

Isolation and Partial Characterization of Allergen C from Kentucky Blue Grass Pollen¹

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Abstract. An allergenic component, designated as Allergen C, was isolated from the retentate (R) of the aqueous extract of defatted Kentucky blue grass (KBG) pollen by a combination of preparative isoelectrofocussing and gel filtration. Allergen C was found to be a glycoprotein with molecular size of the order of 11,000 daltons and had a pI value of ~ 9.7 . It possessed all the allergenic determinants collectively present in R. The allergenic activity was found to be associated with the protein moiety of the molecule. Allergen C was one of the 15 antigenic components detectable in R by crossed immunoelectrophoresis with a hyperimmune sheep antiserum to R. Observations based on enzymatic degradation of the molecule suggested that the determinants recognized by murine IgE antibodies were different from those which combined with rabbit precipitating antibodies.

Introduction

Previous studies on the aqueous extract of Kentucky blue grass pollen (KBG aq. ext.) have revealed the presence of a multiplicity of antigenic components with a wide range of molecular size and charge [3, 4, 9]. Owing to the similarity of the physicochemical properties of many of the components, it has proven difficult to isolate pure allergens from KBG pollen. The impetus for obtaining pure allergens is generated by their re-

quirement for standardization of allergenic potency of allergenic extracts, which are contemplated for therapeutic use in humans and for elucidation of their physical and immunochemical properties.

In one of our studies [3], the nondialysable components, i.e. retentate (R) of KBG aq. ext., were fractionated by preparative isoelectrofocussing in the pH range 3.5-10. The most cathodal fraction, also referred to as focussed fraction 15, which contained only a few components compared to R, was found in a murine reagenic model system to possess all of the allergenic specificities associated with R. The present study was undertaken to further separate the components

¹ This work was supported by grants from the Medical Research Council of Canada.

² The capable technical assistance of Tom Cook is gratefully acknowledged.

of focussed fraction 15, by gel filtration with a view to isolating and characterizing the allergenically active component(s). This fractionation procedure resulted in the isolation of an allergenically active component, referred to as Allergen C.³

Materials and Methods

Preparation of the Nondialysable Components (R) of KBG aq. ext.

R was prepared from an aqueous extract of KBG pollen (Hollister-Stier Laboratory, Mississauga, Ont.) as previously described [5, 6]. Briefly, the aqueous extract of defatted KBG pollen was dialyzed through Visking tubing, No. 20 (Union Carbide Canada Ltd., Lindsay, Ont., Canada) against distilled water at 4 °C for 48 h. The nondialysable components, referred to as the retentate (R), were stored in the lyophilized state.

Preparative and Analytical Isoelectrofocussing Prep-ISO-EF and Anal-ISO-EF

Prep-ISO-EF of R was carried out on Sephadex G-100 superfine gel employing ampholines in the pH range of 3.5–10 as previously described [4]. Fraction 15 was comprised of those components which focussed in a 2.5 cm wide section of gel at the extreme cathodal end. Anal-ISO-EF was performed in polyacrylamide gel also in the pH range of 3.5–10 [4].

Gel Filtration on Bio-Gel P-60

Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, Calif.) was rehydrated and equilibrated with phosphate-buffered saline (7.75 g NaCl,

³ It is generally accepted that the antibodies involved in atopic allergy belong primarily, if not solely, to the IgE class of immunoglobulins. In the context of this report, the term 'allergen' and 'antigen' will be used to designate the moieties which can combine with IgE antibodies or with classes of antibodies other than IgE, respectively. These definitions do not imply that allergenic and antigenic determinants are necessarily distinct and different from one another. They serve only as a conveni-

ence to distinguish the combination of IgE antibodies with their ligands from those involving classes of antibodies other than IgE.

0.54 g KH_2PO_4 , 2.14 g Na_2HPO_4 , in 1 liter of distilled water, pH 7.2; PBS) and packed into a column (85 × 1.7 cm). Focussed fraction 15 of R (10–20 mg) was dissolved in a volume of 2 ml PBS, applied to the column and eluted at 4–5 °C with PBS. Since the allergenic component(s) designated as Allergen C was subsequently found to be stable towards denaturing conditions, a dissociating agent, such as urea, was employed to improve the resolution achieved during gel filtration.

