

Affinity purification of a major *Alternaria* allergen using a monoclonal antibody

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An extract of Alternaria (ALT) was passed through an affinity column containing a monoclonal antibody directed to ALT. After washing the column, a single glycoprotein was eluted using 0.1 M glycine (pH 3.0). This glycoprotein accounted for 13% of the dry weight of our ALT preparation and had a carbohydrate to protein ratio of 0.35 as compared with 3.2 for the whole ALT extract. Its molecular weight was 70 kD by SDS-PAGE and isoelectric point was 3.5 by IEF. Though individual sensitivities varied, 14/16 patients skin reactive to ALT were also reactive to this glycoprotein. Quantitative skin tests of extracts adjusted to the same dry weight per volume showed that it required about a four times greater concentration of the purified glycoprotein to give a wheal size equal to whole Alternaria. The ability to purify large amounts of this allergen with affinity chromatography should make its complete characterization possible.

INTRODUCTION

Spores and mycelial fragments from fungi of the genus *Alternaria* (ALT) are some of the most abundant components of air.¹ They can cause symptoms of rhinitis and asthma in allergic individuals. Despite their clinical importance, complete characterization of the antigenic components of extracts derived from this fungus has not occurred.

Several allergen-containing ALT fractions such as Alt-1 and another heterogeneous fraction with a P.I. of 9.0 have been described previously.^{2,3} These two fractions contain significant allergenic properties, but they do not represent pure materials nor do they represent the total allergen content of ALT extracts. In addition, there are no commonly available assays for their measurement.

We describe the isolation of an electrophoretically pure glycoprotein from ALT that does contain significant allergenic activity. This material has been purified using an ALT-specific monoclonal antibody

in solid phase on an affinity chromatography column.

METHODS

Preparation of Extracts

An ALT culture was obtained from Harriet Burge (University of Michigan, Ann Arbor, MI). This culture had previously been identified as similar to *Alternaria alternata* by Emory Simmons (formerly at the University of Massachusetts in Amherst and now in Crawfordsville, Indiana). The strain used was an atmospheric isolate and does not have an ATCC accession number. Introduction of extraneous antigens was avoided by growing this culture for 4 weeks on a chemically defined sucrose medium [0.3% sodium nitrite, 0.05% magnesium sulfate, 0.05% potassium chloride, 0.1% potassium phosphate (monovalent), 3% sucrose, and trace elements consisting of 1.8 mM Fe (NO₃)₃, 0.97 mM ZnSO₄, 1.8 mM MnSO₄].^{4,5} The cultures were exposed to 12-hour periods of alternating light and dark during their growth. Fungal mats produced this way did contain spores and mycelia so that antigens from both sources were presumably represented in the final extract. The cultures were air dried at 37 °C, and powdered with a mortar and pestle. Each preparation was extracted in 1:10 (wt/vol) phosphate buffer with

0.15 M NaCl (pH 7.4) at 4 °C for 24 hours and then filtered with whatman 4 filter paper followed by progressively finer pore sized membrane filters (Millipore, Bedford Mass.). After passing through a 0.45- μ filter, the clear extracts were dialyzed against 50 mM ammonium bicarbonate (pH 8.2) at 4 °C for 48 hours, and either lyophilized or used as is. The final extract was stored either in a desiccator or in sealed tubes at -20 °C. After the extract was reconstituted, it was stored at 4 °C until used.

Human Volunteers

All clinical studies were performed after obtaining approval from the pediatrics institutional review board of the University of Missouri at Kansas City. Written informed consent was obtained before any experimental procedures were performed. The ages of volunteers ranged between 5 and 18 years old because The Children's Mercy Hospital limits treatment to children as an institutional policy.

The biologic potencies of each extract fraction was determined using the histamine equivalent prick method⁶ on patients with signs and symptoms of atopic disease (allergic rhinitis, conjunctivitis, or asthma). Such patients were initially identified by prick skin test screening with a commercial ALT extract (Center

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Labs, Port Washington NY) as well as several extracts derived from experimental strains of ALT. None of these patients had received immunotherapy with ALT. By using several different preparations, the maximum number of ALT-sensitive patients could be identified. All subsequent studies were performed with the experimental strain of ALT and not with the commercial preparation. Patients with wheal sizes greater than 5 mm were further tested as described below.

