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PURIFICATION AND CHARACTERISATION OF A MAJOR ALLERGEN OF *ALTERNARIA ALTERNATA*

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Abstract—A major allergen of *Alternaria* (Alt a Bd29K) was identified by SDS-PAGE and immunoblotting. It was purified by gel-permeation and ion-exchange HPLC. The apparent molecular mass was 29,000, with a cysteine linked sub-unit of 15,000 M_r. The apparent isoelectric point was 4.2. It was found to be a constituent of the Alt I allergen group.

INTRODUCTION

There is a continuing interest in the isolation of allergens, for use both in the standardisation of allergen extracts and in attempts to understand the underlying mechanism of atopy. The mould *Alternaria alternata* is known to be a significant source of aero-allergens (Budd, 1986). Although there is a number of reports on the fractionation of *Alternaria* extracts (Yunginger *et al.*, 1980; Nyholm and Jansen, 1982; Aukrust *et al.*, 1982; Vijay *et al.*, 1981), many of these fractions have been poorly characterised in terms of their physicochemical or allergenic purity. This paper describes the purification and characterisation of the allergen Alt a and examines its relationship to a previously identified (Yunginger *et al.*, 1980) *Alternaria* allergen Alt I.

MATERIALS AND METHODS

Alternaria culture (mixed spores and mycelia), from the Mycolological Institute strain 49788 supplied by Bencard (UK). It was extracted at 4°C in 20 mM ammonium hydrogen carbonate buffer, pH 7.0 by the addition of 16 M ammonia. The extract was clarified by filtration, and then dialysed against 20 mM ammonium hydrogen carbonate, pH 7.9, using a hollow fibre cartridge (D6, Pharmacia; nominal molecular weight cut-off 10,000), and freeze-dried. A sample of Alt I was kindly supplied by Dr R. Helm and Dr J. Yunginger (Mayo Clinic, U.S.A.).

Sera were obtained from adult individuals known to be RAST and skin-test positive to *Alternaria*.

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Alternaria. A pooled serum, derived from eight *Alternaria*-sensitive subjects, and originally supplied for use in the IUIS *Alternaria* Collaborative Study (Helm *et al.*, 1988), was also tested. Pooled sera either from *Lolium perenne* (rye grass) pollen-sensitive or from normal donors were used as controls.

SDS-PAGE and blotting

Samples were run on a vertical slab apparatus (Protein 2, Bio-Rad) using 12.5% T separating gels (Laemmli, 1970). Sample reduction, when used, was achieved by heating at 100°C for 3 minutes with 1% (w/v) dithiothreitol. After electrophoresis the proteins were electrophoretically transferred (Towbin *et al.*, 1979) on to nitrocellulose (0.45 μ m, Bio-Rad). Part of the nitrocellulose, containing a molecular weight (M_r) marker track, was stained for protein using colloidal gold (Aurodyne, Janssen). The remainder was blocked with 5% bovine serum albumin (BSA) solution then incubated for 24 hr. at 20°C, with human sera diluted 1/5 in 0.5% (w/v) BSA, 0.5% (v/v) Tween 20 in phosphate buffered saline (BTP buffer), pH 7.2. After washing with 0.05% Tween in buffered saline, the blot was probed with ¹²⁵I-anti-human IgE (100,000 d.p.m./ml, Pharmacia) in BTP for 24 hr. The blot was then washed and dried. Autoradiography was performed using pre-flashed X-ray film (Fuji RX) and a calcium tungstate intensifying screen at -70°C, for 72 hr.

I.E.F.

Isoelectric focusing was performed on a pH 3.5-9.5 Ampholine PAGplate (LKB) at 10°C according to the manufacturer's instructions. After fixation, sections of the gel were stained with either Coomassie blue R250 or silver (Merrill *et al.*, 1982). Apparent isoelectric points were determined relative to marker proteins (B.D.H. and Pharmacia). An isoelectric focusing blot was prepared from a pH 3-10 agarose Isogel plate (FMC Bioproducts), run according to the

manufacturer's instructions. It was rinsed with water and then dried down on to polyvinylidene difluoride (PVDF, Millipore) using a vacuum slab-gel dryer (Bio-Rad) at 25°C. The dried gel was separated from the PVDF by soaking in BTP buffer and processed as for the SDS-PAGE blots.