For this purpose, the column of Bio-Gel P-60 was equilibrated with an aqueous solution of urea (0.1 M), the sample dissolved in 2 ml of an aqueous solution of urea (10 M) and elution was accomplished with the equilibrating solution. The absorbance of the eluates was continuously monitored at 280 nm with a Uvicord II Ultraviolet Absorptionmeter, type 8303A (LKB, Bromma, Sweden). Fractions of the separated protein peaks were tested for their allergenic activity before they were pooled. Prior to their lyophilization, the fractions were dialyzed against distilled water overnight using Spectrapor membrane (Spectrum Medical Industries, Inc., Los Angeles, CA) with a molecular size cut-off range of 6,000–8,000 to remove the urea.

Production of Sheep Precipitating Antiserum to R

Sheep (North American Laboratory Supplies, Gunton, Manitoba) were hyperimmunized with an emulsion of R in doses of 0.5 mg (dry weight) prepared with Freund's complete adjuvant. A series of five immunizations were administered intradermally and intramuscularly over a period of 138 days (9). This hyperimmune antiserum was employed for crossed immunoelectrophoresis.

Production of Rabbit Precipitating Antisera to Allergen C

New Zealand white rabbits (Canadian Breeding Laboratories, Montreal, Quebec) were immunized by intradermal injections into multiple sites, each animal receiving a dose of 500 µg Allergen C emulsified with Freund's complete adjuvant. Sera obtained two weeks later were found to elicit a single immunoprecipitin peak on crossed immu-

noelectrophoretic analysis of R. Therefore, to take advantage of the fact that the antisera recognized specifically Allergen C, large volumes of blood were obtained at this time by cardiac puncture. Sera were harvested after the blood had coagulated overnight at 4 °C and the clot removed by centrifugation.

Production of Murine Reaginic Antisera

Male A/HeJ mice (Jackson Laboratories, Bar Harbor, Me.), 6–7 weeks of age, were immunized by the i.p. administration of a dose of 100 µg of an allergenic preparation (R, Allergen C) adsorbed onto 5 mg of freshly prepared $\text{Al}(\text{OH})_3$ gel. 1 month later, the animals were bled from the tail and the sera of any one group of mice were pooled.

Measurement of Protein Content

Protein content was determined by the method of Lowry et al. [10]. Unless stated otherwise, amounts or concentrations of various fractions are expressed in terms of their protein content.

Determination of Carbohydrate Content

The phenol-sulfuric acid method [13] was employed to determine the carbohydrate content of Allergen C. A volume of 0.1 ml of an aqueous solution of phenol (80%) was added to a volume of 2 ml of an aqueous solution containing 10–20 µg of Allergen C. 5 ml of concentrated H_2SO_4 was added to the mixture with constant agitation. After standing at room temperature for 30 min, the absorbance of the solution at 490 nm was measured in a Coleman UV-VIS spectrophotometer. A reference blank solution was similarly prepared by substituting water for the solution of Allergen C. A standard curve was established with mannose in the concentrations ranging from 5 to 40 µg.

Amino Acid Analysis

Allergen C was hydrolyzed with 6 N HCl in the presence of thioglycolic acid and phenol at 110–112 °C for 24 h and analyzed⁴ with a Beckman automatic amino acid analyzer (Model

⁴ Amino acid analysis was kindly performed by Dr. F. Stevens of the Department of Biochemistry, University of Manitoba.

120–139; Beckman Instruments, Inc., Palo Alto, Calif.).

Determination of Molecular Weight

The molecular weight of Allergen C was determined by gel filtration on a Bio-Gel P-60 column calibrated with standard proteins of known molecular weight: ovalbumin, 45,000; chymotrypsinogen A, 25,000 (Pharmacia AB, Uppsala, Sweden) and Cytochrome C, 11,700 (Sigma, St. Louis, Mo.).

Immunodiffusion

Ouchterlony [14] double diffusion analysis was performed in 1% agar in phosphate-buffered saline (PBS), pH 7.4, containing 0.001% sodium azide in plastic Petri dishes. The antiserum was allowed to diffuse into the agar for a period of 3 h prior to the addition of antigens. By this protocol, the formation of the immune precipitates adjacent to the antibody wells was avoided.

