

AB 104

Adverse

23474

Scand. J. Immunol. 8, 437-441, 1978

Isolation and Partial Characterization of the Allergen in Mountain Cedar Pollen

G. N. GROSS, J. M. ZIMBUREAN & J. D. CAPRA

Department of Microbiology and the Pauline and Adolph Weinberger Laboratory for Cardiopulmonary Research, Department of Internal Medicine, University of Texas Health Science Center at Dallas, Dallas, Texas, USA

Gross, G.N., Zimburean, J.M. & Capra, J.D. Isolation and Partial Characterization of the Allergen in Mountain Cedar Pollen. *Scand. J. Immunol.* 8, 437-441, 1978.

A biologically active fraction from a crude extract of mountain cedar pollen has been purified and partially chemically characterized. An ammonium bicarbonate extract of commercial defatted pollen was fractionated by G-100 Sephadex chromatography and the biologically active fraction was found to be homogeneous by polyacrylamide gel electrophoresis and N-terminal amino acid sequence analysis. The biologically active component is a 50,000 dalton protein whose N-terminal amino acid sequence is Asp-Asn-Pro-Ile-Asp. These findings provide a further purified pollen allergen for immunologic studies and the first such purified allergen having clinical significance in a limited geographic region.

Gary N. Gross, Department of Microbiology, Department of Internal Medicine, University of Texas Health Science Center at Dallas, Dallas, TX 75235, USA.

Purification of allergens is a fundamental requirement to complete understanding of IgE-mediated disease. Major advances have been achieved in understanding the IgE response using purified components of short ragweed pollen [8-10, 14, 18], and comparative studies using other pollens will determine the general applicability of these principles. Purified allergens are also essential for optimal standardization of allergenic extracts used in the diagnosis and treatment of allergic diseases [3, 12].

Problems encountered in previous allergen purification have been multiple. Pollens of general clinical significance which have been studied are collected at times when several other pollens are present in the environment, and initial contamination may therefore occur

[13]. Alternatively, the plants may be grown in a hot-house, but this method limits quantities of pure pollen. Other antigenic material such as bee venom may be obtained in greater amounts, but large population studies with such proteins are difficult owing to the relative infrequency of exposure and sensitization [1].

The use of cedar pollen provides a natural method of overcoming these difficulties. The pollen is the only one found in certain collection areas during the winter months [6, 17]. It can be obtained in relatively large quantities without contamination of other pollens.

These studies were undertaken to provide a purified pollen antigen which could be used to study immunologic variables in patients in the Southwest. The relatively restricted geographic distribution of this pollen provides an additional

advantage in that it will allow prospective studies on the acquisition of sensitivity and symptoms in individuals moving into this area.

MATERIALS AND METHODS

Pollen extract. Defatted cedar pollen (*Juniperus sabinooides*) was kindly provided by Greer Laboratories (Lenoir, North Carolina). 10 g of defatted pollen was placed in a 4 litre plastic beaker with 100 ml of 0.125 M ammonium bicarbonate. This suspension was gently shaken overnight at 4°C and then centrifuged at 2500 rpm at 4°C for 20 min. The supernatant was then centrifuged at 10,000 rpm at 4°C for 10 min and this supernatant concentrated by lyophilization. This preparation is referred to as 'crude cedar extract'.

Column chromatography. Crude cedar extract derived from 5 g of pollen was dissolved in 13 ml of buffer and was subjected to gel filtration chromatography, using a 200 × 2.5 cm column of Sephadex G-100 in 0.125 M NH_4HCO_3 . Fractions of 300 drops were collected, and the optical density at 280 nm was determined. Fractions representing individual peaks by optical density were pooled and lyophilized.

Polyacrylamide gel electrophoresis. To assess the homogeneity of each peak and molecular size of the component, polyacrylamide gel electrophoresis (PAGE) was employed [11]. Approximately 50 µg of protein was applied to each well of a 10% polyacrylamide slab gel. Markers of 70,000-dalton ovalbumin, 44,000-dalton chymotrypsinogen, and 25,000-dalton bovine serum albumin were included. Samples were prepared in sodium dodecyl sulphate (SDS) gel buffer, and, where indicated, were reduced in 20 mM dithiothreitol at 56°C for 1 h and alkylated in 50 mM iodoacetamide at room temperature for 30 min. Gels were run on a Bio-Rad Slab gel apparatus (Model 220) using an initial voltage of 50 for 30 min and then 100 V for 240 min, provided by a Savant power supply (Model CVR 300). Gels were stained in Coomassie blue in 50% acetic acid and destained in a 45% ETOH and 6% acetic acid solution.

