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## Purification and Characterization of Alt a-29 from *Alternaria alternata*

### Key Words

*Alternaria*

Alt a-29

Allergen

Purification

N-Terminal sequencing

### Abstract

A major protein component reactive with pooled human atopic sera was isolated from a lyophilized broth extract of *Alternaria alternata* 34-016. By successive chromatography on Whatman DE-52, Sephadex G-100 and Mono Q HR5/5, a low molecular weight antigen was obtained. Comparison with standard proteins on Sephadex G-100 indicated its molecular weight was 31 kD. Non-reduced samples run on SDS-PAGE showed a band at 29.2 kD which reacted strongly with human IgE. After reduction, it produced a doublet pattern on SDS-PAGE with MW 14.5 and 16.0 kD. The doublet pattern was confirmed by Western blotting with pooled human atopic sera. IEF of the protein showed a major component with a PI of 4.15 and two minor components at 4.25 and 4.40. Immunoblots of the IEF bands showed all three were reactive with human IgE. Ion exchange chromatography of the protein on Mono Q HR5/5 resulted in three resolved components, all of which are immunoreactive. Together with the IEF data, this suggests that there are several conformational or structural isoforms of this protein.

### Introduction

The ability of the mold, *Alternaria alternata* to induce human IgE-mediated allergic asthma is well established [1]. One of the accepted treatments of the allergic response in individuals is of immunotherapy with mold allergen extracts. Therefore there is continuing importance in isolating individual allergens for use in standardization of mold allergen extracts and in further studies of the mechanism of allergic response in patients. These extracts are complex mixtures with several allergenic components. There have been several reports of fractionation of *A. alternata* extracts [2-4], however biochemical characterization of specific allergens has been hindered due to lack of

purity. More recently, Deards and Montague [5] and Mathiesen et al. [6] have reported purification of a 29-kD and a 28-kD allergen, respectively, from *Alternaria*. Also, a 70-kD allergen has been isolated by Portnoy et al. [7]. It has thus been envisaged that purified allergens can be prepared utilizing recombinant DNA techniques. This approach may be greatly simplified if partial amino acid sequence data for a specific allergen are available. This paper describes the purification and N-terminal sequence of a 29.2-kD protein (previously described as hypoallergen [8], a doublet of 16 kD and 18 kD under reduced conditions) from the broth of *A. alternata*, which exists in multiple isoforms.

## Materials and Methods

### Antigens

*A. alternata* 34-016 broth material ( $\approx 5$  kD) was prepared from a suspension of a 15-day mold culture [9]. The mycelial mat was removed by filtration through cheese cloth and extracted as previously described [9]. Vijay et al. [9]. The broth filtrate (6.0 litres) was then centrifuged at 16,000  $g$ ,  $-4^{\circ}\text{C}$  for 30 min, followed by filtration through Whatman No. 1 filter paper. The broth was then concentrated by ultra-filtration through an Amicon YM5 membrane (5 kD cutoff), was diafiltered twice with sterile water, and centrifuged at 30,000  $g$ ,  $-4^{\circ}\text{C}$  for 30 min to remove any aggregated material. The supernatant was filtered through a Milipore 0.45  $\mu\text{m}$  HA filter, then lyophilized in 70-ml lots and stored at  $-4^{\circ}\text{C}$  until needed.

For time course studies, broth from mold cultures was harvested at time intervals of 3, 5, 7, 10 and 15 days, and broth extracts were prepared as described above.

### Purification

Lyophilized broth extract (1 g) was dissolved in 20 mM sodium phosphate buffer, pH 7.0 (50 ml) and fractionated on a Whatman DE-52 cellulose ion-exchange column ( $5 \times 45$  cm) at  $4^{\circ}\text{C}$ . The column was initially washed with 20 mM phosphate buffer (250 ml) and then eluted with a linear gradient from 0 to 0.5 M NaCl in the above buffer (600 ml). Eluent was collected in 10 ml fractions and monitored at 280 nm. Fractions were monitored, concentrated and diafiltered by ultra-filtration through an Amicon YM5 membrane.

The concentrated sample was separated on Sephadex G-100 in 20 mM phosphate buffer, pH 7.0 at  $4^{\circ}\text{C}$ . The G-100 column ( $2.5 \times 95$  cm) was eluted at a flow of 0.7 ml/min and 6.0 ml fractions were collected. Eluent containing the 29-kD antigen by SDS-PAGE was pooled and concentrated as mentioned above.

The concentrated eluent (2.5 ml) was further fractionated on a Pharmacia Mono-Q HR5.5 anion exchange column using 20 mM phosphate buffer, pH 7.0. Fractions of 1.0 ml were collected and a flow of 1.0 ml/min was maintained. The cartridge was eluted with phosphate buffer, 10 ml, followed by a linear gradient from 0 to 0.5 M NaCl in phosphate buffer, 30 ml. The eluent was monitored at 280 nm. Eluents containing the 29-kD antigen were detected by Western blot. Fractions containing 29-kD antigen were pooled and concentrated as described above. Samples were stored frozen at  $-20^{\circ}\text{C}$ .