Rocket Immunoelectrophoresis and Crossed Immunoelectrophoresis

Rocket immunoelectrophoresis, performed according to the procedures of Axelsen et al. [1], was used to determine the proportion of Allergen C present in R. For standardization of the method, constant volumes (40 µl) of solutions of Allergen C containing 1–10 µg were electrophoresed into 1% agarose gel (Indubiose A-37 Industrie Biologique Française, Gennevilliers, France) containing a rabbit precipitating antiserum to Allergen C. A linear relationship between rocket heights and Allergen C in the concentration range of 1–6 µg was obtained. This reference curve was employed to determine the amount of Allergen C present in R after the electrophoresis of solutions of R (40 µl containing 20–40 µg) into agarose gel containing the rabbit antiserum to Allergen C.

To analyze the antigenic components present in Allergen C relative to the antigenic components present in R, a sheep hyperimmune precipitating antiserum to R was employed in the crossed immunoelectrophoretic analysis according to the method of Axelsen et al. [1].

Radioallergosorbent Test (RAST)

Inhibition of RAST was performed according to the method of Ceska et al. [2]. Briefly, allergosorbent discs were prepared by coupling R to ccl-

lulose discs activated with cyanogen bromide. Allergen C was used in various doses to inhibit the binding of IgE antibodies to the allergosorbent discs. A pool of human sera obtained from individuals allergic to KBG pollen served as the source of IgE antibodies. The extent to which the IgE antibodies bound to the allergosorbent discs was evaluated by the addition of a preparation of ^{125}I -labelled rabbit antibodies specific for human IgE immunoglobulin (Pharmacia). The levels of ^{125}I bound to the allergosorbent discs were measured with a Beckman Gamma 300 System counter.

The capacity of Allergen C to inhibit RAST was expressed as a percentage of the original RAST value obtained under standardized conditions and was calculated according to the formula:

$\frac{A-B}{A} \times 100$, where A = RAST value (cpm) in absence of Allergen C, and B = RAST value (cpm) in presence of Allergen C.

Stability of Allergen C

The following procedures were carried out to test the stability of the allergenic activity of Allergen C on exposure to extremes of pH, a denaturing agent, and heating at 56°C . Amounts of $500\text{ }\mu\text{g}$ of Allergen C were separately dissolved in aqueous solutions of $0.1\text{ M H}_2\text{SO}_4$ and 0.1 N NaOH (in volumes of 0.5 ml) and held at a temperature of 4°C for 5–6 h. For treatment with denaturing agent, an amount of $500\text{ }\mu\text{g}$ of Allergen C was dissolved in an aqueous solution of 6 M guanidine hydrochloride (0.5 ml) and maintained at 37°C for 25 min. The three samples were then separately dialyzed with Spectrapor membrane (molecular cut-off size of 3,500) against distilled water at 4°C overnight and the samples were recovered by lyophilization of the retentate. To test for the stability of Allergen C to moderate heat, an aqueous solution of Allergen C (1 mg/ml) was held at 56°C for 5 h and then cooled to room temperature. Following the various treatments described, the allergenic activity of Allergen C was assessed by the method of inhibition of RAST.

Proteolytic Digestion of Allergen C

To determine if the allergenic activity of Allergen C was dependent on the integrity of the prote-

in moiety, the allergen was subjected to digestion with protease, which has a broad spectrum of specificity. For this purpose, proteolytic digestion of Allergen C was carried out with insolubilized protease (from *Streptomyces griseus*, covalently bound to carboxymethyl cellulose; Sigma). An amount of 50 mg (155 U/g) of the insoluble protease was washed several times with PBS. 1 mg of Allergen C was added to the enzyme suspension in a volume of 1 ml and incubated at 30°C for 30 min with continuous shaking. The insoluble enzyme was removed by centrifugation at the end of the incubation period. The allergenic activity of Allergen C was assessed by the method of inhibition of RAST following digestion with protease.

