

Allergens of *Alternaria*: Further Characterization of a Basic Allergen Fraction¹

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Abstract. A basic allergen fraction isolated from *Alternaria* extracts by preparative flat-bed gel isoelectric focusing (as previously reported) was subjected to further characterization. The fraction was chromatographed on a Sephadex G-25 column and exhibited two major peaks, the first of which was allergenic and the second of which was nonallergenic but caused a precipitate to form when added to a mixture of sodium dodecyl sulfate (SDS) (1%) and trichloroacetic acid (5%). SDS-PAGE indicated that the allergen component of the basic fraction migrated at about the same rate as insulin (5.8 kdaltons). Both G-25 peaks were carbohydrate rich with a protein:carbohydrate ratio (by weight) of 1:4.5 and 1:5.8 for peak 1 and peak 2 respectively. Heat (100°C for 10 min) and enzyme digestion (trypsin, α -chymotrypsin and pepsin) did not reduce the RAST inhibition values for peak 1 or the original basic fraction. The basic fraction did not bind human IgE specific for birch pollen indicating that the RAST inhibition and IgE (*Alternaria* specific) binding to radioimmuno-electrophoresis plates was not due to nonspecific binding of IgE. We conclude that the allergenicity of the basic fraction is due to a small molecule that is predominantly carbohydrate in nature and is a major allergen of *Alternaria*.

Introduction

There has been significant progress in defining the allergenic components of the mold *Alternaria*. This mold is an important aeroallergen [1] having considerable variability of allergenicity among the 50 or so species. Thus, it is necessary to demonstrate the major allergenic components and their species distribution. Most of the allergenic material from *Alternaria* was shown to be carbohydrate-rich or glycoprotein in nature [2-5]. Hoffman et al. [6] has defined several spore-specific antigens of which only one was an allergen. This allergen was not isolated and characterized. Yunginger et al. [7] isolated a major allergen fraction from mycelial extracts and designated it Alt-1. The characteristics of this fraction indicated that it was acidic (pI = 4.5), carbohydrate-rich and consisted of at least six components. Vijay et al. [8] isolated several overlapping fractions by column chromatography and demonstrated allergenicity to a greater or lesser degree in most of them. None of these allergens were isolated to the purity of Yunginger's Alt-1.

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Although there are many antigens in *Alternaria* extracts, perhaps as many as 40-50 in some species [6, 9], a small percentage of these serve as allergens. The complete allergenic profile of *Alternaria* has been difficult to determine due to variability between species and sources of extracts (mycelia, spores, broth, etc.). Another possible variability exists in the hypersensitivity to select allergens by *Alternaria*-sensitive patients. These difficulties are not insurmountable, but make the job of allergen definition a laborious one.

We have previously documented [10] the isolation and allergenicity of a low molecular weight, basic allergen of *Alternaria* that is one of several allergens that are detectable in vivo and in vitro. The molecular weight had been estimated at 8-9.5 kdaltons but due to the fact that sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the basic fraction produced considerable smearing, this could only be considered an approximation. The pI of the fraction was 9.5-9.8 and it was able to inhibit a standard RAST assay for *Alternaria tenuis* (Phadebas RAST - Pharmacia Diagnostics, Piscataway, N.J.) up to 50% when using a 13-patient serum IgE pool. Al-

lergenicity was also demonstrated by intradermal skin test and by radioimmuno-electrophoresis. The present study sought to further characterize the basic allergen fraction in terms of its molecular properties.

Materials and Methods

Alternaria samples. Mycelia powder and broth lyophilized samples of 3 Iowa air sample isolates of *Alternaria*, designated I-Alt 1803, 1808 and 1809 were used to prepare extracts as previously described [10] and were subjected to preparative flat-bed gel isoelectric focusing (IEF) to isolate the basic allergen fraction of pI = 9.5-9.8 [10]. The gel fractions of this pH range from several IEF runs were eluted with distilled water, pooled, dialyzed against 0.05M NH₄HCO₃ and lyophilized. The resulting white powder was redissolved in either Tris-saline buffer for column chromatography separation or in water for other characterization experiments. The protein concentration of the allergen solutions was monitored with a Coomassie dye procedure [11] throughout this study.

