

Separation of *Alternaria* into protein and carbohydrate fractions with phenyl Sepharose

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To evaluate the contribution of high carbohydrate-containing fractions to the allergen content of Alternaria, samples of an Alternaria extract were bound to a phenyl Sepharose column and eluted with Tris buffer containing 4, 2, and 1 mol/L of NaCl and then distilled water. The fractions were dialyzed and lyophilized. The 4 mol/L fraction accounted for 79% of the dry weight and most of the carbohydrate content. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the fractions demonstrated significant interfraction differences with the largest number of silver-stained bands in the H₂O fraction. Although individual differences in skin reactivity to the fractions were present, geometric means of histamine equivalent pricks were approximately the same with only a 0.5 log dilution higher potency for the 1 mol/L and H₂O fractions. The 50% IgE ELISA-inhibition concentration was 100 µg/ml for the 1 mol/L and distilled water fractions and 10 µg/ml for the other fractions. Since the 4 mol/L fraction accounted for most of the dry weight and had the highest carbohydrate to protein ratio (5.8), purification of Alternaria extracts by removal of carbohydrate fractions may result in significant loss of allergenic material. (J ALLERGY CLIN IMMUNOL 1991;87:789-93.)

Extracts of some fungi have been demonstrated to contain large amounts of carbohydrate.¹ Removal of this carbohydrate has been proposed as a method for concentration of allergenic activity in fungi, such as the oyster mushroom, *Pleurotus ostreatus*²; however, for other fungi, the importance of this carbohydrate-containing material has not been fully determined. Since spores and mycelial fragments from fungi of the genus *Alternaria* are some of the most abundant components of air,³ determination of the relative allergenicity of carbohydrate- and protein-rich components of extracts derived from this fungus might prove important. The simplest method for separation of extracts into these components is with hydrophobic interaction chromatography with phenyl Sepharose. For this reason, the following study was performed.

MATERIAL AND METHODS

Preparation of extracts

The *Alternaria* culture used for this study was the same as described previously.⁴ Introduction of extraneous antigens

Abbreviations used

OD405:	Optical density at 405 nm wavelength
PBS:	Phosphate-buffered saline
PL:	Isoelectric point
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
DW:	Dry weight

was avoided by growing this culture for 4 weeks on a chemically defined sucrose medium described previously.^{5,6} The cultures were exposed to 12-hour periods of alternating light and dark during their growth. Fungal mats produced by this method contained spores and mycelia; therefore, antigens from both sources were represented in the final extract. The cultures were air dried in an incubator at 37° C for 24 hours and powdered with a mortar and pestle. Each preparation was extracted 1:10 (wt/vol) in phosphate buffer with 0.15 mol/L of NaCl (pH 7.4) at 4° C for 24 hours and then filtered with Whatman No. 4 filter paper (Whatman, Inc., Clifton, N.J.) followed by progressively finer pore-sized membrane filters (Millipore Corp., Bedford, Mass.). After clarification through a 0.45 µm filter, the extracts were dialyzed against 50 mmol/L of ammonium bicarbonate (pH 8.2) at 4° C for 48 hours and lyophilized. The final extracts were stored in a desiccator in sealed tubes at -20° C. Reconstituted extracts were stored at 4° C until use.

Patient population and skin testing

All clinical studies were performed after obtaining approval from the pediatrics institutional review board of the

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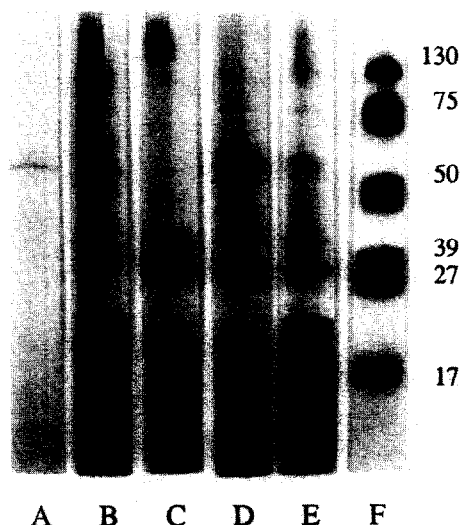


FIG. 1. SDS-PAGE of the 4 mol/L (lane A), 2 mol/L (lane B), 1 mol/L (lane C), H₂O (lane D), and whole *Alternaria* (lane E) fractions. Lane F contains calibration markers.