Trypsin, α -chymotrypsin and papain were employed in the degradation studies of Allergen C in order to gain some insights into the structural integrity of Allergen C responsible for its allergenic and antigenic properties. Preparations of insolubilized trypsin (from bovine pancreas), papain (from papaya latex) which had been insolubilized by their attachment to carboxymethyl cellulose and insolubilized α -chymotrypsin (from bovine pancreas) covalently bound to polyacrylamide, were all purchased from Sigma. An amount of 20 mg of each insolubilized enzyme (235 , 275 and 580 U/g , respectively) was transferred into separate small test tubes and washed several times with PBS. An amount of 1 mg of Allergen C in a total volume of 0.5 ml was added to each of the insolubilized enzyme preparations. The suspensions were then placed in a water bath at 30°C for 5–6 h with continuous shaking. The enzymatic reactions were stopped by removing the enzyme preparations by centrifugation. For control, a solution of Allergen C was processed under the same conditions in the absence of enzyme. The allergenic activity of Allergen C which had undergone the treatments described was evaluated in terms of the ability to elicit PCA reactions in rats sensitized with a murine anti-R reagent serum and also by the ability to neutralize the IgE antibodies present in the murine antisera for the inhibition of PCA reactions on subsequent challenge with R. The ability of the treated forms of Allergen C to form immunoprecipitin bands with a rabbit antiserum to Allergen C was assessed by Ouchterlony double diffusion analysis in agar.

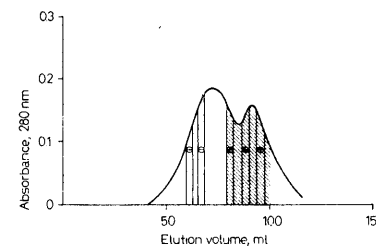


Fig. 1. Elution profile of focussed fraction 15 of R on Bio-Gel P-60 equilibrated with PBS. + refers to fractions that were capable of eliciting PCA reactions and – refers to fractions that were unable to elicit PCA reactions in rats sensitized with murine reagent antisera to R. The shaded area represents the pink-colored fractions, which on pooling, constitute the allergenic material designated as Allergen C.

Results

Isolation of Allergen C by Gel Filtration on Bio-Gel P-60

The chromatographic profile of focussed fraction 15 of R eluted with PBS is illustrated in figure 1. Fractions which corresponded to the ascending portion of the optical density profile of the first peak were found to be unable to elicit PCA reactions in rats sensitized with a murine anti-R reagent serum. The allergenic activity was found to be associated with fractions of the second peak, which coincided with the presence of pink-colored material; hence, their designation as Allergen C. A better resolution between the two peaks was obtained by gel filtration of focussed fraction 15 of R in the presence of urea (fig. 2).

Since allergenic activity was associated with the presence of colored material, the protocol for the isolation of Allergen C was modified to take advantage of the color as a

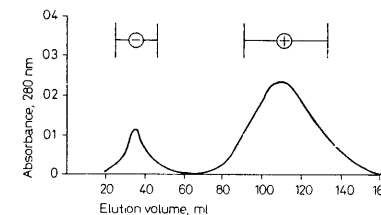


Fig. 2. Elution profile of focussed fraction 15 of R on Bio-Gel P-60 equilibrated with 0.1 M urea. Focussed fraction 15 of R was dissolved in 2 ml 10 M urea, applied to the column and eluted with 0.1 M urea. + refers to the recovered protein fraction (Allergen C) that was capable of eliciting PCA reactions in rats sensitized with a murine reagent anti-R sera and – refers to the recovered protein fraction that was unable to elicit the PCA reactions.

visual marker. Instead of using the whole focussed fraction 15 or R (i.e. a 2.5-cm segment of the preparative gel) as a source of Allergen C rich fraction, only that area of fraction 15 which corresponded to the pink focussed band following Prep-ISO-EF of R was cut out and eluted. Ampholines were removed by dialysis through Spectrapor membrane with a molecular weight cut-off range of 6,000–8,000. The elution profile of Allergen C on Bio-Gel P-60 prepared in this manner was similar to that illustrated in figure 2 with the notable difference in that the protein peaks were sharper than those seen in figure 2. Experiments reported in this study were performed employing Allergen C prepared in this manner.