Amino acid analysis

The sample was hydrolysed for 24 hr at 110°C with 5.5 M HCl in the vapour phase. The products were derivatised using phenylisothiocyanate. Analysis was performed on a Pico-Tag (Waters) system using an acetate/acetonitrile gradient. Peaks were detected at 254 nm.

CRIE

Crossed radio-immunoelectrophoresis was carried out essentially as described by Weeke and Lowenstein (1975), except that the electrophoresis buffer was 80 mM Tris 24 mM Tricine 0.28 mM calcium lactate in water, pH 8.6. The second dimension serum was a pool from two rabbits, immunised with *Alternaria* extract, used at 50 μ l ml⁻¹ of agarose solution. The plates were incubated successively with human serum and with ¹²⁵I-anti-IgE for 24 hr periods at 4°C and then autoradiographed as above.

Protein measurement

Protein concns were determined using a modification (Hartree, 1972) of the Lowry colorimetric method. Results were expressed relative to a B.S.A. (Cohn V, Pierce) standard.

Purification

All the high performance liquid chromatography equipment was supplied by LKB. *Alternaria* extract was dissolved (21 mg protein/ml) in 0.2 M sodium phosphate buffer, pH 7.0. For each run, 2 ml of extract solution were injected on to a TSKG3000 SWG gel-permeation column (21.5 × 600 mm) with a GSWP pre-column (21.5 × 75 mm). It was eluted isocratically with 0.2 M phosphate buffer at 5 ml/min. The eluate was monitored at 280 nm. Fractions (5 ml) were collected from four runs and pooled. A fraction (see results) was dialysed against 0.02 M Tris/HCl pH 8 using an ultrafiltration membrane of 10,000 M_r cut-off (PM10, Amicon). It was concentrated to 5.25 mg protein/ml and then fractionated on a TSK DEAE-5PW ion-exchange column (7.5 × 75 mm). The injection volume was 0.2 ml per run, with a flow rate of 0.6 ml/min. After injection, the column was washed for 10 min with 0.02 M Tris/HCl pH 8. It was then eluted with a

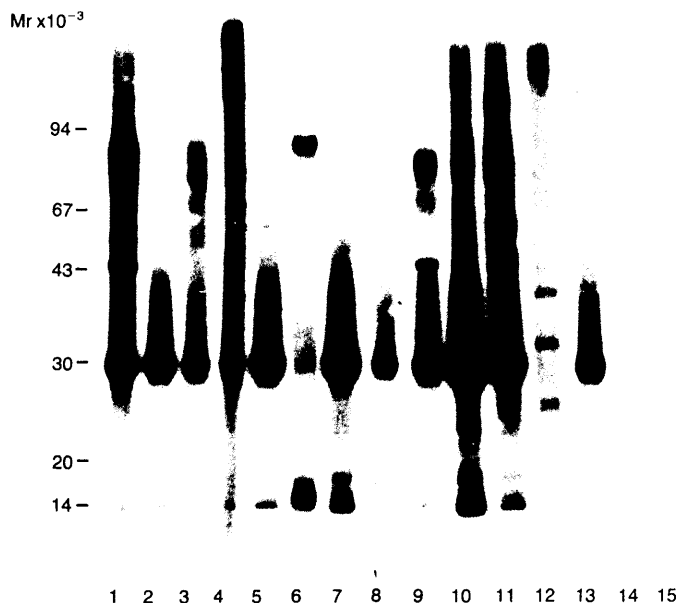


Fig. 1. Human IgE responses to *Alternaria* allergens revealed by SDS-PAGE blotting with non-reduced extract. 1-12: sera from different *Alternaria*-sensitive individuals. 13: Pooled serum from eight other *Alternaria*-sensitive subjects. 14: Normal serum pool. 15: Pooled serum from rye-grass sensitive subjects.

linear gradient of 0 to 0.2 M NaCl in the above buffer over 55 min. The column was cleaned with 1 M NaCl in Tris buffer and then re-equilibrated. Fractions were collected from 7 runs and the peaks pooled. One peak (see results) was concentrated and dialysed against 0.05 M phosphate buffer pH 7 using an ultrafiltration membrane of 10,000 M_r cut-off (YM10, Amicon). It was stored frozen at -20°C.