University of Missouri-Kansas City. Written informed consent was obtained before any experimental procedures were performed. Volunteers for skin testing and venipuncture consisted of children between the ages of 6 and 18 years because those are the ages observed at The Children's Mercy Hospital allergic clinic. Most of the serum samples used in this study were obtained from the skin test subjects, but several samples were from patients who had been tested previously. Also, a few pediatric patients refused to permit their blood to be drawn.

The biologic potency of each extract was determined by prick skin testing of patients with signs and symptoms of atopic disease (allergic rhinitis, conjunctivitis, or asthma). These patients were initially screened with a 1:10 dilution of a commercial *Alternaria* extract (Center Laboratories, Port Washington, N.Y.) as well as several extracts derived from experimental strains of *Alternaria*. All subsequent skin tests were performed with experimental extracts and not with the commercial extract. Skin-reactive patients were defined as atopic and received further tests as described below.

Skin testing materials were prepared by adding 10 mg of lyophilized antigen to 1 ml of PBS and mixing with equal parts of glycerol, eliciting a final concentration of 5 mg/ml. Serial fivefold dilutions of this material were made, and the patient was tested with each dilution. Wheal diameter versus log (dilution) was compared to a standard histamine preparation (5.43 mmol/L or 1 mg/ml of histamine hydrochloride) to determine the histamine-equivalent dose of the extract.⁷ The concentration of antigen (in milligrams per milliliter) that produced a wheal size equal to the standard concentration of histamine was determined by linear regression. This number is lower as the allergen becomes more potent, and it has been demonstrated to correlate with biologic activity.⁹ Serum samples were obtained from volunteer skin-reactive (>5 mm wheal by prick after 20 min-

utes) patients after testing with either crude *Alternaria* extracts or the experimental antigens.

Chromatography

Lyophilized *Alternaria* was reconstituted in 0.05 mol/L of Tris buffer (pH 7.4) with 4 mol/L of NaCl at a concentration of 10 mg of DW per milliliter. A 2 ml aliquot of this material was applied to a 20 cc bed volume phenyl Sepharose column equilibrated with the same buffer. A step-wise elution with five bed volumes of 4, 2, and 1 mol/L of NaCl in 0.05 mol/L of Tris and distilled water was then used to recover the fractions. The progress of elution was continuously monitored with an ultraviolet detector set at 280 nm. The effluents were dialyzed extensively against 50 mmol/L of ammonium bicarbonate and lyophilized.

ELISA inhibition

Antigen dissolved in 50 mmol/L bicarbonate buffer, pH 9.6 (200 μ l per well of 10 μ g DW per milliliter), was adsorbed to wells of microtiter plates (Immulon II, Dynatech Industries, Alexandria, Va.) overnight at 37°C followed by addition of 3% bovine serum albumin in PBS (pH 7.4) to block nonspecific binding. Serial dilutions of each phenyl Sepharose fraction were then added to the antigen-coated wells along with pooled sera (1:10) from 10 patients who were skin sensitive to *Alternaria*, and the mixture was incubated for 4 hours. Antigen-specific IgE was subsequently detected with 1:1000 rabbit antihuman IgE (Sigma Chemical Co., St Louis, Mo.) dissolved in PBS with 1% bovine serum albumin. This was followed by a 4-hour incubation with 1:1000 alkaline phosphatase-conjugated goat anti-rabbit (Sigma Chemical Co.). After addition of 1 mg/ml of p-nitrophenyl phosphate, the OD405 was measured with a Titertek Multiscan (Flow Laboratories, McLean, Va.). The inhibited OD405s, decreased relative to the uninhibited values, were then expressed as percent inhibition.⁸