Immunochemical and Immunological Properties of Allergen C

Crossed immunoelectrophoretic analysis of Allergen C employing a hyperimmune sheep precipitating antiserum to R revealed

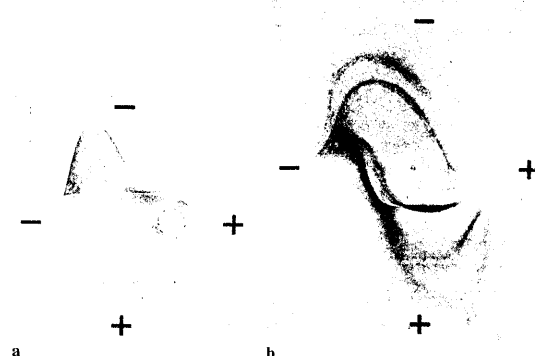


Fig. 3. Crossed immunoelectrophoretic analysis in agarose gel employing a sheep-precipitating antiserum to R. - refers to the cathodal ends of the gel and + refers to the anodal ends in the 2 dimension electrophoresis as described in Methods. **A** Analysis of Allergen C. **B** Analysis of R.

one cathodally migrating component (fig. 3).

Amount of Allergen C in R

By means of rocket immunoelectrophoresis employing a rabbit precipitating antiserum to Allergen C, it was determined that Allergen C was present in R in the order of 10%.

Allergenicity

The allergenic properties of Allergen C were established in terms of its ability to (i) elicit PCA reactions in rats passively sensitized with a murine reagenic antiserum to R; (ii) neutralize the PCA reactions elicitable with R; and (iii) inhibit RAST. It was found that (i) Allergen C at a dose of 200 μ g could elicit PCA reactions which corresponded to a titer of 640, with the murine reagenic antiserum to R. In animals sensitized in an identical manner, R at a dose of 50 μ g elicited a PCA titer of 1,280 (table I); (ii) Allergen C, employed at a dose of 500 μ g/ml, was found to be able to inhibit completely and specifically the PCA reactions in rats normally elicitable with R and

Table I. Elicitation of PCA by Allergen C

Dose of Challenging Allergen C	Allergen-reagenic antiserum system PCA Titer	
	R-anti-R	OA-anti-OA
100 μ g	320	nil
200 μ g	640	nil
500 μ g	640	nil
R 50 μ g	1,280	nil

Hooded rats were passively sensitized with 50 μ l of a serially diluted murine anti-R and anti-OA reagenic sera. 24 h later, two of the rats sensitized in an identical manner were challenged with one of the doses of Allergen C in 0.25% Evan's blue dye, injected intravenously. The PCA titers of the murine reagenic anti-R and anti-OA sera determined with R and OA (1 mg dry weight) separately were 1,280.

the murine reagenic anti-R serum system. By comparison, R could inhibit the PCA reactions in this system at a dose of 50 μ g/ml (table II); (iii) Allergen C could inhibit RAST to the extent of 78%. By comparison, both R and focussed fraction 15 of R could achieve 100% inhibition (fig. 4).

Table II. Inhibition of PCA by Allergen C

Concentration of Allergen C incubated with murine reagenic antisera¹

anti-R			anti-OA		
100 μ g/ml	500 μ g/ml	1 mg/ml	100 μ g/ml	500 μ g/ml	1 mg/ml
+	-	-	+	+	+

¹ Each reagenic antisera at a final dilution of 1:500 was incubated with the appropriate amount of Allergen C for 2-3 h before intradermal sensitization of rats. 24 h later the rats were challenged intravenously with a solution containing 500 μ g of R, 500 μ g of OA (dry weights) and Evan's blue dye, 0.25%.

+ refers to a positive PCA reaction; - refers to an absence of PCA reaction.

The titers of the murine anti-R and anti-OA reagenic sera were both 1,280, as determined with 500 μ g (dry weights) of R and OA, respectively.

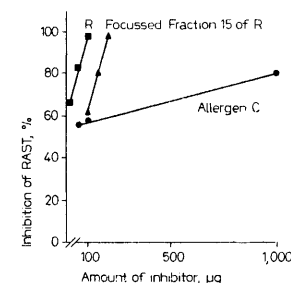


Fig. 4. Measurement of the allergenic potency of R, focussed fraction 15 of R and Allergen C, by inhibition of RAST. Allergosorbent discs were prepared by coupling R to cellulose discs. The potency was measured in terms of the ability of these allergens to inhibit the binding of the human IgE antibodies present in the pool of allergic sera to the allergosorbent discs.

Stability of Allergen C

Based on the observations that the degree of inhibition of RAST by Allergen C was virtually unchanged (table III) following exposure to acid and guanidine hydrochloride, it can be concluded that Allergen C was remarkably stable to these conditions. There

Table III. Inhibition of RAST by Allergen C after various treatments

Treatments ¹	% inhibition of RAST normalized values ²
None	100
56 °C	90
H ₂ SO ₄	92
NaOH	90
Guanidine HCl	100
Protease	0

Amounts of 50 μ g of Allergen C (both native and treated) were tested for their abilities to inhibit RAST.