RESULTS

Allergen identification and isolation

The mol. wt profile of allergens detected by different sera using SDS PAGE blotting, using non-reduced extract, is shown in Fig. 1. A band of 29,000 M_r (Alt a Bd29K) was recognised by 10 of the 12 (83%) individual sera. The *Alternaria*-specific pooled serum showed the majority of its IgE binding to be centred on this region. With reduced *Alternaria* extract (Fig. 2), the most commonly recognised band was that at 15,000 M_r. Certain sera also showed binding at the 29,000 M_r region with the reduced extract. Control sera showed no evidence of non-specific binding.

The Alt a Bd29K (reducible) allergen was purified (see Methods) and relevant column fractions were identified by SDS-PAGE. For the G3000 chromatogram these were between 15,000 and 30,000 M_r (F1, Fig. 3), as determined from the column calibration plot of retention time versus log M_r. The

allergen was eluted from the ion-exchange column in 0.16 M NaCl (F2, Fig. 4). The protein concn of the final product was 17 μ g/ml, representing a yield of 0.04% of the initial protein load.

For comparison of the protein values presented here with other published data, a 100 μ g/ml Alt I solution gave a value of 46 μ g protein ml⁻¹ in the Hartree-Lowry protein assay.

Characterisation

Samples of Alt I and Alt a Bd29K were run, both reduced and non-reduced, on SDS-PAGE then blotted. The blot was probed with serum from an individual that responded to a broad range of *Alternaria* allergens (serum 1, Fig. 1). Figure 5 shows that, following reduction, the purified Alt a Bd29K only showed IgE binding activity at about 15,000 M_r, and was thus composed of cysteine-linked sub-units. Although the same component was present in Alt I, it was also clear that Alt I contained additional allergens.

This apparent heterogeneity of Alt I was confirmed by crossed radio-immunoelectrophoresis, (Fig. 6). This showed that the most intense radio-stained precipitin peak seen with whole *Alternaria* extract was Alt a Bd29K. Furthermore this was the predominant peak in Alt I, although another peak was clearly visible. When all the sera tested by SDS-PAGE blotting (Fig. 1) were tested by CRIE (data not shown), the same (83%) frequency of binding to the Alt a Bd29K peak was observed.



Fig. 2. SDS-PAGE blot using *Alternaria* extract reduced with dithiothreitol. Sera numbered as Fig. 1.

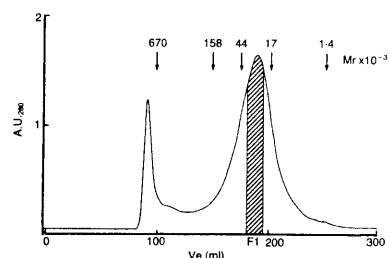


Fig. 3. Gel-permeation HPLC chromatogram of *Alternaria* extract showing retained fraction, F1. Elution volumes of mol. wt markers are also shown.

The *Alternaria* fractions were subjected to isoelectric focusing using a pH 3.5–9.5 polyacrylamide gel (Fig. 7). The apparent isoelectric point of Alt a Bd29K was found to be 4.2, by comparison with marker proteins. It ran as one distinct band. This band was discernable in the Alt I track but was not predominant. An agarose isoelectric focusing blot (Fig. 8) again showed Alt a Bd29K focused as one band at pH 4.2. Alt I showed four bands, 2 of which focused around pH 4.2. The whole extract

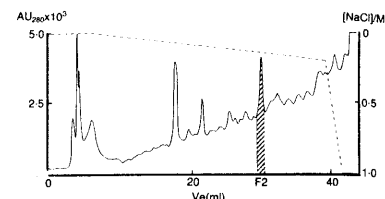


Fig. 4. DEAE ion-exchange HPLC chromatogram of *Alternaria* fraction F1, showing Alt a Bd29K peak (F2).

displayed at least thirteen bands. The greatest radio-staining was in the 7–8 pH region. This was not due to precipitation of allergens at the sample application point, because the samples were applied on filter paper rectangles spanning the 5.7–6.5 pH range.