The protein concentration of samples was determined by the dye binding method of Bradford [10], using bovine serum albumin (BSA) as a standard.

### SDS-PAGE and Western Blotting

Protein components in fractions were separated by SDS-PAGE as described by Laemmli [11] and electroblotted onto Immobilon-P, PVDF (Millipore) membrane in a Bio-Rad mini-transblot apparatus as described by Matsudaira [12]. Proteins were visualized on the membrane by staining with Coomassie Brilliant Blue R-250.

The IgE antibody preparation was made by mixing equal volumes of sera from 7 patients who were highly allergic to *A. alternata* as judged by a skin test reaction of 4+, and a direct RAST titre of 3+ using discs coupled with *A. alternata*  $> 5$  kD [14].

Proteins binding to specific human IgE antibodies against *A. alternata* were detected by incubating the PVDF membranes with atopic human sera (a 7-patient pool), followed by incubation with: (a)  $^{125}\text{I}$ -labelled anti-human IgE autoradiography as reported by Vijay et al. [9]; or (b) alkaline phosphatase conjugated goat anti-human IgE,

followed by chemiluminescent detection using the substrate 3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)-1,2-dioxetane (AMPPD) [13].

For chemiluminescent detection of antigens in Western blots [13], membranes were blocked by incubation in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) containing 3% Blocking Agent (Boehringer-Mannheim) for 2 h at room temperature. The membrane was incubated with the atopic sera diluted 1:7 in TBST overnight. The membrane was washed 3 $\times$  with TBST, followed by incubation with goat anti-human IgE conjugated with alkaline phosphatase (AP) (1:2,000 dilution) for 2 h. It was then washed 3 $\times$  with TBST, 1 $\times$  with 100 mM Tris-HCl (pH 9.5), 100 mM NaCl and 5 mM  $\text{MgCl}_2$  (AP buffer), and incubated with the substrate AMPPD (0.05 mg/ml in AP buffer) for 2 min. Excess substrate solution was drained away, and the membrane sealed in a zip-lock bag. Antigenic proteins were observed by chemiluminescence after exposure with a XOMAT AR film (Kodak) for 30–60 min. Multiple film exposures were possible for the duration of a 12-hour period.

### Protein Sequencing and Amino Acid Analysis

Proteins to be sequenced were run on SDS-PAGE as above, and then transferred electrophoretically to Immobilon-P in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11.0 as described by Matsudaira [12]. The membrane was stained with Coomassie Brilliant Blue R-250 and the appropriate protein bands were excised with a razor blade. The N-terminal amino acid sequences of the protein were obtained directly from the excised membrane using an Applied Biosystems protein sequencer, model 470A.

For amino acid analysis 5  $\mu\text{g}$  of purified protein was placed in a vial containing 6.0 M HCl and hydrolysed at  $200^{\circ}\text{C}$  for 75 min. The sample was then processed and analyzed on an automated derivatiser and amino acid analyzer (model 420H, Applied Biosystems).

### Enzymatic Deglycosylation

Enzymatic deglycosylation was accomplished using N-glycosidase F [15] and O-glycosidase [16] from Boehringer-Mannheim. A 20- $\mu\text{l}$  sample of 29 kD protein ( $\approx 50$   $\mu\text{g}$ ) was denatured by addition of 1  $\mu\text{l}$  of 20% SDS followed by incubation at  $95^{\circ}\text{C}$  for 5 min. After cooling for 10 min, 171  $\mu\text{l}$  of 20 mM sodium phosphate buffer (pH 7.3) containing 0.5% CHAPS (Sigma) was added to the denatured protein mixture. N-glycosidase F (0.4 units) and O-glycosidase (2.5 milliunits) were added, bringing the final volume to 200  $\mu\text{l}$  and incubated overnight at  $37^{\circ}\text{C}$ .

Treated and untreated protein samples were separated on SDS-PAGE and protein bands were stained with Coomassie Brilliant Blue R-250 or immunoblots were prepared using PVDF membrane.

### Deglycosylation by Trifluoromethanesulphonic Acid

Chemical deglycosylation with trifluoromethanesulphonic acid (TFMS) followed the procedure of Sojar and Bahl [17]. Purified 29-kD protein (100  $\mu\text{g}$ ) was lyophilized overnight. The lyophilized protein was treated on ice with 40  $\mu\text{l}$  of anhydrous TFMS, and maintained on ice in a nitrogen atmosphere for 2 h with occasional shaking. Subsequently, the reaction was cooled in a dry ice/ethanol bath and the acid neutralized by the slow addition of 60% pyridine (120  $\mu\text{l}$ ). The reaction mixture was then diluted to 10 ml with 100 mM sodium phosphate buffer, pH 7.0. The diluted sample was concentrated and diafiltered using an Amicon ultrafiltration unit with a YM5 membrane. Protein samples were analyzed using 12% polyacrylamide gel.