SDS-PAGE

Glycoproteins were separated on the basis of molecular weight with SDS-PAGE on 8% to 18% gradient slab gels according to the method of Laemmli.⁹ Lyophilized samples were reconstituted in distilled water at a concentration of 1 mg/ml of DW, and 10 μ l, diluted in 30 μ l of sample buffer, was applied to each lane. These samples were run under nonreducing conditions, that is, without the addition of mercaptoethanol to the sample buffer. SDS-treated samples were denatured by exposure to boiling water for 1 minute. The gels were silver stained according to the method of Wray et al.¹⁰ Calibration markers were run with each gel.

Biochemical characterization of glycoproteins

Protein concentrations were measured according to the method of Lowry et al.¹¹ with a commercial kit (Sigma Chemical Co.). Carbohydrate concentrations were measured by the phenol-sulfuric acid method.¹² Briefly, serial dilutions of a standard glucose solution (0.5 mg/ml) and of the unknowns were added to 2 ml of distilled water followed by 50 μ l of a 90% phenol solution. Next, 5 ml of sulfuric acid

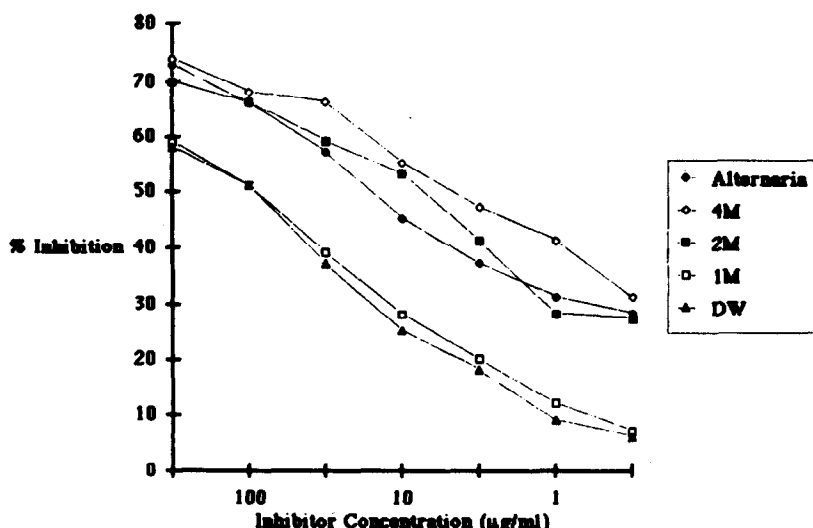


FIG. 2. IgE ELISA inhibition of the four recovered fractions and *Alternaria* with whole *Alternaria* in solid phase.

TABLE I. Total dry weight recovered from *Alternaria* fractions separated by phenyl-Sepharose and the carbohydrate and protein contents of 10 mg DW per milliliter

Antigen	DW (mg)	Protein (mg/ml)	Carbohydrate (mg/ml)	Carbohydrate to protein ratio
Fraction (mol/L)				
4	52	2.2	12.7	5.8
2	10	6.9	4.2	0.6
1	1.5	2.8	0.8	0.3
H ₂ O	2.5	4.4	1.1	0.2
Whole <i>Alternaria</i>		2.7	7.5	2.8

was rapidly pipetted in, and the resulting mixture was allowed to sit for 30 minutes. The OD485 of each tube was next measured, and a calibration curve was plotted. The unknowns were compared to the calibration standards by linear regression, and the carbohydrate content could then be expressed in milligrams per milliliter.