Skin testing. Multiple samples of whole cedar extract were separated by column chromatography during initial purification. Fractions from each column were tested on the volar surface of the forearm in an individual known to be sensitive to cedar pollen [2]. A panel of ten subjects who had symptoms of cedar pollenosis was later tested using pooled fractions separated by column chromatography and concentrated by lyophilization. Protein concentrations of these final test solutions were calculated using an extinction coefficient of 1.0 and optical density (O.D.) at 280 nm. Fractions A, B, C and D contained approximately 105, 40, 160 and 25 µg protein per millilitre. Fraction E was composed of a low molecular weight chromophore and contained the majority of O.D. units from the crude extract. 15 min after the extract was applied to the skin, the largest diameter of each wheal was determined by a technician unaware of the allergen content of the test material. Histamine (0.1 mg/ml) and saline control tests were included.

Sequencing procedure. The fraction obtained from

G-100 chromatography, which was shown to be homogeneous by PAGE and active by skin testing, was dissolved in 25% acetic acid. Approximately 1.0 mg of this material was studied in an automated protein-peptide sequencer (Beckman Model 890C), utilizing techniques and programs previously described from this laboratory [4]. Phenylthiohydantoin derivatives were identified by gas chromatography, high-pressure liquid chromatography, and by back hydrolysis and subsequent amino acid analysis.

Amino acid analysis. Purified cedar pollen allergen was lyophilized *in vacuo* with HCP and analysed on a Durram D-500 amino acid analyser equipped with a 30 min program.

RESULTS

Sephadex G-100 separation of crude cedar extract by column chromatography resulted in five identifiable peaks. Fractions were pooled as indicated in Fig. 1 and tested for activity by skin test.

The five chromatographic fractions were analysed by polyacrylamide gel electrophoresis. Only fractions C and D stained for protein, with fraction C showing two identifiable bands (approximately 50,000 and 100,000 daltons) and fraction D a single band near the dye front. Whole crude cedar extract showed similar bands. A polyacrylamide gel of whole cedar extract and fractions C and D is shown in Fig. 2. Also shown is completely reduced and alkylated fraction C, which resulted in a single band of approximately 50,000 daltons.

During initial testing of unconcentrated fractions of chromatographed cedar extract, peak C was found to give consistently positive skin test results in one subject. Ten patients with positive reactions to crude cedar extract and symptoms of cedar pollenosis were therefore tested (Table I). Skin test results are expressed as wheal size in millimetres greater than saline control and indicate that the major reactivity resides in fraction C. Fraction A did not give a positive result in any of the patients tested. In only one patient (J.C.) did another of the fractions produce a wheal of comparable size to that of fraction C. A small group of normal controls was tested and found to have no cutaneous reactivity to any of the fractions.

The amino acid composition of fraction C is shown in Table II, and preliminary amino acid sequence analysis of 20 nmol of this material revealed a homogeneous sequence: Asp-Asn-Pro-Ile-Asp.

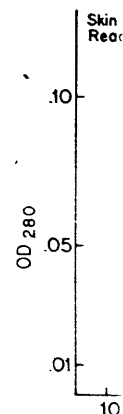


FIG. 1. Elution reactivity indi



FIG. 2. 10% poly cedar extract, fraction column and fraction alkylation (CRA). M chymotrypsinogen albumin (MW 70,000)

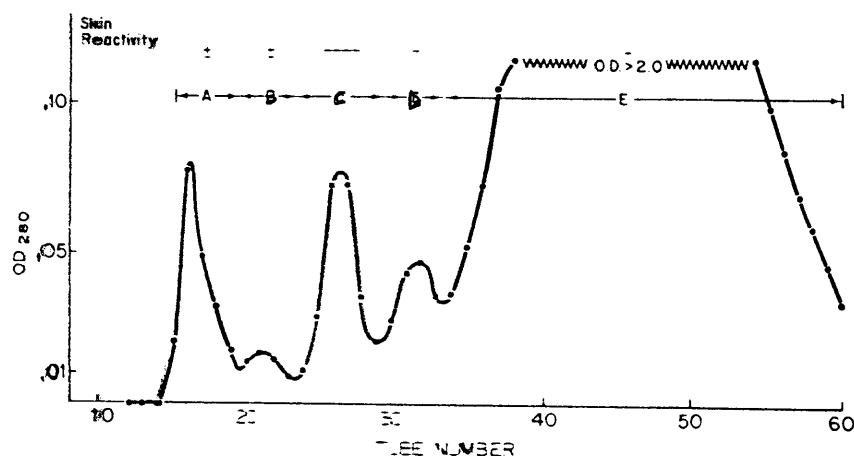


FIG. 1. Elution profile of crude cedar extract from a 2.5 x 100 cm G-100 Sephadex column. Skin reactivity indicates results of testing with concentrated pooled fractions.