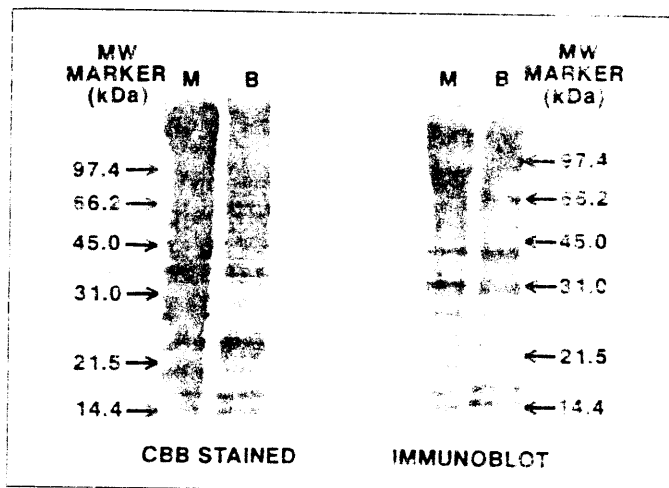


Fig. 1. Comparison of broth and mycelial extracts after reduction with DTT by SDS-PAGE and immunoblot. 250  $\mu$ g dry weight of extract per lane; M = mycelial extract; B = broth extract.

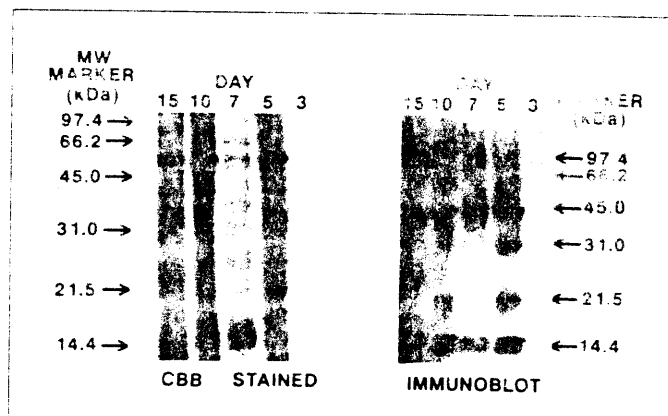


Fig. 2. SDS-PAGE and Western immunoblot of broth extract from *A. alternata* grown for 3 to 15 days.

#### Isoelectric Focusing

Isoelectric focusing was performed on a pH 4.0–6.5 Ampholine PAGplate (LKB) at 4°C according to manufacturer's protocols. For immunoblots, the gels were then contact blotted onto Immobilon-P, PVDF membrane (Millipore) for 2 h. The membrane was briefly washed with water to remove excess ampholyte, then either: (a) stained with Coomassie Brilliant Blue R-250 or (b) probed with human atopic sera as described above. All incubations and washes were conducted at room temperature. The membrane was washed for 3  $\times$  5 min in phosphate-buffered saline (PBS) containing 0.05% Tween-20, followed by addition of  $^{125}$ I-labelled anti-human IgE from Pharmacia (10<sup>6</sup> cpm/ml) in PBS containing 3% BSA and incubated overnight. Excess antibody was removed by washing with PBS containing 0.5% Tween-20 3  $\times$  for 10 min each and the membrane was dried and autoradiographed.

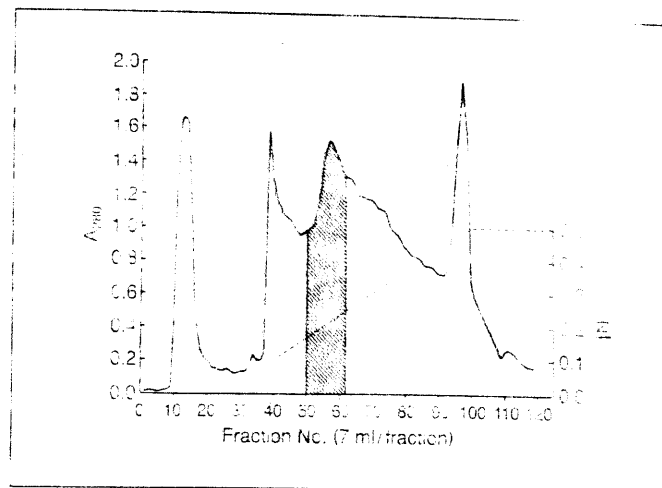


Fig. 3. Anion exchange chromatography of *A. alternata* extract ( $> 5$  kD) on Whatman DE-52 cellulose. Dashed line represents NaCl gradient. Hatched area indicates fractions containing 29-kD antigen as detected by immunoblots. Elution buffer A: 20 mM sodium phosphate, pH 7.0; and elution buffer B: 20 mM sodium phosphate, pH 7.0, 1.0 M NaCl; flow rate, 1.0 ml/min; detection, UV absorbance at 280 nm; column dimensions, 2.5 cm  $\times$  45 cm.

## Results

### Purification

Broth and mycelia extracts prepared from a 15-day culture of *A. alternata* were compared by SDS-PAGE and western immunoblot (fig. 1). The protein content of the broth extract was 173  $\mu$ g of protein/mg dry weight and the mycelia extract was 146  $\mu$ g of protein/mg dry weight as determined using the dye binding assay of Bradford [10]. The 14.5- and 16.0-kD antigen doublet was reported previously in mycelia extracts [2, 8] after reduction with  $\beta$ -mercaptoethanol. This doublet was also prominently visible in the SDS-PAGE of the broth extract, both in the stained gel and in the immunoblot.