Skin testing materials were prepared by adding 10 mg of antigen to 1 mL of phosphate buffered saline, pH 7.4 (PBS) and mixing with equal parts of glycerol giving a final concentration of 5 mg/mL. Serial 5-fold dilutions of this material were made and the patients were tested with each one. Wheal diameter versus log (dilution) was compared with a standard histamine preparation (5.43 mmol/L or 1 mg/mL of histamine hydrochloride) to determine the histamine equivalent dose of the extract. The concentration of antigen (in mg dry weight) that produced a wheal size equal to the standard concentration of histamine was determined by linear regression. The antigen concentration was divided by the histamine concentration to give a number expressed as mg antigen per mg histamine. This number is lower as the allergen becomes more potent, and it has been shown to correlate with biologic activity.⁶ Serum samples were obtained from willing skin-reactive (>5-mm wheal) patients after testing with either crude ALT extracts or the purified antigen. Since many of the younger volunteers were not willing to undergo venipuncture, the serum samples did not always come from patients who were tested with the purified antigen.

Production of Monoclonal Antibodies

Balb/C mice were immunized IP with 50 µg of antigen in complete

Freund's adjuvant and twice more at 2-week intervals with antigen in complete Freund's adjuvant. The mice were again immunized with aqueous antigen five days before sacrifice. After sacrifice, spleen cells were fused with HAT-sensitive mouse myeloma cells (653s) in the presence of 40% polyethylene glycol followed by incubation in HAT medium. Surviving colonies were screened for ALT-specific antibody production using direct ELISA with ALT in solid phase. Cells that produced antibody were cloned twice by limiting dilution and expanded. Hybridoma cells were then injected IP into pristane-prepared mice resulting in the production of ascites fluid which contained large amounts of antibody.⁷ The mAbs were purified using protein A (BioRAD) on an affinity column.⁸

Preparation of the Affinity Column
CNBr-activated sepharose 4B (Sigma) was washed with 10^{-3} M HCl, mixed with 10 mg/mL lyophilized antigen in a bicarbonate buffer (pH 9.6) per gram sepharose, washed in alternating Na acetate/Na borate three times, blocked with 3% BSA, and stored at 4 °C in PBS with 0.02% sodium azide until used. This material was then put into an affinity column (Econocolumn, BioRAD) and washed extensively with PBS and with 0.1 M glycine (pH 3.0) before use.

Affinity Purification

For purification of the antigen, crude ALT extract was dialyzed against PBS and then passed through the affinity column either with a peristaltic pump or by gravity. The protein content of the effluent was monitored continuously with a UV detector set at 280 nm and a chart recorder. Once the OD280 returned to baseline, the bound material was eluted with 0.1 M glycine, pH 3.0, into 0.1 M Tris, pH 11.0, in order to minimize the exposure of the eluted protein to acid. The effluent was dialyzed against 50 mM ammonium bicarbonate and lyophilized.

Biochemical Characterization of Glycoproteins

Protein was measured by the method of Lowry⁹ using a commercial kit (Sigma, St. Louis, MO). These concentrations were measured at 2-fold dilutions of each sample to insure that the curves were parallel to the standard curves. All samples were dilute enough that they were not visibly pigmented. Carbohydrate was measured by the phenol-sulfuric acid method.¹⁰ Briefly, several volumes of a standard glucose solution (0.5 mg/mL) and of the unknowns were added to 2 mL distilled water followed by 50 µL of a 90% phenol solution. Next, 5 mL of sulfuric acid was rapidly pipetted in and the resulting solution was allowed to sit for 30 minutes. The OD485 of each tube was measured and a calibration curve plotted. The unknowns were compared with the calibration standards by linear regression, and the carbohydrate content was expressed in µg/mL.

