room temperature. After centrifugation, 1–5 μl of the supernatant was injected into the gas chromatography column at the starting temperature of 140 °C; the temperature was then raised to 200 °C at the rate of 0.5 °C/min.

The Radioallergosorbent Test (RAST)

The test was performed according to Ceska *et al.* [7]. Briefly, R was insolubilized by attachment

through covalent bonds to cellulose discs (7 mm diameter) punched out from Whatman 3 MM paper. For this purpose, the discs were first treated for 1 h with an equal weight of cyanogen bromide in aqueous solution at pH 10, which was kept constant by the addition of the required volumes of a solution of 1 N NaOH. The discs were then washed with copious volumes of a solution of 0.1 M NaHCO_3 and then incubated with the allergenic sample for 24 h at 4 °C with constant agitation. To block any residual, active sites on the cellulose, the discs were reacted with a solution of β -ethanolamine (0.5 M in 0.1 M NaHCO_3) for 3 h and then washed sequentially with a solution of 0.1 M NaHCO_3 and 0.1 M acetate buffer (pH 4.0). The allergosorbent discs were then maintained in PBS (0.9% NaCl, 0.05 M phosphate buffer, pH 7.4) containing 0.3% human serum albumin and 0.05% sodium azide. Volumes of 0.05 ml of a pool of human sera from patients allergic to KBG were incubated overnight at 4 °C with the allergosorbent discs and then washed with the PBS. The extent to which the IgE antibodies bound to the insolubilized allergens was evaluated by the addition of ^{125}I -labeled sheep anti-human IgE antibodies which had been purified with appropriate immunosorbent(s). These antibodies had been kindly provided by Dr. K. Ishizaka and labeled with ^{125}I by the procedure of Hunter and Greenwood [19]. The discs were then thoroughly washed in order to remove any unbound labeled antibodies and the amount of ^{125}I sheep anti-IgE antibodies bound to the discs was measured with a Beckman Gamma 300 system counter. Since maximal binding of IgE antibodies to the allergosorbent occurred when an amount of 100 μg of R was coupled to the activated discs, this amount of R was used for all the subsequent RAST experiments described in this paper; each RAST was performed in triplicate.

Neutralization of Passive Cutaneous Anaphylaxis (PCA)

This procedure was employed to establish the allergenic activity of the dialysable fractions relative to that of the retentate of the aqueous extract of KBG pollen. For this purpose, IgE antibodies to the allergens present in R were produced in mice and were determined in terms of their PCA titers in the skin of rats, as previously described [13]; the PCA titer of the reagent anti-R serum used in this experiment was 2,560. The capacity of

the dialysable allergens to combine with the murine anti-R IgE antibodies was evaluated in terms of the degree of neutralization of the PCA reactions. This procedure consisted in mixing the allergenic fractions, in different amounts, with an appropriate volume of the murine reagent anti-R serum and maintaining the mixture for a period of 1 h at room temperature. (The final dilution of this serum was chosen as 1:1,280 since, at this dilution, only minimal amounts of allergenic fractions were required for neutralization of the anti-R IgE antibodies.) For the actual determination of the degree of neutralization, hooded rats were locally sensitized by the intradermal injection of a volume of 50 μl of the mixture and 24 h later the PCA reaction, if any, was elicited by intravenous (i.v.) administration of 1 mg of R in the presence of Evan's blue dye (1% in saline); a positive PCA reaction was due to the residual free IgE antibodies. A PCA reaction was considered positive when the extent of the extravasation of the dye, as measured on the underside of the skin, was 5 mm in diameter or greater.