RESULTS

The total eluted contents of fractions 4, 2, and 1 mol/L, H₂O, and whole *Alternaria*, as well as their relative concentrations of carbohydrate and protein, are presented in Table I. Most of the carbohydrate was recovered in the 4 mol/L fraction. Total recovery was complete with the above elutions, since only trace amounts of glycoprotein were found in an ethylene glycol wash that was used to regenerate the phenyl Sepharose column. These results confirm that this *Alternaria* extract does contain a relatively large amount of carbohydrate as compared to pollen extracts that contain primarily proteins.

SDS-PAGE of the fractions (Fig. 1) resolved a number of bands as detected with the silver stain. Distinct

differences were observed between the fractions; however, the 4 mol/L fraction demonstrated a relative lack of staining with only one band at 50 kd being clearly observed. Lanes containing other fractions revealed multiple, clearly resolved bands. Numerous bands were observed in the lane containing whole *Alternaria*, which has been our experience in the past.

IgE ELISA inhibition of the fractions with whole *Alternaria* in solid phase is illustrated in Fig. 2. The four inhibition curves were parallel to each other. The concentration required for 50% inhibition was tenfold less for the whole *Alternaria* preparation and for the 4 and 2 mol/L fractions. All the fractions did demonstrate significant inhibition.

The results of skin testing are presented in Table II. All 12 patients were skin reactive to a commercial *Alternaria* preparation (Center Laboratories) before testing with the experimental extract. Patients 4 and 5 were only minimally reactive to the experimental whole *Alternaria*, and they reacted only to the 1 and 2 mol/L and H₂O fractions, respectively. This finding

TABLE II. Age, sex, diagnosis, and Log₅ concentration of antigen (milligrams per milliliter) that elicits a wheal size equivalent to 1 mg/ml of histamine HCl

Patient	Age (yr)	Sex	Diagnosis	Fractions (mol/L)				<i>Alternaria</i>
				4	2	1	H ₂ O	
1	15	M	AR, AS	0.25	0.21	0.15	0.20	0.20
2	8	M	AR	1.43	1.43	1.43	1.00	0.83
3	7	F	AR, AS	0.59	0.59	0.77	0.59	0.19
4	13	F	AR, AS	5.00	5.00	0.67	5.00	5.00
5	12	M	AR	NR	1.00	NR	1.42	NR
6	9	F	AR, AS	2.50	2.50	1.67	1.00	0.50
7	5	M	AR, AS	2.00	2.00	1.42	1.00	0.83
8	8	F	AR	3.33	1.43	1.43	0.77	0.50
9	3	M	AR, AS	0.83	1.00	1.00	1.25	0.42
10	5	M	AR, AS	1.00	1.00	1.25	1.25	0.45
11	8	M	AR, AS	0.23	0.50	0.77	1.00	0.33
12	43	F	AR	1.43	0.71	0.45	0.59	0.71
Geometric Mean				1.15	1.07	0.86	0.96	0.55

Smaller numbers indicate greater potency.

AR, Allergic rhinitis; AS, asthma; NR, not reactive.

suggests that the least *Alternaria*-sensitive individuals were more selective with respect to allergens to which they react and supports a qualitative difference between these fractions with individual allergens being uniquely represented in each. Subjects 1, 4, and 12 had greater skin reactivity to fraction 1 mol/L than to whole *Alternaria*, and subject 11 had greater sensitivity to the 4 mol/L fraction. The other patients were less reactive to each of the fractions than to the whole *Alternaria* preparation, suggesting that purification and concentration of allergens relevant to these patients did not occur. The geometric mean histamine equivalent potency was greatest for whole *Alternaria* with the 1 mol/L fraction being next in potency and the 4 mol/L fraction being the least potent. Since differences between fractions were <0.5 log dilutions, the importance of this finding is questionable.