Electrophoresis

Glycoproteins were separated on the basis of molecular weight using SDS-PAGE on 8% to 18% gradient slab gels (BioRAD) using the method of Laemmli.¹¹ Lyophilized samples were reconstituted in distilled water at a concentration of 1 mg/mL dry weight. Ten microliters was diluted in 30 µL of sample buffer and applied to each lane. These were run under reducing conditions by addition of mercaptoethanol to the sample buffer followed by exposure to boiling water for one minute. Isoelectric focusing was performed in 0.25-mm acrylamide gels using a mini isoelectric focusing cell (BioRAD) and a pH gradient of 3 to 10 with 1% Biolyte (BioRAD). Ten microliters of each sample was applied to the surface of the gel at a concentration of 1 mg/mL. Isoelectric focusing and SDS-PAGE gels were stained with a commercial silver stain (BioRAD). Calibration markers obtained from BioRAD were run with each gel.

Direct ELISA

Antigen-specific immunoglobulin concentrations were measured using an enzyme-linked immunosorbent assay (ELISA). Antigen dissolved in a bicarbonate buffer, pH 9.6 (200 μ L per well of 5 μ g dry weight/mL) was adsorbed to wells of microtiter plates (Immulon-2, Dynatech) overnight at 37 °C followed by addition of 1% BSA in PBS to block nonspecific binding. After washing three times with PBS/Tween-20, aliquots of antibody-containing serum diluted 1:10 were then placed in the antigen-coated wells in quadruplicate. Antigen-specific IgE was subsequently detected with 100 μ L per well of 1:1000 rabbit anti-human IgE (Sigma, St. Louis, MO) dissolved in PBS with 1% BSA and 1 μ L/mL human serum containing less than 5 ng/mL total IgE to inhibit crossreactivity with IgG. This was followed by 100 μ L per well of 1:1000 alkaline phosphatase-conjugated goat anti-rabbit (Sigma, St. Louis, MO). After addition of 100 μ L per well of 1 mg/mL *p*-nitrophenyl phosphate, the OD405 was measured with a Titertek Multiscan (Flow Labs, McLean, VA). Since many patients who volunteered for quantitative skin testing were unwilling to undergo venipuncture, a direct correlation between skin reactivity and antigen-specific IgE could not be determined. As a result, most of the sera used for direct ELISA were from patients who had previously been tested with whole ALT but not with gp70.

Inhibition ELISA

Serial dilutions of the inhibiting antigen were added to antigen-coated wells of a microtiter plate. Pooled sera (1:10) from ten ALT-sensitive patients was then added to each well and the mixture incubated for four hours. Eight of these samples were the same as those measured in Table 3. The two additional samples were not available when the direct ELISAs were run. The plate was washed and the bound IgE was detected as described above. After washing and

addition of enzyme substrate, the optical densities were measured. The inhibited OD405s, which were decreased relative to the uninhibited values, were then expressed as percent inhibition.¹²

RESULTS

An antigen from the crude ALT extract was purified by affinity chromatography using a monoclonal antibody designated F8F11 in our laboratory. The purity of this material was confirmed using both SDS-PAGE and isoelectric focusing (Fig 1 and 2). These procedures revealed that it consists of a single glycoprotein that has a molecular weight of 70 kD and a pI of 3.5. For this reason, we have designated the antigen gp70.

Table 1 shows the contents of 1 mg dry weight of the bound antigen, the remaining unbound antigen that was exhaustively absorbed by the F8F11 antibody column, and total extract dissolved in PBS. The glycoprotein [gp70] is primarily composed of protein, though it does con-

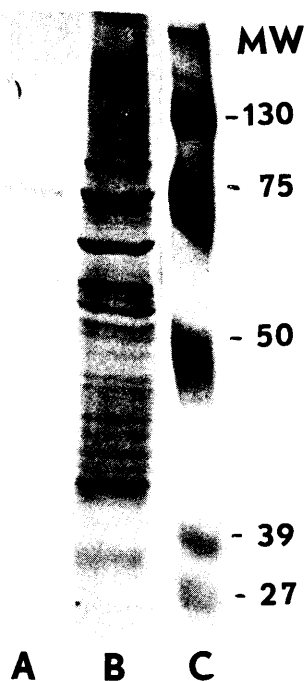


Figure 1. Silver-stained SDS-PAGE of purified antigen (A), whole *Alternaria* (B), and calibration (C).