Crossed Immunoelectrophoresis (CIE)

The CIE procedure originally described by Axelsen *et al.* [4] was employed for resolving the identifying antigenic components present in the aqueous extract of KBG pollen. Briefly, glass plates (10.1 $\times$ 8.2 cm) were overlaid with a volume of 20 ml of 1% agarose (Indubiose A-37, L'Industrie biologique française, Gennevilliers, France), which was prepared by dissolving the agarose at 100 °C in a buffer at pH 8.6 (containing 8.86 g tris (hydroxy methyl)-amino methane, 4.48 g barbital 0.106 g calcium lactate, 0.2 g sodium azide, dissolved in distilled water and adjusted to a final volume of 1 liter). The electrophoresis of the KBG pollen extract in the first direction was performed for a period of 2 h at a potential of 100 V using the Gelman electrophoresis chamber (Gelman Instrument Company, Ann Arbor, Mich., USA). Electrophoresis in a direction perpendicular to the first was carried out for a period of 24 h at a potential of 25 V after the addition of a 1% agarose gel, which contained the sheep anti-R serum (5.6% v/v). Following electrophoresis, the gel was dried by pressing between several layers of paper towels. The dried plate was stained with 0.5% Coomassie blue [methanol/acetic acid:water (4.5:1:4.5)].

Results

Chromatography

Fraction D-I of the dialysate, isolated by filtration through Sephadex G-25, as previously described [12], was resolved by ion-exchange chromatography on CM-Sephadex C-25 with sodium phosphate buffer (0.01 M, pH 6.8) into at least four fractions designated arbitrarily as C-I, C-II, C-III and C-IV (fig. 1). No additional material detectable by absorption at 280 nm emerged from the column on increasing the concentration of the eluting buffer from 0.01 to 0.15 M and only a trace of material emerged on increasing the molarity of the eluting buffer to 1 M with NaCl. Since C-II, C-III and C-IV were isolated in very small amounts, only fraction C-I was used for further fractionation by gel filtration on Sephadex G-50, which led to the subfractions, C-I-1, C-I-2 and C-I-3; these three subfractions were recovered in the ratios of

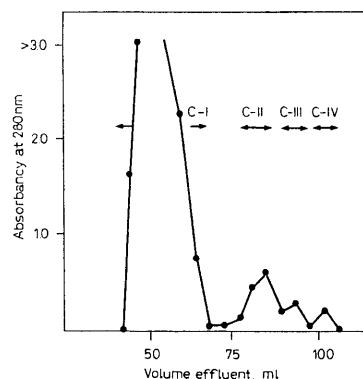


Fig. 1. Cation-exchange chromatography of fraction D-I on CM-Sephadex C-25. Column size: 1.6 × 40 cm; eluting buffer: sodium phosphate buffer, 0.01 M, pH 6.8.

1:1.6:11.7, as determined from their optical densities at 280 nm (fig. 2).

Allergic Activity

The allergic activity of subfractions C-I-1, C-I-2 and C-I-3 was evaluated in terms of their capacity to inhibit either the RAST using a pool of human reaginic serum, as described by Gleich and Jones [18], or the PCA reactions with the aid of a murine anti-R reaginic serum [13].

For the experiments involving the inhibition of RAST, 0.5-ml volumes of a pool of sera from four KBG-allergic patients were mixed with different amounts of each of the three subfractions and maintained for 1 h at 37 °C and the overnight at 4 °C prior to addition of the allergosorbent discs. The results given in figure 3 indicate that the capacities of the subfractions C-I-1 and C-I-2 to inhibit the binding of IgE antibodies to the allergosorbent discs were comparable to that of R and it is, therefore, concluded that these fractions contained essentially all the allergenic determinants of R. By contrast, the subfraction C-I-3, which constituted the bulk (82%) of the material present in the parent fraction C-I, did not inhibit the bind-

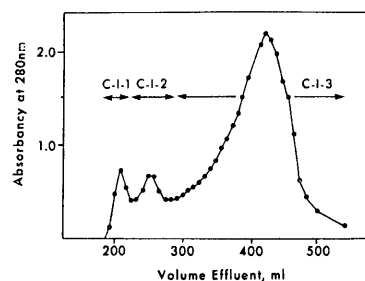


Fig. 2. Gel filtration of fraction C-I on Sephadex G-50 (particle size, 20–80 μm). Column size: 2.5 × 90 cm; eluting buffer: PBS, pH 7.2.