DISCUSSION

Extracts of *Alternaria* have been previously separated into fractions by gel filtration and ion exchange chromatography. With these techniques, several allergen-containing *Alternaria* fractions have been described by investigators. Alt-I, for instance, is a carbohydrate-rich, heterogeneous fraction with molecular weight between 25 and 50 kd and pI between 4.0 to 4.5.¹³ IgE RAST inhibition revealed significant allergen activity, but there was still activity left in other *Alternaria* fractions. Another heterogeneous fraction has been reported with a pI of 9.0 and a molecular weight of 6 kd.¹⁴ This material was purified by preparative isoelectric focusing.

Phenyl Sepharose is a chromatography medium that

separates glycoproteins on the basis of hydrophobicity. At high salt concentrations, proteins, which are relatively hydrophobic, tend to bind, whereas carbohydrates, which tend to be more hydrophilic, do not bind. The protein and carbohydrate measurements demonstrated a separation into carbohydrate and protein-rich fractions. SDS-PAGE of these fractions demonstrated a large number of prominent protein bands to be in the H₂O fraction. The recognition of antigens in each fraction by human IgE was demonstrated by skin tests as well as by IgE ELISA inhibition.

ELISA-inhibition curves that are parallel suggest that the fractions either contain different glycoproteins or that they have the same components in different concentrations. Since qualitative differences between the fractions were observed by electrophoresis and by skin test reactivities and since the carbohydrate to protein ratios differed between fractions, the variation in inhibition potency probably represents a true qualitative difference between the fractions.

The ELISA-inhibition data support lower potency of the distilled water and 1 mol/L fractions, whereas skin test data suggest that patients were more sensitive to those same fractions. On the surface this appears to be a contradiction; however, these are different tests, which is why they were both done. The ELISA depends strongly on the nature of the pooled serum IgE used to probe the allergens and on which allergens were immobilized on the plastic solid phase. Skin testing, in contrast, depends on mast cell-bound IgE and the ability of allergens to trigger degranulation.

This observation that the whole *Alternaria* was

more allergenic than the individual fractions is not surprising. The whole preparation contains all the allergens, including some allergens that may cross-react with each other and that might be synergistic. Individual fractions do not contain as many different allergens and would therefore not be expected to display as much biologic potency. In particular, IgE directed to *Alternaria* allergens in fraction 4 mol/L would not necessarily be inhibited as well by those in fraction 1 mol/L, even if they were cross-reactive.

Similar hydrophobic separation has been performed for *Aspergillus* extracts.¹⁵ After phenyl Sepharose separation of protein from carbohydrate, human IgG directed to each of the *Aspergillus* fractions was found to be present, although a larger number of fractions tended to be recognized by patients with aspergillosis. Measurements of IgE were not made in that study.

More recently, allergens from spores of the oyster mushroom, *Pleurotus ostreatus*, were separated by hydrophobic chromatography.² In contrast to the results with *Alternaria*, these authors found that the 4 mol/L fraction did not contain significant allergen activity and that removal of this fraction resulted in concentration of allergens. Since the carbohydrate-rich fraction of *Alternaria* does contain allergenic activity, routine hydrophobic fractionation of *Alternaria* extracts does not appear to be an effective method for concentration of allergenic activity.

Our conclusion is that *Alternaria* extracts contain large amounts of carbohydrate. Fractions containing large amounts of carbohydrate can be separated from those with relatively more protein with phenyl Sepharose, but this separation is not complete. Total allergen activity is greater in the 4 mol/L fraction because there is more of the fraction. On a per weight basis, the carbohydrate-rich 4 mol/L fraction is more or less potent, depending on whether potency is measured by ELISA inhibition or by skin testing. Since there appear to be qualitative differences between the fractions, potency will depend on the individual patient against whom it is measured.

The effect that this high carbohydrate content has on the response to *Alternaria* immunization that occurs with immunotherapy remains to be determined. Since hydrophobic interaction chromatography does permit separation of complex glycoprotein mixtures such as *Alternaria* into distinct fractions, its use may prove to be an effective initial step for purification of

individual glycoproteins with other technologies, such as affinity chromatography with monoclonal antibodies.⁴

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