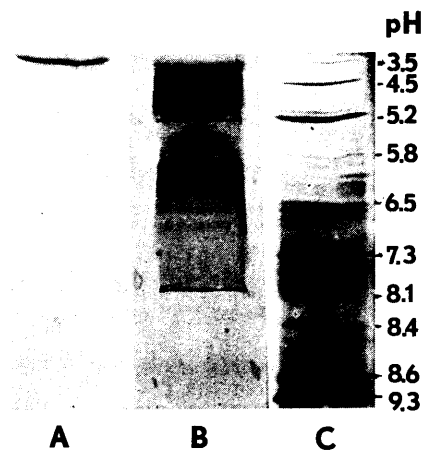


Figure 2. Silver-stained isoelectric focusing of purified antigen (A), whole *Alternaria* (B), and calibration (C).

Table 1. Analysis of F8F11 Ag: Significant Affinity Purified Glycoprotein Allergen Derived from an *Alternaria* Extract

Material	Protein, mg/mL	Carbohydrate, mg/mL	CHO/Prot
gp70 antigen	0.71	0.25	0.35
gp70-depleted	0.09	0.92	10.67
Whole Alt	0.25	0.80	3.22

tain 26% carbohydrate. When gp70 is removed from the ALT extract, the remaining material is enriched for carbohydrate. On a dry weight basis, the purified glycoprotein accounted for 13% of the total weight of the extract.

Sixteen ALT-sensitive volunteers were tested epicutaneously with whole ALT and with purified glycoprotein gp70. All but three of these subjects were patients undergoing skin testing for the presence of allergic disease at The Children's Mercy Hospital allergy clinic. Of those tested, 14 were skin-reactive to gp70 as well as to whole ALT. No subjects were skin-reactive to gp70 without reacting to whole ALT. The results of quantitative skin testing of the ALT-sensitive volunteers are shown in Table 2. On average, the purified glycoprotein needed to be about four times as concentrated on a per weight basis as the whole ALT to give an equivalent wheal size.

Direct IgE ELISA OD405 values

Table 2. Log₅ Concentrations of Purified Fraction (GP70) and Whole *Alternaria* Required to Give a Wheal Equivalent to Histamine (1 mg/mL). Smaller Numbers Indicate Greater Potency

Patient	GP70	Whole <i>Alternaria</i>
1	1.3	0.5
2	1.2	0.5
3	0.8	0.7
4	1.5	0.4
5	1.2	0.5
6	1.0	0.8
7	0.8	0.6
8	0.8	0.2
9	1.7	0.4
10	1.0	0.4
11	1.5	0.5
12	1.0	0.6
13	1.0	0.4
14	0.4	0.4
15	NR*	1.0
16	NR*	0.8
Mean	1.1	0.5

* No reaction.

of 15 serum samples obtained from ALT allergic and nonallergic individuals is shown in Table 3. The amount of whole ALT-specific IgE, as measured by the OD405s, was generally lower for nonallergic individuals (mean 25.8 ± 7.4) than for allergic ones (mean 409 ± 128). This was a statistically significant difference ($P < .05$ by the unpaired *t* test). This difference was not seen for IgE directed to gp70 where the mean for skin nonreactive patients was 101 ± 13 and for skin-reactive ones was 262 ± 128 ($P = \text{NS}$). Two of these reactive patients did have large amounts of IgE to gp70 that did not correlate with the amount of IgE directed to the total ALT. Three patients with ALT-specific IgE greater than 100 had large amounts of gp70 IgE.

IgE ELISA inhibition with whole ALT in solid phase showed 35% inhibition by gp70 at a 1 mg/mL concentration as compared with 80% for ALT and 65% for the gp70-depleted preparation (Fig 3). The uninhibited OD405 value of the positive serum pool was 0.557 and the negative control value of pooled serum from the seven nonallergic individuals was 0.086. The inhibition curves all had statistically significant correlations. Since 50% inhibition was not obtained for gp70,

it was not possible to determine a 50% inhibition concentration.