The amino acid composition of Alt a Bd29K was determined in triplicate, following acid hydrolysis. Results are shown in Table 1. The values for serine and threonine were probably underestimated by 20% and 10%, respectively, and tryptophan was not detectable, because of hydrolytic losses.

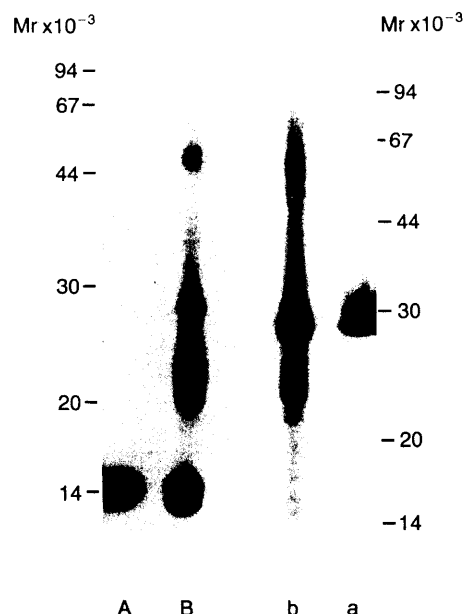


Fig. 5. SDS-PAGE blot of Alt a Bd29K (A, a) and Alt I (B, b) loaded at 0.43 and 23 µg protein per track, respectively. Capital letters signify reduced and lower case letters non-reduced samples. The blot was probed using serum 1, Fig. 1.

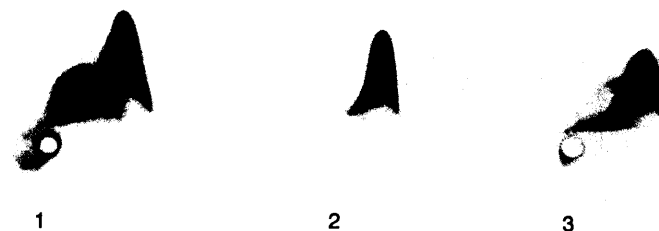


Fig. 6. Crossed radio-immuno-electrophoresis: 1, whole *Alternaria* extract; 2, Alt a Bd29K; 3, Alt I. Loadings for 1, 2, 3 were 15, 0.05, 0.7 µg protein, respectively. Human serum was number 1, Fig. 1.

DISCUSSION

The multi-component nature of many of the *Alternaria* allergen fractions previously described (Yunginger *et al.*, 1980; Nyholm and Lowenstein, 1982; Aukrust *et al.*, 1982; Vijay *et al.*, 1981) makes it difficult to determine whether different laboratories have been isolating the same allergen. Allergen fractions have been stated to be the same, such as Alt I and Ag 1 (Nyholm *et al.*, 1983), but inconsistencies remain. Nyholm *et al.* have stated that the apparent molecular mass of Ag 1 is 60,000 as determined by soft-gel permeation chromatography. However our

study, using SDS-PAGE blotting has revealed only weak IgE binding activity in Alt I in the 60,000 M_r region. It may perhaps be argued that Alt I contains a 60,000 M_r allergen, the epitopes of which are extensively denatured during the electrophoretic processing. However, crossed radio-immuno-electrophoresis shows that the most intense radio-stained peak in Alt I is Alt a Bd29K. Furthermore, IEF blotting, in non-denaturing conditions, shows that the activity which focuses at the pI of Alt a Bd29K is predominant amongst the allergens of Alt I.