¹ Details of the treatment procedures are described under Methods.

² These values have been normalized with respect to the degree of inhibition of RAST achieved with the native Allergen C, which was 76%.

appeared to be a slight loss of RAST inhibitory activity (a decrease of 10%) after exposure to NaOH and to a temperature of 56 °C.

Enzymatic digestion of Allergen C with a protease possessing broad specificity resulted in complete loss of its capacity to inhibit RAST (table III).

Table IV. Effects of enzyme treatment of allergen C on its ability to elicit PCA reactions

Treatment	Allergen-reaginic antisera system PCA titer	
	R-anti-R	OA-anti-OA
Trypsin	nil	nil
α -Chymotrypsin	40	nil
Papain	320	nil
Control ¹	320	nil

Hooded rats were passively sensitized by injecting intradermally volumes of 50 μ l of a serially diluted murine anti-R and anti-OA reaginic sera. 24 h later, 2 of the rats, sensitized in an identical manner, were challenged by an intravenous injection of one of the enzyme-treated Allergen C at a dose of 200 μ g/rat in a solution of Evan's blue dye (0.25%). The PCA titer of the murine reaginic anti-R and anti-OA sera as determined separately on challenge with a dose of 500 μ g of R or OA (dry weight) was of the order of 1,280.

¹ For control, Allergen C was incubated at 30 °C for 5-6 h in the absence of enzyme. A dose of 200 μ g was used to challenge sensitized animals.

The ability of Allergen C to elicit PCA reactions in rats sensitized with a murine reaginic anti-R serum after treatments with the insolubilized enzyme preparations is illustrated in table IV. Treatment with trypsin eliminated completely the ability of Allergen C to elicit the PCA reaction. The PCA titer, elicited with Allergen C following treatment with α -chymotrypsin, was several-fold lower as compared with the PCA titer elicited with the native Allergen C. Treatment with papain had no detectable effect on the ability of Allergen C to elicit PCA reactions. All preparations of enzymatically digested Allergen C were capable of inhibit-

Table V. Inhibition of PCA by enzyme-treated Allergen C

Treatment	Concentration of enzyme-treated Allergen C incubated with murine anti-R reaginic sera ¹		
	100 μ g/ml	500 μ g/ml	1 mg/ml
Trypsin	+	+	-
α -Chymotrypsin	+	+	-
Papain	+	+	-

For this study the murine anti-R reaginic sera at a final dilution of 1:500 was incubated with the indicated amounts of Allergen C for 2-3 h before intradermal sensitization of rats. 24 h later the rats were challenged intravenously with a solution containing 1 mg of R in Evan's blue dye (0.25%).

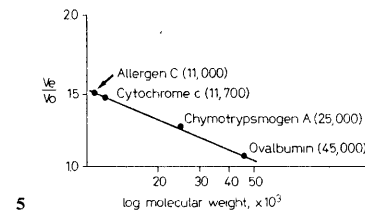
+ refers to a positive PCA reaction; - refers to an absence of PCA reaction.

¹ The titer of the murine anti-R reaginic sera evaluated with R at a dose of 1 mg (dry weight) was 1,280.

ing the PCA reactions elicitable with R and the murine reaginic anti-R sera (table V).

Antigenicity

Antigenic analysis of enzyme-treated Allergen C by immunodiffusion employing a rabbit-precipitating antiserum to Allergen C revealed that the ability of trypsin and α -chymotrypsin-treated Allergen C to form precipitin bands with rabbit anti-Allergen C was unaffected, i.e. reactions of identity were formed with native Allergen C. On the other hand, treatment of Allergen C with papain resulted in a preparation which did



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not form a detectable precipitin band with the rabbit antiserum.

Physicochemical Properties of Allergen C

Molecular Weight. The molecular weight of Allergen C was of the order of 11,000 daltons as determined by gel filtration on a calibrated column of Bio-Gel P-60 (fig. 5).