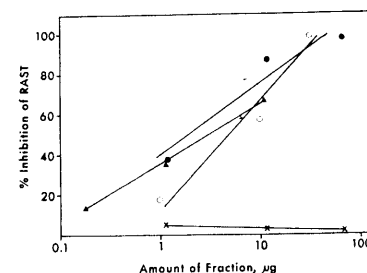


Fig. 3. Inhibitions of the radioallergosorbent test by fractions of D-I with a pool of sera obtained from 4 individuals allergic to KBG pollen. Aliquots of the pool of sera were mixed with different amounts of the fractions C-I-1, C-I-2 or C-I-3 and maintained for 1 h at 37 °C and then overnight at 4 °C, prior to incubation with the allergosorbent discs prepared with R. The degree of binding of IgE antibodies to the allergosorbent discs was established with an ¹²⁵I-labeled anti-IgE antibody preparation: ○ = C-I-1; ● = C-I-2; × = C-I-3; ▲ = R.

ing of the human anti-KBG IgE antibodies to the allergosorbent discs and was, therefore, considered to be devoid of allergenic determinants.

The evidence for the shared allergenic determinants between the subfractions C-I-1, C-I-2 and R is further strengthened by the findings that (i) these subfractions elicited PCA reactions in rats sensitized with a murine reaginic antiserum to R, and (ii) on mixing C-I-1 or C-I-2 with this reaginic anti-R serum, these subfractions, at a concentration of 50 μg/ml, neutralized the capacity of the serum to give PCA reactions. On the other hand, subfraction C-I-3, even at a concentration as high as 10 mg/ml, was not capable of either eliciting PCA reactions, or of neutralizing the murine anti-R IgE antibodies; this finding further supports the conclusion that this subfraction

was devoid of allergenic determinants capable of combining with IgE antibodies directed to the allergen(s) of KBG. As would be expected, the PCA reactions were completely neutralized by R at a concentration of 5 μg/ml.

Physicochemical Characterization of Chromatographic Fractions – C-I-1, C-I-2 and C-I-3

The molecular weights of C-I-1, C-I-2, and C-I-3, which were deduced in terms of their elution volumes by gel filtration through a column of Sephadex G-50 calibrated with proteins of known molecular weights, were of the order of 31,600, 19,000 and 4,800, respectively.

Since one of the objectives of this study was to isolate and to characterize the lowest molecular weight allergen(s) present in the aqueous extract of KBG pollen, subfraction C-I-2 was subjected to SDS-PAGE. It was thus confirmed that C-I-2 contained a component with a molecular weight of the order of 19,000 daltons. Moreover, this technique revealed the presence of an additional component in C-I-2 with a molecular weight of the order of 14,500 daltons, which had not been resolved by gel filtration. Hence, C-I-2 was rechromatographed on Sephadex G-50 and the eluates were arbitrarily pooled into two subfractions, i.e. C-I-2a and C-I-2d, which were respectively the ascending and descending portions of the chromatographic profile. By analysis with SDS-PAGE, it was confirmed that subfractions C-I-2a and C-I-2d consisted primarily of components with dimensions corresponding to substances with molecular weights of 19,000 and 14,500 daltons, respectively. Moreover, when these two subfractions of C-I-2 were covalently coupled to CNBr-activated cellulose discs, the resulting allergosorbents were

shown to be capable of binding human IgE antibodies to KBG; these data are in agreement with the results of the experiments demonstrating that C-I-2 inhibited the RAST.

As illustrated in figure 5b, the subfraction C-I-2d was further resolved by analytical ISO-EF on polyacrylamide gel into three major components with pI values of 3.2, 4.7 and 5.1, and a minor component with a pI value of 3.9. On the other hand, R was resolved into at least 30 components with pI values ranging from 3 to 10 (fig. 5a).