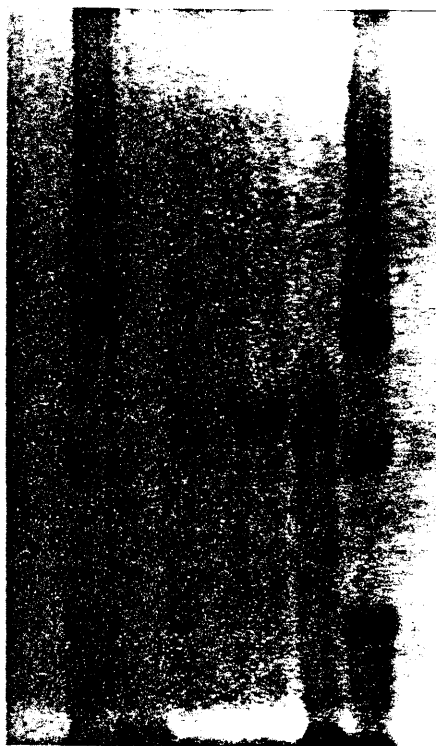


FIG. 2. 10% polyacrylamide slab gel showing crude cedar extract, fractions C and D from G-100 Sephadex column and fraction C after complete reduction and alkylation (CRA). Markers of ovalbumin (MW 25,000), chymotrypsinogen (MW 44,000) and bovine serum albumin (MW 70,000) are included on either side.

TABLE I. Skin tests with cedar fractions

Patient	Wheal size of skin test greater than control (mm)			
	Fraction B	Fraction C	Fraction D	Fraction E
M.S.	3	6	3	2
L.S.	4	19	6	5
F.S.	0	7.5	0	0
C.S.	3	6	3	0
E.M.	0	6	0	0
E.N.	3	10	3	0
C.J.	1	4	0	0
G.G.	1.5	7	5.5	2
M.E.	1.5	4	1.5	0
J.C.	0	5	5	5

DISCUSSION

This study demonstrates that a relatively small (50,000 daltons) fraction of crude mountain cedar pollen extract accounts for the pollen's major allergenicity. The results of polyacrylamide gels indicate that the active fraction consists of a single polypeptide chain after reduction and alkylation, but may form dimers under other conditions. This active fraction has been subjected to preliminary amino acid sequencing, which indicates its N-terminal structure to be homogeneous.

TABLE II. Estimated amino acid analysis of the purified allergen from cedar pollen

Amino acid	Residues/molecule	Percentage
CMC	11.0	2.45
Asp	66.3	14.77
Thr	30.4	6.77
Ser	38.5	8.57
Glu	33.4	7.44
Pro	8.4	1.87
Gly	47.8	10.65
Ala	35.9	7.99
Val	29.5	6.57
Met	13.4	2.98
Ile	22.4	4.99
Leu	27.3	6.08
Tyr	12.6	2.81
Phe	18.4	4.10
His	19.5	4.34
Lys	21.6	4.81
Arg	12.6	2.81
Trp	ND	ND
Total	449	100.00

CMC = carboxymethyl cysteine.

The application of such methods of protein isolation and purification have been used previously for other allergens [13], and the availability of purified allergens has led to a clearer understanding of the basic immunologic mechanisms of IgE-mediated events [3, 7].

Optimal diagnosis and treatment of allergic disease is dependent upon the availability of such purified, standardized reagents [5, 12], and modifications of specific antigenic sites may lead to improved modalities in therapy [15]. Cedar pollen extract has been shown to be effective in limiting symptoms of patients having cedar allergy [16] and this antigen can therefore be used in the study of immunologic events associated with beneficial immunotherapy.