DISCUSSION

The complete characterization of ALT has been a difficult problem due to several factors including disparities in spore, mycelial, and metabolic antigens, batch-to-batch antigenic variation, inter-strain and intra-strain differences, and variations introduced during extraction and processing.^{13,14} As a result of this variability, characterization of ALT antigens from different sources is like comparing apples and oranges. It is necessary that some consistency

Table 3. Direct IgE ELISA of 15 Sera with *Alternaria* or gp70 in Solid Phase*

Patient	Skin Reaction	<i>Alternaria</i>	gp70
1	Negative	4	75
2	Negative	4	98
3	Negative	14	98
4	Negative	27	172
5	Negative	37	70
6	Negative	39	84
7	Negative	56	113
8	Positive	107	41
9	Positive	157	78
10	Positive	173	77
11	Positive	239	1397
12	Positive	320	71
13	Positive	352	124
14	Positive	810	59
15	Positive	1119	250

* The Numbers are in OD405 after the background values were subtracted. A positive prick skin test is defined as the presence of >5 -mm wheal.

be obtained before a coherent picture can be produced.

One solution to the problem of antigen characterization has been to purify individual allergens. In the past, this has been technically very difficult to accomplish. Despite the difficulties, a number of major purified allergens have been described including ragweed antigen E (Amb a 1),¹⁵ group #1 antigen (Lol p 1) in grass pollen,¹⁶ antigens P1 (Der p 1) and F1 (Der f 1) in house dust mite,¹⁷ cat 1 (Fel d 1) in the domestic cat,¹⁸ phospholipase-A in honeybee venom,¹⁹ peanut I in peanut extract,²⁰ and many more. Most of these antigens contribute the majority of allergenic activity to their respective extracts and may be useful as standards for total biologic activity when measured. The significance of less active materials in the same extracts is unknown.²¹

Several allergen-containing ALT fractions have been described by investigators, however, the fractions do not consist of individual glycoproteins. Alt-1, for instance, is a carbohydrate-rich, heterogeneous fraction with molecular weight between 25 to 50 kD and P.I between 4.0 to 4.5. In general, the richest ALT ex-

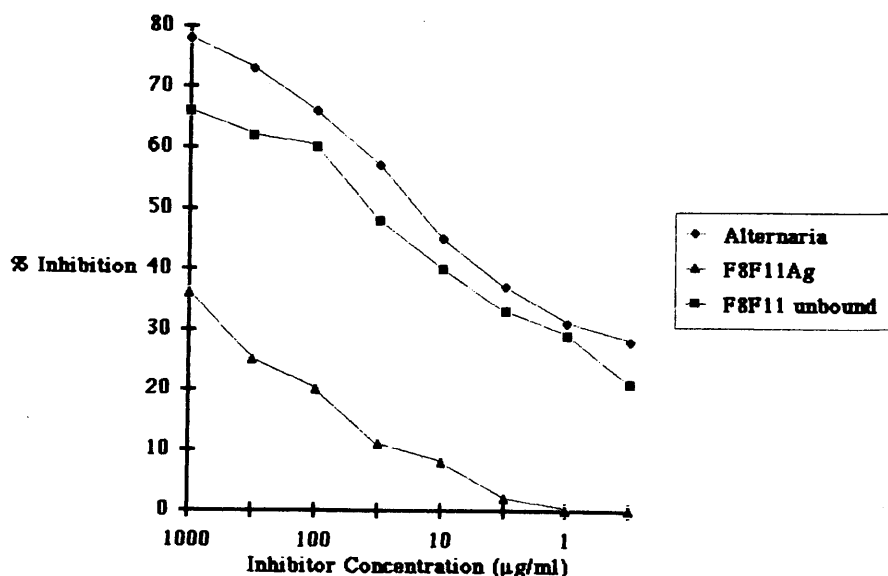


Figure 3. IgE ELISA inhibition of whole *Alternaria*, *Alternaria* depleted of the purified antigen, and the purified antigen (F8F11 bound).

tract was felt to contain less than 0.7% of Alt-1 based on dry weight. This material, which was produced using ion exchange chromatography, has been shown to have biologic activity by skin test, leukocyte histamine release, and broncho-provocation.²² IgE RAST inhibition revealed significant allergen activity, but there was still activity left in other ALT fractions. Different groups have also isolated this material from other strains of ALT.²³ Extracts of *Stemphylium*, another important allergenic fungus, have been shown to contain Alt-1 suggesting that distinct fungal extracts do indeed share cross-reacting materials.²⁴ Unfortunately, although attempts have been made to purify further Alt-1, none has been successful to date. In addition, subsequent investigators have had difficulty reproducing this material with the result that it has been in short supply and therefore not generally available for further study.