Sephadex G-25 column chromatography of the basic fraction. The basic fraction concentrate (0.5 mg lots - 2.5 mg total) from IEF was applied to a Sephadex G-25 column (1.5 x 100 cm) equilibrated with Tris-NaCl (0.05 + 1.0 M, pH = 7.5) buffer. 2-ml fractions were collected at a flow rate of 16 ml/h and the OD₂₈₀, OD₂₀₈, and protein content (Coomassie dye assay) were monitored. Resulting peak fractions were pooled, dialyzed against distilled water, lyophilized, and redissolved in 2 ml of water. Characterization was done on the two major peaks and the basic fraction starting material.

Carbohydrate determination. The basic fraction (from IEF) and the two G-25 column peak concentrates were assayed for carbohydrate (CHO) content by the α -naphthol reaction [12]. Briefly, 100 μ l of test solution dilutions, a water control, a protein control (trypsin) and glucose standards were added to 2.5 ml of cold concentrated sulfuric acid and mixed. This mixture was warmed to room temperature and then placed in a boiling water bath for 3 min. The tubes were then cooled to room temperature and 200 μ l of a freshly prepared 2% alcoholic solution of α -naphthol were added with mixing. After 6 h, OD₅₄₀ and γ_{max} were recorded using the water blank as reference. The trypsin protein control indicated no color reaction (trypsin is not a glycoprotein), thus any color reaction with the test solutions would be attributable entirely to CHO. An absorption maximum at 570 nm would indicate hexose sugars, 550 nm for pentose and 560 nm for heptose and methyl pentose sugars. At 560 nm, ribose, arabinose, and fructose have molar extinction coefficients to within 5% of that for glucose [12]. The test solutions were compared to the glucose standards for quantitation.

Polyacrylamide gel electrophoresis. The G-25 column peaks and the basic fraction were subjected to standard SDS-PAGE [13]. 10 or 12% slab running gels were used with a sample load of 5-12 μ g (by protein assay). The gels were run at 30-40 mA/gel at 4°C and stained with Coomassie brilliant blue. Molecular weight marker proteins (low molecular weight kit - BioRad, and insulin - Sigma) were used for SDS-PAGE calibration. Also, columns from the slab SDS-PAGE of the basic fraction were cut into 5-mm fractions and eluted in 150 μ l of distilled water. 50 μ l of the eluate were used for RAST inhibition analysis to identify the position of the allergen(s) in the gel.

RAST inhibition. A competitive RAST inhibition assay was used to confirm the allergenicity of the basic fraction and column chromatography peak fractions. It was also used in subsequent heat- and enzyme-sensitivity assays. A standard RAST procedure (Phadebas - Pharmacia) was used as previously described [10] with incubation of 50 μ l of varying dilutions of the suspected allergens with commercial *Alternaria* allergen discs and pooled human anti-*Alternaria* sera. The use of commercial RAST discs was valid since most of the allergens in our crude extracts were represented on the commercial discs. Competitive inhibition values using our crude extracts ranged as high as 80%. Inhibition of the RAST assay by the basic allergen fractions also indicated its presence on the commercial discs. The human anti-Alt sera was a pool of 13 patients having *Alternaria*-specific IgE levels of RAST class 3 or above and not having undergone immunotherapy. The tests were run in triplicate and the results were plotted as percent inhibition (compared to known sera controls).

Heat stability. Aliquots of the basic fraction were subjected to heat (boiling water bath) for 10 min and tested by the RAST inhibition assay. Unheated controls were used for comparison.

Enzyme sensitivity. The basic fraction and the allergen peak from the G-25 column chromatography were subjected to digestion with trypsin, α -chymotrypsin, and pepsin (all at 0.25% in PBS) for 60 min at 37°C. The enzymes were deactivated by heating in a boiling water bath for 10 min. The test was carried out in 1-ml capped microcentrifuge tubes so that condensation of the solution on the walls of the tubes after heating could be briefly centrifuged to the bottom of the tube, thus preventing concentrating the allergens by evaporation. The digested preparations were then tested for RAST inhibition. Any reduction in the RAST inhibition values would indicate that the allergen was sensitive to the enzyme.

Precipitability with trichloroacetic acid (TCA). Both peak fractions from the G-25 column chromatography (as well as the basic fraction starting material) gave positive reactions with the Coomassie dye protein assay. This assay was used to determine whether the two major components of the basic allergen fraction were precipitable with 5% TCA. Both peak fractions were made to 5% TCA by the addition of an equal volume of 10% TCA and centrifuged in a microcentrifuge (Fisher model 235 - Fisher Scientific) for 10 min. An aliquot of each supernatant was then tested for protein by the Coomassie dye assay. TCA alone gave no color reaction with this test nor did it interfere in the protein measurement of BSA standards when the test solutions were not centrifuged (i.e., the assay measures denatured proteins).