A further major role of purified allergens is the study of the genetics of the immune response [13]. In this regard a purified cedar allergen offers exceptional advantages. The relatively limited distribution of this antigen to the southwestern United States provided an opportunity for the sensitivity to and subsequent sensitization by the allergen to be examined in large groups of individuals moving into this area. The advantages of a pure allergen, previously shown to be clinically effective and having limited distribution, should allow further studies of both clinical and immunological events of importance in allergic diseases.

ACKNOWLEDGMENTS

Supported in part by grants from the National Institutes of Health (AI 12127 and AI 12796) 22411). Mike Zimburean was supported by the Morgan Good Foundation (51310).

REFERENCES

- 1 Benton, A.W., Morse, R.A. & Stewart, J.D. Venom collection from honey bees. *Science*, **142**, 228, 1963.
- 2 Booth, B.H., III. Diagnosis of immediate hypersensitivity. p. 77 in Patterson, R. (ed.) *Allergic Diseases*. Lippincott, Philadelphia, 1972.
- 3 Buckley, R.H., Seymour, F., Sanal, S.O., Ownby, D.R. & Becker, W.G. Lymphocyte responses to purified ragweed allergens *in vitro*. I. Proliferative responses in normal, newborn, agammaglobulinemic, and atopic subjects. *J. Allergy clin. Immunol.* **59**, 70, 1977.
- 4 Capra, J.D., Tung, A. & Nisonoff, A. Structural studies on induced antibodies with defined idiotype specificities. I. The heavy chain of A/J mouse anti-p-azophenylarsonate antibodies bearing the cross-reactive idiotype. *J. Immunol.* **114**, 1548, 1975.
- 5 Desbordes, J. The need for standardization of allergic extracts utilized in humans for diagnosis and therapy. In *International WHO-IABS Symposium on Standardization and Control of Allergens Administered to Man*. Develop. Biol. Standard. **29**, 267, 1975.
- 6 Fein, B.T. & Karmin, P.B. A 10 year survey of the hay fever plants and important atmospheric allergens in the San Antonio, Texas metropolitan area. *J. Allergy*, **33**, 141, 1962.
- 7 Ishizaka, K. Cellular events in IgE antibody response. *Advanc. Immunol.* **23**, 1, 1976.
- 8 Ishizaka, K., Okudaira, H. & King, T.P. Immunogenic properties of modified antigen E. II. Ability of unreacted antigen and a polypeptide chain to prime T cells specific for antigen E. *J. Immunol.* **114**, 110, 1975.
- 9 Kishimoto, T. & Ishizaka, K. Regulation of antibody response *in vitro*. VII. Enhancing soluble factor for IgE and IgG antibody response. *J. Immunol.* **111**, 1194, 1973.
- 10 Kojima, S. & Ovary, Z. Effect of *Nippostrongylus brasiliensis* infection on anti-hapten IgE antibody response in the mouse. II. Mechanism of potentiation of the IgE antibody response to a heterologous hapten-carrier conjugate. *Cell. Immunol.* **17**, 383, 1975.
- 11 Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680, 1970.
- 12 Lichenstein, L.M. Standardization and efficacy of allergen extracts. *New Engl. J. Med.* **295**, 1195, 1976.
- 13 Marsh, D.G. Allergens and the genetics of allergy. In Sela, M. (ed.) *The Antigens*. Vol. III. Academic Press, New York, 1975.

Warrick, D.G. *Allergol, Proc. 8th Int. Congr.* 1973, 381-393. Excerpta Medica. Amsterdam.

Warrick, D.G., Patterson, R., Zeiss, C.R., Irons, J., Krensky, J.J., Suszko, I.M. & Levitz, D. Isolation of polymerized and unpolymerized antigen E for immunotherapy of ragweed allergy. *New Engl. J. Med.* 295, 1160, 1976.

Warrick, D.G., Mitchell, D.Q., Greely, R.L., Undergraff, B.R. & Selfridge, H.A. Immunotherapy for mountain cedar pollinosis. *J. Allergy clin. Immunol.* 58, 39, 1976.

17 Schatz, M. An approach to diagnosis and treatment in the migrant allergic population. *J. Allergy clin. Immunol.* 59, 254, 1977.

18 Takatsu, K., Ishizaka, K. & King, T.P. Immunogenic properties of modified antigen E. III. Effect of repeated injections of modified antigen on immunocompetent cells specific for native antigen. *J. Immunol.* 115, 1469, 1975.

Received 10 April 1978

Received in revised form 8 August 1978