Another heterogeneous fraction has been reported with a pI of 9.0 and a molecular weight of 6 kD.³ This material was purified by preparative isoelectric focusing. Skin tests were positive in 90% of ALT-sensitive patients tested with this material and IgE RAST inhibition showed about 20% inhibition of whole ALT. A major limitation of studies involving these two fractions is that the fractions do not represent pure materials nor do they contain the total allergen content of ALT extracts. In addition, there are no commonly available assays for their measurement nor procedures for their large-scale production.

We now describe the isolation of a pure glycoprotein by a method that will enable large-scale and reproducible purification. This material does not contain all, or even most of the allergenic activity of ALT, but it is a homogeneous material. It is an allergen as demonstrated by the fact that 14/16 whole ALT-sensitive patients are skin-reactive to it, and it gives 35% inhi-

bition of human IgE directed to ALT. The lack of gp70-specific IgE in the sera of several ALT-sensitive patients may be due to low absolute amounts of IgE to this particular glycoprotein despite the presence of large amounts of IgE to whole ALT. It may also relate to the affinity of the antibody or to other factors that are unknown. The fact that direct ELISA does not necessarily correlate with skin reactivity demonstrates the need for *in vivo* biologic activity measurements as is provided by quantitative skin testing.

Though we have not yet defined its role in ALT allergy, the ability to produce large amounts of gp70 for study will hopefully permit further understanding of some of its biologic and immunologic properties.

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VEHICLES AFFECT ACTIVITY AND PENETRATION OF CORTICOSTEROID CREAMS

In order to evaluate certain compounding variables, the authors studied the effects of addition of each of four chemicals or chemical combinations to each of four adrenal corticosteroid creams: 0.2% hydrocortisone 17-valerate (Westcort cream), 0.1% triamcinolone acetonide (Kenalog), 0.05% fluocinonide (Lidex), and 0.25% desoximetasone (Topicort). The chemicals added were 2% salicylic acid, 10% urea, 5% USP coal tar solution (liquor carbonis detergens), and the combination of 0.25% camphor, 0.25% menthol, and 0.25% phenol. Compatibility was evaluated 24 hours after compounding and at 1 and 2 months by determination of steroid content by high-performance liquid chromatography and measurement of penetration through human skin samples *in vitro*.

Following compounding with 10% urea after 2 months at ambient conditions steroid content had decreased by 11% for hydrocortisone 17-valerate, 19% for desoximetasone, 34% for fluocinonide, and 45% for triamcinolone acetonide. Decreases of 9% to 25% within 24 hours after compounding suggested the likelihood of chemical degradation, but no significant degradation followed compounding any of the steroid creams with 2% salicylic acid, 5% liquor carbonis detergens, or the combination of 0.25% camphor, 0.25% menthol, and 0.25% phenol.

Twofold to threefold enhancement of skin penetration by the steroids followed compounding with 2% salicylic acid. Compounding with 10% urea also enhanced penetration of triamcinolone acetonide, but no penetration of fluocinonide with urea was detectable. Only 2% to 3% active fluocinonide was recoverable from the skin surface after compounding with 10% urea, suggesting chemical instability.

These observations indicate results of compounding of corticosteroid creams with different vehicles can vary, depending on the specific steroid and the vehicle. Salicylic acid may enhance penetration due to its keratolytic effect on the horny layer. Urea enhanced penetration of only triamcinolone acetonide, and urea apparently caused degradation of the steroids, especially triamcinolone and fluocinonide.

Compounding of corticosteroid creams is wise only with vehicles known to have no adverse effect on stability and skin penetration of the specific steroid.

—RMS

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