Fractionation of C-I-2 by Preparative ISO-EF and the Allergenic Activity of the Subfractions of C-I-2

In an attempt to achieve a better resolution of fraction C-I-2, it was subjected to preparative ISO-EF on Sephadex G-100 in the pH range of 3.5–10. The gel containing the components was arbitrarily subdivided into 15 subfractions. 75% percent of the focussed components were localized on the gel within a relatively narrow region corresponding to pI values in the range of 4.1–5.3 (fig. 4).

The allergenic activity of these 15 subfractions of C-I-2 was evaluated by inhibition

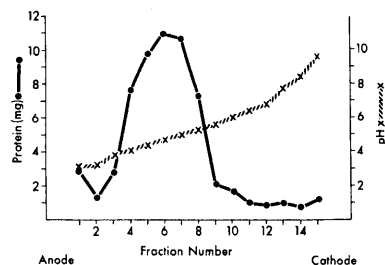


Fig. 4. Preparative ISO-EF of C-I-2 on Sephadex G-100 (Ampholine: pH 3.5–10; 96 h).

of the RAST utilizing allergosorbent discs prepared with R and the pool of human anti-KBG reagenic sera, and in terms of their ability to elicit PCA reactions in the skin of rats sensitized with murine anti-IgE antibodies. The results of these tests, listed in table I, indicate that whereas all 15 subfractions inhibited the RAST to varying degrees, only the subfractions 6–14 elicited PCA reactions. These results demonstrate that (i) all 15 subfractions possessed allergenic determinants capable of blocking the

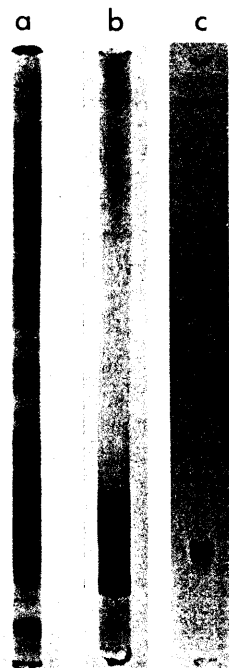


Fig. 5. Analytical ISO-EF of R(a), C-I-2d(b) and C-I-2-6(c) in polyacrylamide gel (Ampholine: pH 3.5–10; 22 h the anodal end of the gel is at the bottom).

Table I. Allergenic activity of focussed fractions of C-I-2

Fraction No.	PCA titer obtained ¹	Inhibition of RAST ² , %
1	nil	11
2	nil	43
3	nil	49
4	nil	38
5	nil	28
6	80	49
7	320	65
8	640	77
9	640	85
10	640	87
11	640	96
12	320	87
13	320	97
14	640	94
15	nil	73
R	640	96.6

¹ 100 µg (protein) of each fraction and of R was used for i.v. challenge of rats passively sensitized with serial dilutions of a murine reagenic anti-R antiserum.

² 100 µg (protein) of each fraction and of R was incubated with a pool of sera from humans allergic to KBG pollen, prior to addition to the allergosorbent disc prepared with R.

interaction between human anti-KBG IgE antibodies and the allergosorbent, and (ii) each of the fractions 6–14) possessed multiple allergenic determinants capable of triggering PCA reactions by cross-linking of the murine IgE antibodies fixed to the rat mast cells.

Demonstration of Hapten-Like Material in Subfractions of C-I-2

Although subfractions 1, 3, 4 and 5 did not elicit PCA reactions (table I; subfractions 2 and 15 were not tested because of their presence in only minute amounts), these subfractions completely inhibited

these reactions. Thus, addition of 500 µg of each of these 4 subfractions to 1 ml of the murine anti-R reagenic serum (which was used at a dilution of 1:640) completely inhibited the PCA reactions elicitable with R. The inhibition of these PCA reactions was deemed specific, since mixing any of these subfractions with a murine reagenic serum containing anti-OA IgE antibodies, prior to using this serum for a PCA reaction in rats, did not interfere with this reaction on subsequent i.v. challenge of the rats with ovalbumin. Hence, these findings – taken in conjunction with the results of the preceding experiments – support the interpretation that (i) all 15 subfractions possessed allergenic determinants capable of blocking the interaction between IgE antibodies produced by patients allergic to KBG and the allergosorbent, and (ii) whereas each of the subfractions 6–14 possessed multiple allergenic determinants capable of triggering PCA reactions by cross-linking the murine IgE antibodies fixed to the rat mast cells, the subfractions 1, 3, 4 and 5 possessed hapten-like properties capable of binding to both human and murine IgE antibodies, the formation of which had been elicited by exposure of the allergic patients to KBG pollen and by sensitization of mice to R.