Amino Acid Analysis and Carbohydrate Determination. Allergen C was found to contain all the naturally occurring amino acids with the notable exception of cysteine, which was not detected. Tryptophan content was not determined. 1 mg of protein was found to be associated with 500 μ g of carbohydrate.

Isoelectrofocussing Profile. Anal-ISO-EF of Allergen C revealed the presence of basic component(s) that focussed at the cathodal end of the gel (fig. 6). Any components that focussed at a higher pH than the maximum obtained in the gel could have migrated out of the gel into the cathodal electrolyte solution. By comparison, R was resolved into at least 30 components that focussed over the entire gel.

pI Value. The pI value of Allergen C was established by (i) measuring the pH of the gel following Prep-ISO-EF and (ii) eval-



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Fig. 5. Estimation of the molecular weight of Allergen C. Molecular weight was estimated by gel filtration on a Bio-Gel P-60 column calibrated with standard proteins of known molecular weight. V_e = Elution volume; V_o = void volume.

Fig. 6. Anal-ISO-EF of Allergen C and R on polyacrylamide gel employing ampholine in the pH range of 3.5-10. + refers to the anodal end of the gel and - refers to the cathodal end of the gel. The protein bands were stained with Coomassie blue as described under Methods.

uating the relative amount (if any) of Allergen C eluted from the focussed gel; this was accomplished by rocket electrophoresis in agarose gel into which the rabbit antiserum to Allergen C had been incorporated. By these procedures, Allergen C was detected in fractions ranging in pH from 8.6 to 9.7 and the highest concentration of Allergen C was found to focus at pH 9.7. Since the pH of 9.7 represents also the highest pH achieved on focussing, the uppermost pI values of Allergen C could not be established from these experiments.

Discussion

A glycoprotein designated as Allergen C was isolated from the retentate of KBG aq. ext. by a combination of techniques involving Prep-ISO-EF and gel filtration. From the observation that the resolution of protein fractions of Bio-Gel P-60 was improved in the presence of a dissociating agent, such as urea, it may be suggested that some components are held together by noncovalent forces.

Allergen C had a pI value of ~ 9.7 , a molecular weight of 11,000 daltons and contained 500 μ g carbohydrate/mg protein. It possessed all the naturally occurring amino acids with the exception of cysteine. A more precise chemical composition must await further studies. Anal-ISO-EF revealed the presence of only one cathodally focussing component. However, other components, if any, that possessed a higher pI value than the maximum pH (pH 10) established on the analytical gel, would have migrated out of the gel into the cathodal electrolyte solution and, consequently, would not have been visualized. Due to the diffi-

culties in achieving gradients with pH values greater than 10, it was not possible to resolve whether or not Allergen C was indeed a single component with respect to its isoelectric form.

Crossed immunoelectrophoretic analysis of Allergen C employing a hyperimmune sheep-precipitating antiserum to R revealed the presence of one cathodally migrating antigenic component. By comparison, 15 antigens could be detected on R by this method. Thus, it may be said that Allergen C consisted of one antigenic component. In a parallel study [7] an allergenically active cytochrome c isolated from KBG pollen possessed certain properties such as color, size and a pI value that were similar to those of Allergen C. These comparisons made in retrospect, suggest that Allergen C may have been contaminated with cytochrome c. However, the absorption spectrum characteristic for cytochrome c was not detected in the preparation of Allergen C. The contamination, if any, of Allergen C by cytochrome c would have to be at a level below that detectable by the absorption study. The immunological relationships between the two allergens must await the availability of appropriate antisera to KBG cytochrome c.

On the basis that Allergen C could neutralize completely and specifically the PCA reactions elicitable with R and the murine anti-R sera, it was concluded that Allergen C possessed all the allergenic specificities present in R that were recognized by the murine sera used in this study. It should be noted that the experimental protocol for the inhibition of PCA employed murine reaginic sera (titer 1,280) at a final dilution of 1:500. This dilution was chosen in order to minimize the amounts of purified Allergen C which would be required to neutralize

completely the IgE antibodies present in the murine reaginic antisera to R. Consequently, it is conceivable that antibodies of certain specificities might be present in the diluted antiserum at levels below those required to elicit PCA reactions. As a result, the possibility existed that the presence of IgE antibodies with specificities other than those capable of combining with Allergen C might have not been detected by PCA in the protocol employed. Ideally, the experiments for the inhibition of PCA should be performed with undiluted reaginic sera. These experiments could not be performed because of the unavailability of the large amounts of purified allergen that would have been required to neutralize all the IgE antibodies present in the undiluted reaginic sera.