Test for nonspecific IgE binding. A RAST inhibition assay using birch pollen allergen discs and human IgE antibody pollen reference serum was used to determine if the basic allergen fraction of *Alternaria* bound human IgE nonspecifically. Any inhibition of the RAST assay would indicate either nonspecific binding of IgE or cross-reactivity between birch pollen and the *Alternaria* basic allergen.

Results

The basic allergen of *Alternaria* was isolated from all three Iowa isolates as well as from the broth in which they grew. The basic allergen fractions from IEF were similar in their column chromatographic

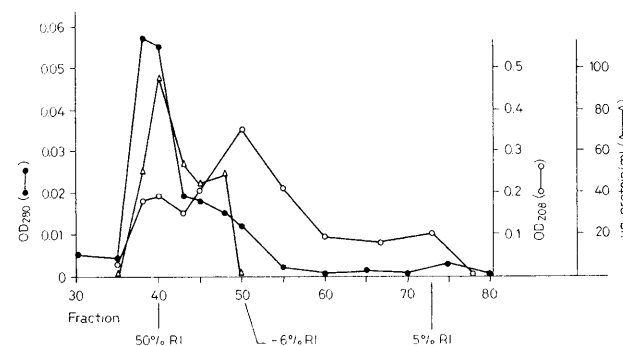


Fig. 1. Sephadex G-25 column chromatography of the IEF basic fraction. OD₂₈₀, OD₂₀₈ and protein content of 2-ml fractions were monitored. RAST inhibition assays for allergen activity was performed on the peak fractions prior to concentration by lyophilization.

Table 1. Characterization of the basic fraction and G-25 column peaks

Parameter	Peak 1	Peak 2	IEF FRXN
μ g protein/ml ¹	210 ²	80	120
μ g hexose/ml	995 ²	470	450
Protein:CHO ratio	1:4.5	1:5.8	1:2.7
% RAST inhibition	48.4	4.9	48.6 $\pm$ 2.2 ⁴
% RAST inhibition after treatment with:			
Heat	-	-	37.1 ⁵
Trypsin	52.3	-	34.1
α -Chymotrypsin	48.2	-	38.0
Pepsin	44.6	-	39.1
TCA-precipitable	yes	no	-

¹ Starting material = 2.5 mg IEF basic fraction, recovery = 70%.

² Coomassie dye assay, bovine γ -globulin standard.

³ α -Naphthol/sulfuric acid test, glucose standard.

⁴ n = 8

⁵ Sensitivity test done at 90 μ g/ml concentration.

profile and in their ability to inhibit the standard RAST assay for *Alternaria*.

Figure 1 shows the results of Sephadex G-25 column chromatography. As indicated, the two major peaks differed in their absorbance of light at 208 and 280 nm. The lower absorbance at 208 nm and higher absorbance at 280 nm of the first peak (relative to peak 2) could be attributed to the higher protein content. As shown in table 1, the peak 1 and 2 concentrates measured protein:CHO ratios of 1:4.5 and 1:5.8 respectively. Peak 2, with a higher proportion of CHO showed greater absorbance at 208 nm. The CHO determinations indicated hexose content by the color reaction giving an absorption maximum of 570 nm of

all the samples. As indicated in both figure 1 and table 1, the allergen of the basic fraction is confined to the first peak. The RAST inhibition dose response of the peak 1 concentrate was comparative to the original basic fraction (fig. 2). Both gave maximum RAST inhibition values close to 50%. The allergen (peak 1 and the original basic fraction) was very stable to heat and resistant to proteolytic enzyme digestion (table 1). Neither 10 min in a boiling water bath nor trypsin, α -chymotrypsin, or pepsin reduced the inhibition of human IgE binding to allergen discs in the RAST inhibition assay.

Coomassie dye detectable protein was removed from the allergen peak (peak 1) by TCA precipitation (table 1). When examined with SDS-PAGE (fig. 3), the allergen activity of the IEF basic fraction was eluted from the gel fraction (fig. 3, asterisk) at about the same position as the peak 1 material and was at about the same position as insulin that was used as a molecular weight marker. As seen in figure 3, the smearing effect found with SDS-PAGE of the IEF basic fraction was attributable to the material of the second G-25 column peak which was very CHO rich and nonallergenic. This had made previous attempts to approximate molecular weight difficult. The molecular weight of the basic allergen is thus estimated at about 5.5-6 kdaltons.