Physicochemical Properties of Subfraction C-I-2-6

The 15 subfractions of C-I-2 were subjected to analytical ISO-EF. Subfraction 6, referred to hereafter as C-I-2-6, appeared to be the most homogeneous since, as shown in figure 5c, it was resolved into two closely associated components with pI values 4.5 and 4.9. However, on SDS-PAGE, subfraction C-I-2-6 showed only one diffuse band corresponding to a molecular weight of the order of 10,000 daltons.

Table II. Amino acid composition of fraction C-I-2-6

Amino acid	Residue per molecule
Lysine	8
Histidine	2
Arginine	1
Aspartic acid	10
Threonine	6
Serine	5
Glutamic acid	15
Proline	5
Glycine	9
Alanine	13
Half cystine	0
Valine	9
Methionine	1
Isoleucine	3
Leucine	4
Tyrosine	1
Phenylalanine	4
Tryptophan	1

The amino acid analysis of subfraction C-I-2-6 revealed that it contained all the naturally occurring amino acids except cysteine (table II). Moreover, it gave a positive test for carbohydrate. However, the lack of a sufficient amount of C-I-2-6 precluded the determination of the nature of its sugar residues. Nevertheless, since the sugar residues of the parent fraction, C-I-2, were identified by GLC as glucose and galactose, one may make the reasonable assumption that C-I-2-6 contained either or both of these sugars.

Immunological Properties of Subfraction C-I-2-6

Injection of 100 μ g of the subfraction C-I-2-6 with 5 mg $\text{Al}(\text{OH})_3$ into A/HeJ mice resulted in the production of IgE antibodies, which could be revealed by PCA in rats, using either C-I-2-6 or R for i.v. chal-

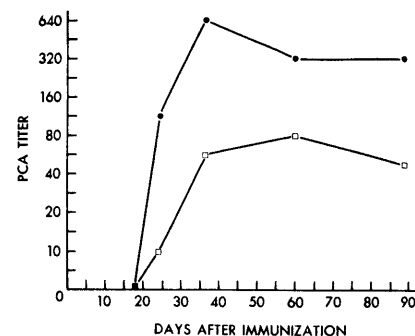


Fig. 6. IgE antibody response to C-I-2-6 in A/HeJ mice. The mice were immunized by an intraperitoneal injection of 100 μ g of C-I-2-6 with 5 mg $\text{Al}(\text{OH})_3$. $\square$ = PCA reactions elicited with C-I-2-6; $\bullet$ = PCA reactions elicited with R.

lenge. The kinetics of the anti-C-I-2-6 IgE response is illustrated in figure 6. It is interesting to note that the PCA reactions elicited by challenge with R were considerably higher, i.e. by a factor of at least 4, than the PCA titers obtained by i.v. challenge with C-I-2-6.

On the other hand, for the neutralization of anti-C-I-2-6 IgE antibodies in PCA reactions, C-I-2-6 proved to bind more effectively to these antibodies than R; thus, the amount of C-I-2-6 required for neutralization was only one-tenth of that of R. By contrast, in neutralization experiments using anti-R IgE antibodies, the amount of R required to inhibit the PCA reactions was one-tenth of that of C-I-2-6. The apparent discrepancy in the manifestation of the relative allergenic potency of these two preparations, as reflected by the results obtained by these two different methods, will be discussed later.