This limitation on the interpretation of the results of the inhibition of PCA experiments notwithstanding, it would appear that the number of different allergenic specificities present in R which were detectable with the murine antiserum to R was of a very low order. This conclusion is consistent with those reported in earlier studies [3, 4], in which it was demonstrated that purified components or fractions isolated from KBG aq. ext. possessed essentially all of the allergenic determinants collectively present in R. The studies of Malley et al. [11] have also suggested that the principal allergenic specificity of the aqueous extract of Timothy grass pollen was born by a well-defined allergen referred to as D3, which was composed of flavonoid pigment, a sugar moiety of cellobiose and at least one amino acid, threonine.

It ought to be noted that Allergen C and R were not equally effective in their ability either to elicit or to neutralize PCA reactions. Thus, on the basis of an equal protein

concentration, it was found that the concentration of Allergen C was 4-fold higher than that of R, which was required to elicit comparable PCA titers. Similarly, a 10-fold higher concentration of Allergen C was required to inhibit PCA reactions to the same extent as those inhibited with R. These observations have been interpreted to mean that the allergenic epitope density is significantly lower in Allergen C than that density which is collectively present on the various components of R.

Although studies on the allergenic composition of Allergen C carried out with the murine reaginic antibody system indicated that this allergen possessed all of the allergenic specificities present collectively in R, the RAST studies carried out with the pool of human allergic sera indicated that Allergen C lacked some of the allergenic specificities present in R. The reasons for the apparent difference in the allergenic specificities detected by these sera from two different species have been discussed previously [3, 4]. Thus, Allergen C was not capable of inhibiting the RAST beyond a maximum degree of 78%. Moreover, the slope of the RAST inhibition profile of Allergen C was markedly lower than that obtained with either focussed fraction 15 or R. These results suggest that Allergen C was not as efficient as the parent fractions in neutralizing the human IgE antibodies to the allergens of KBG pollen.

The observations that Allergen C proved to be remarkably stable to extremes of pH, moderate heat, and to guanidine hydrochloride is consistent with observations on the stability of most grass pollen allergens [12]. Although the allergenic activity of Allergen C appeared to be stable to denaturing agents, it is noteworthy to recall that another

er pollen allergen, such as ragweed antigen E, is labile in the presence of urea [8].

The finding that the allergenic activity was completely lost after enzymatic degradation of the protein moiety suggests that the carbohydrate moiety plays a minor role, if indeed any, in the allergenicity of Allergen C. Digestion of Allergen C with other enzymes has also provided some insights into the allergenic and antigenic properties of the allergen. Thus, trypsin apparently cleaved the allergen into components with hapten-like activity capable of inhibiting the PCA reactions normally elicitable with the reaginic anti-R sera on challenge with R. The products of the trypsin digest were, on the other hand, at least antigenically divalent since they retained their ability to precipitate with rabbit antiserum to Allergen C. Thus, allergenic activity appeared to depend on the integrity of that segment(s) of the polypeptide chain of Allergen C involving arginine and lysine residues. From the observations that digestion with chymotrypsin resulted in only a moderate loss of allergenic activity, it may be suggested that aromatic amino acid residues may also contribute to a moderate extent to the integrity of the determinant(s) responsible for the allergenic activity.

The antigenic properties of Allergen C were apparently unaffected by digestion with trypsin or chymotrypsin. By contrast, digestion of Allergen C with papain, which has a broad specificity, had no detectable effect on its allergenic activity; however, the antigenic determinants were degraded as indicated by its inability to form a precipitin reaction with rabbit anti-Allergen C serum.

Moreover, owing to the uncertainty as to whether or not Allergen C consists of a single isoelectric form, it is not clear if the al-

lergenic and antigenic specificities are both associated with one molecular species or if the two properties may each be found on separate molecular species that might constitute Allergen C. Nevertheless, it is clear that the allergenic determinants recognized by the murine IgE antibodies are different from the antigenic determinants recognized by the rabbit-precipitating antibodies. It remains to be determined if the IgE class of antibodies recognize the identical determinant to which other classes of antibodies may be formed in any one species.

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Received: October 22, 1980

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