There was no inhibition of the birch pollen reference RAST assay by the basic allergen of *Alternaria*. This indicated no cross-reactivity between the basic allergen and birch pollen and also that the basic allergen does not bind IgE nonspecifically. Thus, the RAST inhibition using the *Alternaria* allergen discs by the basic allergen is due to specific binding of the available *Alternaria*-specific IgE.

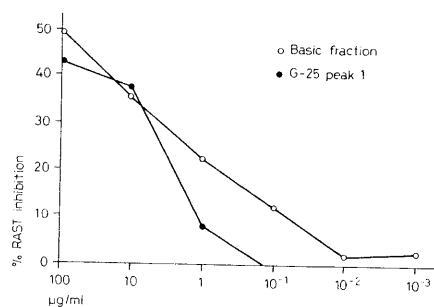


Fig. 2. RAST inhibition dose-response curve of the basic fraction and of peak 1 from the G-25 column chromatography, commercial allergen discs plus 50 µg of the allergen dilutions were coincubated with 50 µg of *Alternaria*-sensitive patient serum (13-patient pool, RAST class 3-4, 2x concentrated). ¹²⁵I-anti-HuIgE was used to quantitate bound IgE.

Discussion

Previous observations [10] indicated that the basic allergen containing fraction isolated by IEF from *Alternaria* extracts contained peptides. In this study we have determined that the basic allergen component of that fraction was mostly CHO (protein:CHO ratio 1:4.5), and that it migrated in SDS-PAGE at a rate corresponding to an approximate molecular weight of 5.5-6 kdaltons. Others [2-4] have demonstrated the CHO nature and low molecular weight of some *Alternaria* allergen fractions. However, this is the first report of a basic allergen isolated in relatively pure form and with significant potency. The Alt-1 fraction reported by Yunginger et al. [7] was also CHO rich but had a pI of 4.5. It was found in mycelial extracts as well as in spore extracts [6]. The pI = 9.5 allergen was isolated from mycelia and broth extracts and has not been looked for in spore material. We have also detected it in commercial extracts of *A. tenuis* [manuscript in preparation]. The fact that the basic allergen is small and shed into the culture broth suggested that this fraction could be a cleavage product of a larger molecule. Other experiments in our lab [manuscript submitted] have demonstrated a high incidence of pa-

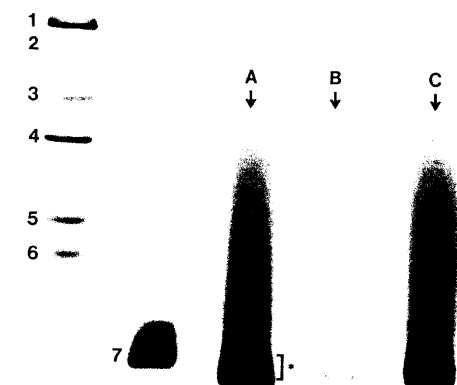


Fig. 3. SDS-PAGE (12% running gel) of the IEF basic allergen fraction (A) and G-25 column peaks 1 (B) and 2 (C). Molecular weight markers were used for reference: (1) phosphorylase B (92.5 k); (2) BSA (66 k); (3) ovalbumin (45 k); (4) carbonic anhydrase (31 k); (5) soybean trypsin inhibitor (21.5 k); (6) lysozyme (14.4 k), and (7) insulin (5.8 k). A second column containing the IEF basic fraction (same as 'A') was cut into 1x0.5 cm fractions and tested for allergen content by RAST inhibition. The asterisk indicated the position of allergen activity.

tient hypersensitivity to the basic allergen. Thus, regardless of the origin, the basic allergen is undoubtedly of biological importance.

Since the allergenicity of the original basic fraction and the G-25 column purified allergen peak (peak 1) were not degraded by heat or proteolytic enzymes (table I), the molecular characterization of the allergen will probably be determined by CHO chemistry. Preliminary experiments have indicated that the allergen was sensitive to acid conditions (data not shown). Whether the small amount of protein associated with the allergen would be involved with (or necessary for) the allergenicity is not known. The proteolytic enzyme data suggested not. It is feasible that a small peptide moiety could be stabilized against heat when complexed with large amounts of CHO.

The properties of this allergen point out the need to standardize the preparation of *Alternaria* extracts. Excessive washing of mycelia would remove much of this allergen as would excessive dialysis of extracts with membranes having a high molecular weight cut-off. Further studies on the molecular structure of this allergen are needed.

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