In spite of the fact that C-I-2-6 elicited in the presence of $\text{Al}(\text{OH})_3$ the production

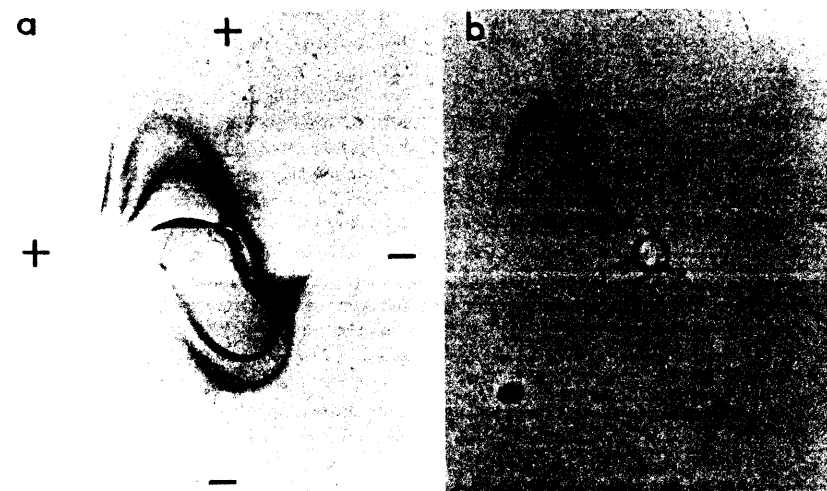


Fig. 7. CIE of R **a** and C-I-2-6 **b**. The electrophoresis in the first direction was performed for a period of 2 h at a potential of 100 V and in

the second direction for a period of 24 h at a potential of 25 V.

of murine IgE antibodies, it did not induce the formation of a detectable precipitating antibody on immunization of rabbits using FCA as an adjuvant, as was the case for R [12]. Nevertheless, C-I-2-6 was shown to share some of the antigenic determinants of R as demonstrated by CIE. Thus, as is evident from the CIE pattern of C-I-2-6 (illustrated in fig. 7b), using a sheep anti-R antiserum, two essentially superimposable precipitin peaks were revealed. By contrast, one could detect at least 15 antigenic components in R.

Discussion

One of the main objectives of this study was to isolate low-molecular-weight allergens from KBG pollen extract since, obviously,

the task of establishing the chemical structure of the determinants of an allergen molecule would be simpler, the smaller the size of the molecule. Hence, the dialysable fraction, D, was selected as a potential source of low-molecular-weight allergens and, on further fractionation by gel filtration on Sephadex G-25 [12], it yielded the skin-active subfraction D-I, which was, in turn, subjected to ion-exchange chromatography and gel filtration. In this manner, two allergenically active fractions, designated as C-I-1 and C-I-2, and an inactive fraction, C-I-3, were obtained. Although C-I-3 was considered allergenically inactive, as determined by RAST, it ought to be noted that the RAST assay may not have detected the entire spectrum of possible allergenic specificities present in various fractions, since it may be visualized that had one employed a larger ser-

um pool for the RAST, one may have detected IgE antibodies to C-I-3.

In view of the fact that the parent fraction had originated from the dialysable preparation of the aqueous extract of KBG, it was surprising to find that fraction C-I-1 possessed a component with an apparent molecular weight as high as 31,000 daltons, as estimated from its elution pattern on Sephadex G-50 and confirmed independently by SDS-PAGE. This apparently high molecular weight of C-I-1 may be attributable to its high carbohydrate content, which is known to vitiate molecular weight determinations based on methods which had been calibrated with proteins of known molecular weights. In view of the indications that C-I-1 possessed a high-molecular-weight component and since the original objective was to isolate low-molecular-weight allergen, C-I-2, with an apparently lower molecular weight of 14,500 daltons, was selected for further fractionation.