The presence of the low molecular weight doublet in broth from 15-day mold cultures prompted an investigation of shorter-term mold cultures. Analysis of time course broth samples after various growth times (fig. 2) showed that the 14.5- and 16.0-kD doublet was evident in immunoblots from day 3 onwards.

The low molecular weight antigens were purified by successive chromatography on DE-52 anion exchange cellulose, Sephadex G-100 and Mono-Q HR5/5 anion exchange matrix. Antigen purification was monitored by probing western blots with atopic human IgE. DE-52 chromatography (fig. 3) served to separate the protein from neutral carbohydrate present in the broth as well as re-

move a large portion of the pigment materials associated with the extract. Size exclusion chromatography on Sephadex G-100 indicated the antigen has an apparent MW of 31 kD in size by comparison with standard proteins (fig. 4). A typical Mono Q anion exchange chromatography profile is



**Fig. 4.** Sephadex G-100 chromatography of 29-kD antigen pool eluted from DE-52 cellulose. Arrows indicate standard proteins: Bovine Serum Albumin (BSA), chymotrypsinogen (CH) and lysozyme (LY). Hatched area indicates fractions containing 29-kD antigen, as determined by Western blots. Elution buffer: 20 mM sodium phosphate, pH 7.0; flow rate, 0.7 ml/min; detection, UV absorption at 280 nm; column dimensions, 2.5 cm  $\times$  95 cm.

illustrated in figure 5. The column separated the antigen into three distinct species (based on OD at 280 nm) which were indistinguishable by SDS-PAGE or immunoblot and were designated F1, F2 and F3. The fractions F1, F2, and F3 were recovered from the anion exchange column at NaCl concentrations of 120, 170 and 210 mM, respectively. Table 1 shows the purification of each individual fraction (0.12% yield per fraction) as well as the pooled fractions (0.36% yield) in comparison to the initial protein load. Figure 6 demonstrates the purification achieved from the initial broth extract via Coomassie stained SDS-PAGE and Western blots.

#### Characterization

The molecular weight of the antigen was determined using SDS-PAGE prior to and after reduction with dithiothreitol (1%). In non-reduced protein samples, a protein band at 29.2 kD was observed and was identical for all the fractions (fig. 7A). After reduction of the protein samples, a doublet of 14.5 and 16.6 kD in all samples was observed, indicating the protein was composed of cystine cross-linked polypeptides. Murine human IgE recognized both the reduced and non-reduced protein in Western blots, as shown in figure 7B, C.

The Mono Q fractions were characterized further by isoelectric focusing on a pH 4.0–6.5 polyacrylamide gel (fig. 8). The IEF of the crude broth extract after staining with Coomassie gave multiple protein bands in the region from pH 4.0 to 5.2. Immunoblotting of the IEF of the broth extract showed (lane 2) 5 major bands at pH 4.1, 4.2, 5.2, 5.25 and 5.45 and at least 12 minor bands. The fractions

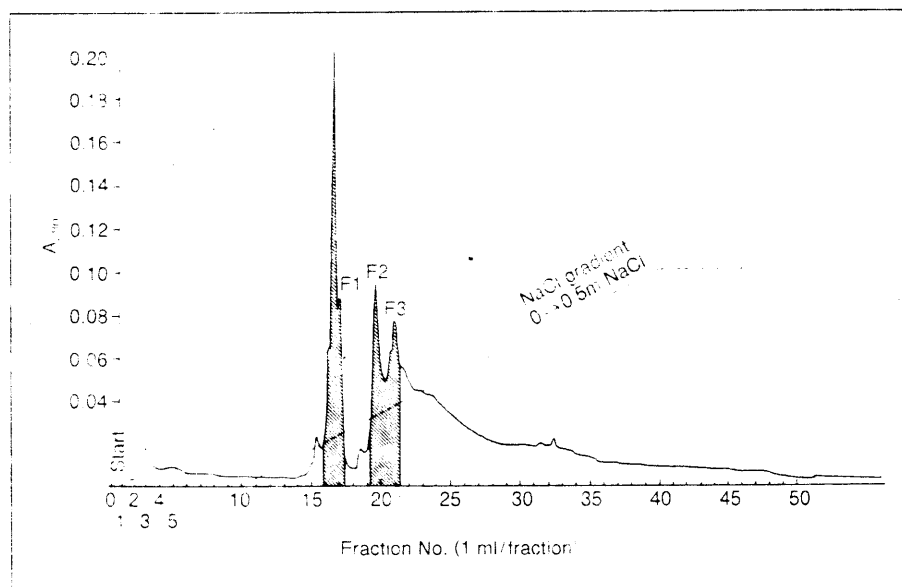


**Fig. 5.** Anion exchange chromatography of 29-kD antigen eluted from Sephadex G-100. The dashed line represents the NaCl gradient. The hatched area indicates fractions containing 29-kD antigen as determined by Western blots. Elution buffer A: 20 mM sodium phosphate, pH 7.0; elution buffer B: 20 mM sodium phosphate, pH 7.0 and 1.0 M NaCl; flow rate, 1.0 ml/min; detection, UV absorption at 280 nm; column: Mono Q HR5.5 (Pharmacia).