Fraction C-I-2 yielded thus, on further preparative ISO-EF, the still lower-molecular-weight allergenic component, C-I-2-6, which contained carbohydrate and all the naturally occurring amino acids except cysteine. The lack of cysteine suggests that allergen C-I-2-6 may be a single polypeptide chain. Glutamic acid, alanine, lysine and aspartic acid were present in relatively large amounts. It is interesting to note that the presence of these amino acids in relatively large amounts had been previously also found in allergenic fraction C-IV, isolated from timothy grass pollen [14].

Remarkably, the results of the experiments involving the neutralization of PCA reactions indicate that the low-molecular-weight allergen C-I-2-6 shared apparently all the allergenic determinants of R. One may contend that reaginic antibodies were

not elicited to all potentially allergenic specificities present in R by immunization of the inbred mice. Nevertheless, these results justify investing further efforts for the isolation of this allergen in larger amounts and for a more detailed characterization of its immunochemical properties.

ISO-EF analysis of the allergen C-I-2-6 revealed the presence of two components only, whereas R was shown to contain at least 30 components with pI values over the broad range from 3 to 10. By CIE, 15 of the components in R were shown to be antigenic, whereas under identical conditions allergen C-I-2-6 possessed only two antigenic components (fig. 7b). This is reminiscent of the findings of Aukrust *et al.* [3], who reported that the purified codfish allergen, DS22, which consisted of two antigenic components, had all the allergenic specificities of the crude extract containing 17 antigenic components.

During this study, it was observed that pollen fractions capable of inhibiting RAST were also capable of neutralizing PCA reactions and/or eliciting PCA reactions in rats passively sensitized with murine anti-R IgE antibody. Therefore, the neutralization of PCA reactions by fractions isolated during a particular fractionation procedure was routinely used to test their potency. Moreover, subfractions 1, 3, 4 and 5 of C-I-2 which could not elicit PCA reactions but which could completely inhibit PCA reactions normally elicitable with R, were regarded as possessing hapten-like properties. However, considering the dilution of the serum, these subfractions required for the neutralization of PCA reaction are very large. Hence, either affinity of hapten-like constituents for antibodies is extremely low or their concentration is very low.

The allergen C-I-2-6 was shown to neu-

tralize *in vitro* the anti-C-I-2-6 reaginic serum more effectively than R. Therefore, the observation (fig. 6) that injection of R elicited a stronger PCA reaction than that of C-I-2-6 in rats passively sensitized with murine anti-C-I-2-6 reaginic serum is at first slightly surprising, since one would have expected to obtain a stronger PCA reaction with the homologous allergen C-I-2-6. This apparent discrepancy in the relative allergenic potencies of C-I-2-6 and R, as determined by the two variations of the PCA method, may be reconciled by the reasonable suggestion that R, having a higher molecular weight, possesses a higher epitope density and would, therefore, bridge more effectively the IgE antibodies fixed to the mast cells, which is a prerequisite step for the triggering of their degranulation. Hence, R would be expected to be a better elicitor of PCA reactions than C-I-2-6.

The original aim of this study to isolate low-molecular-weight allergenic constituents of KBG has been achieved and the finding that several components of C-I-2, isolated by ISO-EF, had hapten-like properties, opens up the possibility of establishing the chemical composition of at least some of the allergenic determinants of KBG. However, it must be stressed that the hapten-like nature of these components has so far been demonstrated only with respect to the IgE antibodies present in the murine reaginic serum. The findings of inhibition of RAST indicate that these components can combine with human IgE antibodies, but the inhibition of RAST could not distinguish between a haptenic fraction from a polyvalent allergenic fraction. Hence, a definite identification of these components as haptens with respect to the IgE antibodies produced by KBG-sensitive patients must await the results of experiments involving the inhibition

of P-K reactions with serum of allergic patients. Obviously it is essential to establish the chemical structure of the individual allergenic determinants before their syntheses can be attempted by the application of modern methods of organic chemistry. The availability of these determinants will make it possible to convert them to tolerogenic conjugates capable of abrogating specifically the corresponding IgE antibodies, as was recently successfully achieved in this and other laboratories for well-defined haptens [21, 24, 26, 41] and higher-molecular-weight allergens [25].

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