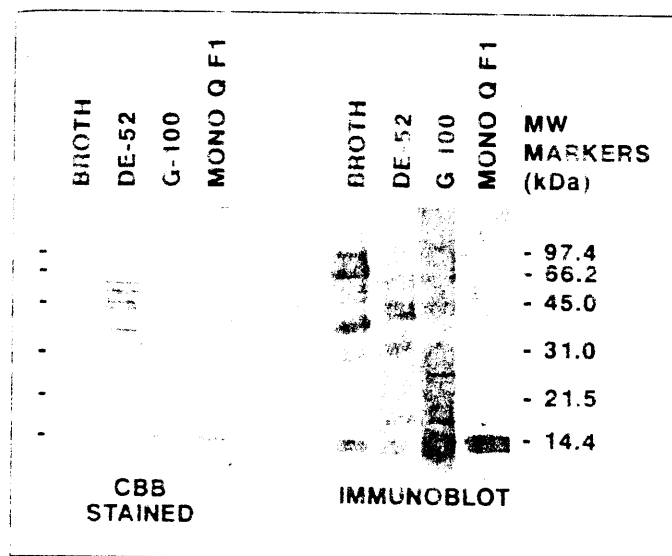


**Fig. 6.** SDS-PAGE (12%) and Western immunoblots of *A. alternata* broth extract and fractions containing 29-kD antigen purified on Whatman DE-52, Sephadex G-100, and Mono Q HR5/5.

**Table 1.** Yield of Alt a-29 from *A. alternata* 34-016 broth extract

| Sample                         | Total protein, mg    | Purification       | Yield %             |
|--------------------------------|----------------------|--------------------|---------------------|
| 1.056 g broth extract (> 5 kD) | 166                  | —                  | —                   |
| DE-52, fractions 50–62         | 21.2                 | 7.8                | 12.7                |
| G-100, fractions 44–52         | 1.22                 | 136                | 0.73                |
| Mono Q F1                      | 0.228                | 732                | 0.13                |
| F2                             | 0.208                | 798                | 0.12                |
| F3                             | 0.178                | 932                | 0.11                |
|                                | (0.614) <sup>1</sup> | (270) <sup>2</sup> | (0.36) <sup>2</sup> |

<sup>1</sup> Sum of Alt a-29 antigen purified.

<sup>2</sup> Calculations based on '1'.

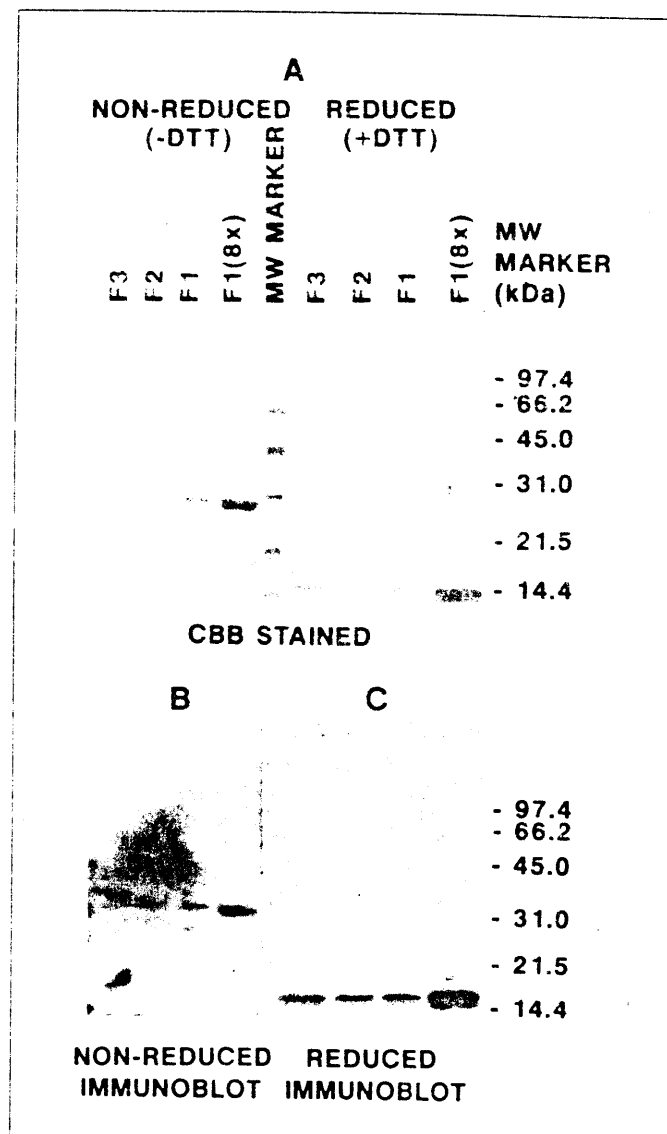


**Fig. 7.** SDS-PAGE (12%) and Western Immunoblot of purified 29-kD antigen in the absence (non-reduced) and presence (reduced) of 1% dithiothreitol.

prepared from the Mono-Q column had similar profiles, with a major band at pH 4.1, followed by minor bands at pH 4.2 and 4.25. Immunoprobings of blotted IEF gels showed all three bands bound atopic human IgE.

The amino acid composition of fraction F1 (table 2) was determined after hydrolysis with acid. Cysteine residue was detected, consistent with a disulphide crosslink between the polypeptides. Tryptophan is not detectable under these hydrolysis conditions.

N-terminal amino acid sequencing was performed on the 16.0 and 14.5-kD bands of Sephadex G-100 fraction

44–52 and Mono Q fraction F1. After SDS-PAGE, the two bands were blotted on to PVDF membrane. The first 20 amino acids (fig. 9) were determined by automated sequencing and the sequence of both the 16- and 14.5-kD polypeptides were identical, with the exception of position 18 where phenylalanine and tyrosine were observed at this position in both peptides.

Glycan content was estimated by relative mobility of the 29.2-kD protein in SDS-PAGE after cleavage via enzymatic or chemical methods, to remove O-linked and/or N-linked glycans from proteins. In SDS-PAGE of non-re-

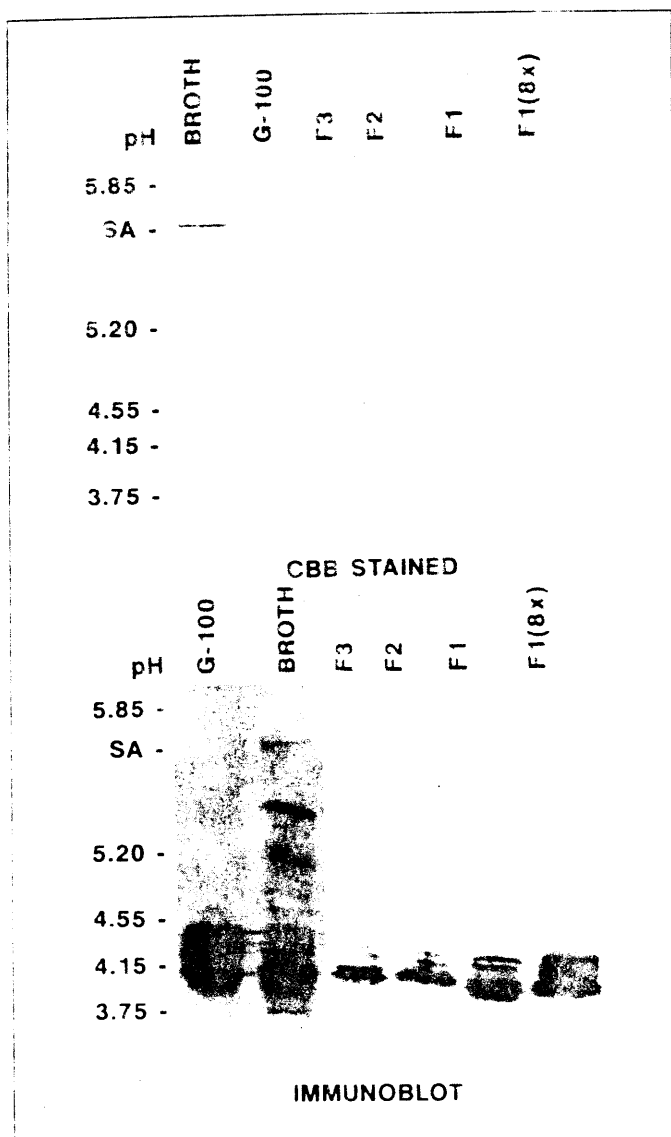


Fig. 8. Native IEF and immunoblot of broth extract and fractions using a pH 4.0–6.5 polyacrylamide gel.

1 Ala-Asp-Pro-Val-Thr-Thr-Glu-Gly-Asp-Tyr-  
10  
11 Val-Val-Lys-Ile-Ser-Glu-Phe-Phe<sup>Tyr</sup>-Gly-Arg  
20

Fig. 9. N-terminal sequence of 14.5 kD and 16.0 kD polypeptides from reduced 29.2 kD antigen.

Table 2. Amino acid composition of purified Alt a-2<sup>9</sup> from *A. alternaria* 34-106

| Amino acid    | Composition (residues/100) |
|---------------|----------------------------|
| Aspartic acid | 12.18                      |
| Threonine     | 9.78                       |
| Serine        | 8.64                       |
| Glutamic acid | 7.75                       |
| Proline       | 7.20                       |
| Glycine       | 8.77                       |
| Alanine       | 9.73                       |
| Cystine       | 1.01                       |
| Valine        | 6.03                       |
| Methionine    | 0.65                       |
| Isoleucine    | 3.03                       |
| Leucine       | 7.71                       |
| Tyrosine      | 4.53                       |
| Phenylalanine | 5.37                       |
| Histidine     | 0.51                       |
| Lysine        | 5.44                       |
| Arginine      | 2.00                       |
| Tryptophan    | n.d.                       |

n.d. = Not determined.

duced protein no change was observed in the mobility of the 29.2-kD protein after enzymatic treatment (fig. 10A) or TFMS treatment (fig. 10B) in comparison with untreated 29.2-kD protein. Immunoblots of the treated protein samples showed the 29.2-kD protein was still capable of binding IgE from atopic sera (fig. 10). Protein samples demonstrated no change in relative mobility in reducing SDS-PAGE (data not shown).

The potential clinical significance of the 29.2-kD protein was examined with immunoblots of the non-reduced protein probed with individual atopic human sera. The western blots of the purified protein were incubated with a 1:6 dilution of individual sera as described above. Analysis of immunoblots (fig. 11) found in 10 of 13 individuals high levels of IgE antibodies which bound the 29.2-kD protein; 2 individuals had a reduced level IgE antibodies to the antigen; and 1 person had no detectable IgE antibodies to the purified protein. Normal sera did not bind to the 29.2-kD protein.

## Discussion

*Alternaria* species possess a number of low molecular weight allergens which are prominent in both mycelial and broth extracts [8]. The most prominent antigen observed was characterized by a doublet in the 14.5- to 18-kD region in SDS-PAGE after reduction of protein. It was found that

the relative proportion of antigen which binds human IgE in Western blots was higher in broth preparations than in mycelial preparations, thus it was decided to isolate the allergenic component from the broth extract of *A. alternata* 24-016. The N-terminal sequences of antigen prepared from broth or mycelia were identical (data not shown).

A major problem in purifying proteins from *Alternaria* is the presence of large amounts of pigmented material which hampers chromatography. This problem was largely alleviated by using ion exchange matrix, such as DE-52 cellulose, which irreversibly binds a large portion of pigmented material. The pigment was not eluted with 0.5 N NaOH or 2.0 M NaCl. The antigen mixture isolated by DE-52 chromatography was separated on Sephadex G-100 and the monitoring of fractions by SDS-PAGE showed that the components with the typical 14.5- and 16.0-kD doublet eluted with an apparent MW of 31 kD. Further fractionation by high-resolution anion exchange chromatography gave the major fractions, F1, F2 and F3. SDS-PAGE of these fractions under non-reducing conditions produced a single band at 29.2 kD and the 14.5- and 16.0-kD doublet after reduction. Their IEF patterns with bands in the range pH 4.1–4.25 are consistent with those values observed from allergenic fractions prepared by Helm et al. [18] and Vijay et al. [14]. The evidence indicates the antigen is a 29.2-kD protein which is a heterodimer of two polypeptide chains of 14.5 and 16.0 kD MW. The two polypeptide chains are evidently closely related since their N-terminal sequences are virtually identical. The relative areas of the 14.5- and 16.0-kD bands, approximately 1:2.5, were consistent in densitometry measurements of the three fractions. This 29.2-kD protein has been designated Alt a-29.

The possibility that the heterogeneity observed with Alt a-29 in purification and IEF could be a result of glycan

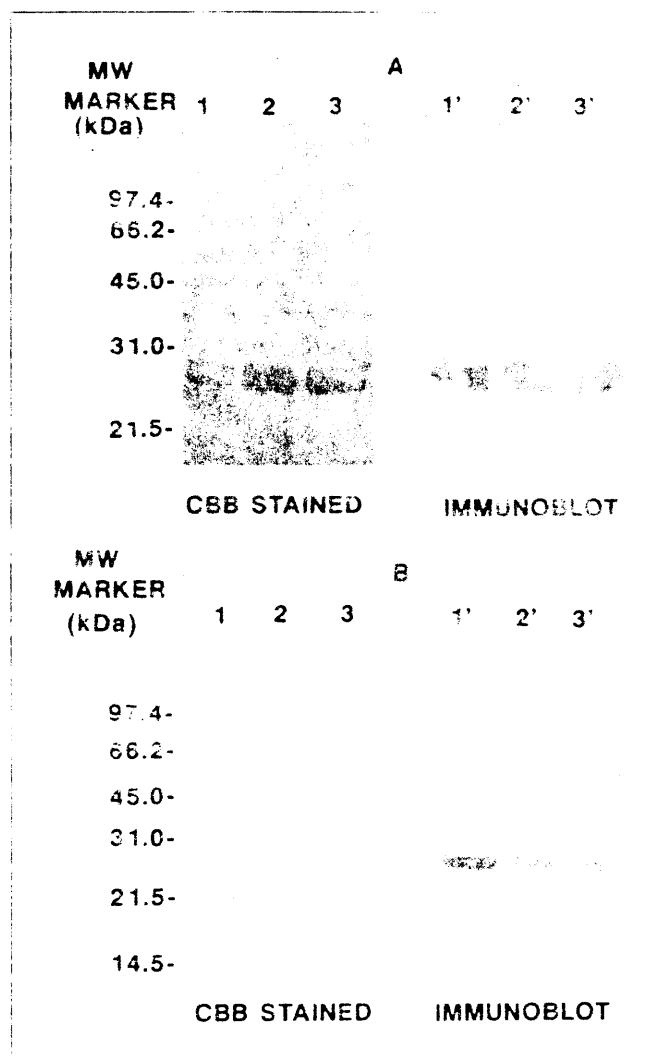
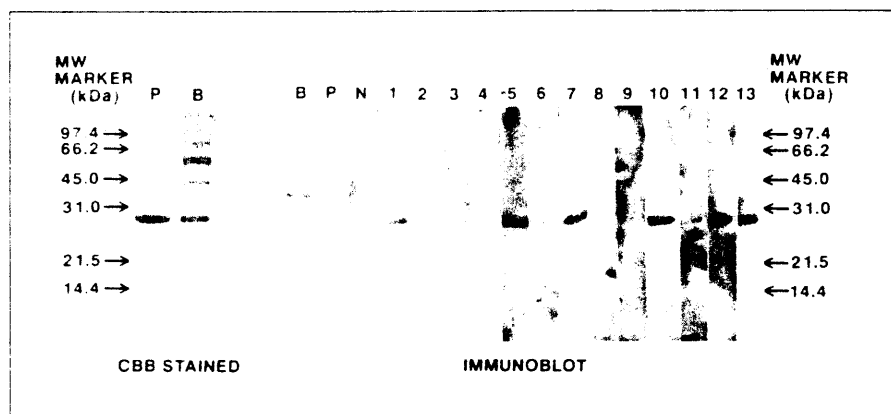


**Fig. 10.** SDS-PAGE and Western immunoblot of non-reduced fractions F1 and F2 after incubation with N-glycosidase F and O-glycosidase for 18 h at 37 °C (A) and treatment with TFMS (B). Lane 1 and 1', untreated F1; lane 2 and 2', treated F1; lane 3 and 3', treated F2.

**Fig. 11.** SDS-PAGE and immunoblot of non-reduced 29.2 kD protein probed with 7 pool atopic human sera or individual atopic human sera. Purified 29.2 kD protein (P) and broth protein (B) were probed with 7 pool atopic sera, while individual western blots were probed with normal human sera (N) or individual atopic human sera (1–13).



residues was examined by incubation of Alt a-29 with N-glycosidase F and O-glycosidase, which enzymatically removes glycan residues from proteins. The relative mobility of the protein in SDS-PAGE remained unchanged after treatment with glycosidases. Similarly, chemical removal of glycans with TFMS failed to change the mobility of the protein in SDS-PAGE, indicating that if glycan residues were present they totalled less than 5% of the protein's mass. Furthermore, immunoblots showed the presence of glycan residues (if any) does not affect the binding of IgE antibodies from atopic sera.

The identical sequence obtained from the 14.5- and 16.0-kD bands and the three observed species of antigen obtained by Mono Q ion exchange chromatography are all indicative of isoallergen forms, which have been well documented for grass pollen allergens [19]. Attempts to match the N-terminal sequence obtained with databases of known protein sequences gave no significant matches.

The clinical significance of Alt a-29 has previously been described as a major allergenic component in *A. alternata* extracts [3, 5, 8, 20]. The immunoblots of non-reduced 29.2 kD protein probed with individual human atopic sera demonstrate Alt a-29 reacted with 92% of the sera tested. These immunoblot results support Alt a-29 is a major *Alternaria* allergen.

Recently, a number of groups have reported purification of similar antigen from *Alternaria* mycelia. Matthiesen et al. [6] have reported a protein which they termed Alt a 1. Alt a 1 was a 28-kD protein which after reduction produced a 14-kD band in SDS-PAGE. These authors demonstrated that Alt a 1 was capable of binding specific IgE in western immunoblot experiments. Comparison of Alt a-29

N-terminal sequence with the sequence recently presented by Matthiesen et al. [personal commun.] for Alt a 1 shows these proteins have 85% homology over the first 20 amino acids.

Deards and Montague [5] have also reported a 29-kD allergen from *Alternaria*, which they have called Alt a Bd 29K. SDS-PAGE after reduction of Alt a Bd 29K was consistent with it being a dimeric protein with two 15-kD subunits. Paris et al. [20] have reported a 31-kD allergen (Alt a 1<sub>1563</sub>) with biochemical characteristics similar to those reported here for Alt a-29. SDS-PAGE of this antigen after reduction was not performed, so the quaternary structure of this protein has yet to be reported. Although the N-terminal sequences of Alt a Bd 29K and Alt a 1<sub>1563</sub> are not available, they are possibly the same protein as Alt a-29. It should be noted that Alt a-29 has been prepared from the extracellular broth and not by mycelial extraction as used for the other proteins mentioned above, though Alt a-29 was present in both broth and mycelial sources. The greater number of isoforms detected for Alt a-29 have suggested that the heterogeneity may be a consequence of processing in the broth, possibly by proteases present in it. The presence of Alt a-29 in broth extracts seems to indicate this 29-kD protein plays an extracellular role, which is unknown at this time.

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