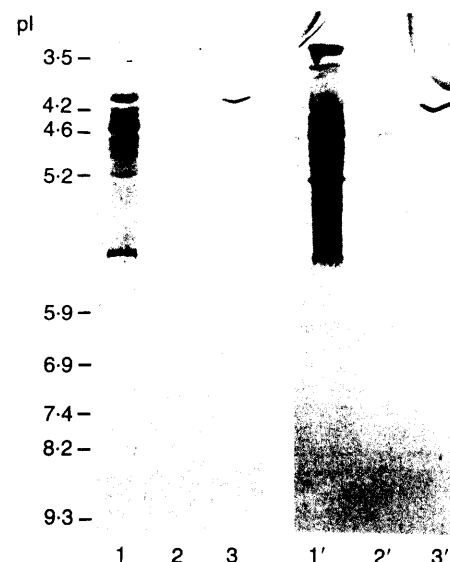


Fig. 7. Isoelectric focusing with a pH 3.5 to 9.5 gradient: 1, whole *Alternaria* extract; 2, Alt I; 3, Alt a Bd29K loaded at 15, 1.4 and 0.3 µg protein per track, Coomassie stained. Tracks 1', 2', 3' were the same samples loaded at 77, 7 and 0.6 µg protein per track, silver stained.

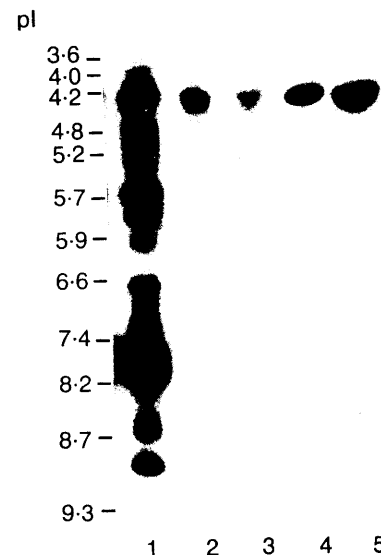


Fig. 8. Blot of pH 3 to 10 agarose isoelectric focusing gel: 1, whole *Alternaria* extract (51); 2, 3, Alt I (5, 2.5); 4, 5, Alt a Bd29K (0.2, 0.4). Values in brackets are micrograms protein loaded per track. The blot was probed with serum 1, Fig. 1.

The observed molecular mass of Alt a Bd29K on SDS-PAGE was not artefactual, as is shown by its elution in the 15,000 to 30,000 M_r range on gel-permeation HPLC. The antigen 8 allergen group (Aukrust *et al.*, 1982) has been described (Nyholm *et al.*, 1983) as a variant of Alt I and has a stated 30,000 to 40,000 M_r range. The apparent isoelectric points for these fractions have been reported as 4–4.5 (Alt I), 4.0 (Ag 1) and 4.3–4.7 (Ag 8). These values are similar to that of 4.2 observed with Alt a Bd29K. Recently, an allergen fraction with a pI of 4.2 has been isolated from the same *Alternaria* strain that was used to prepare Alt I (Helm *et al.*, 1989).

Some of the observed physicochemical variations may be due to differences in synthesis, or degradation, of the same allergen in different *Alternaria* strains. Published reports of SDS-PAGE blots of *Alternaria* have been restricted to those produced with chemically-reduced extracts. Kroutil and Bush (1980) observed binding activity in the 15,000–20,000 M_r region, but this was not the band most commonly recognised by different sera. Possibly the DEAE-cellulose chromatography used to prepare the extract had depleted it of Alt a Bd29K. Also, Tween 20 was not used in their incubation buffers. We have found this to be an essential ingredient for

Table 1. Amino acid composition of Alt a Bd29K

Amino acid	Composition (residues/100)
Asx	8.6
Glx	7.5
His	0.9
Arg	4.4
Lys	5.8
Gly	8.6
Thr	8.8
Tyr	6.3
Cys	2.3
Ser	7.3
Ala	8.6
Pro	6.6
Val	5.9
Met	1.6
Ile	3.6
Leu	7.7
Phe	5.5

satisfactory IgE binding to Alt a Bd29K, perhaps because detergent exchange with the SDS leads to some refolding of the molecule. A similar effect has been observed with *D. pteronyssinus* allergens (Tovey *et al.*, 1989). Bengtsson *et al.* (1986) used a reduced *Alternaria* blot to test a human serum sample said to have a high amount of Alt I-specific IgE.

They observed binding to two bands in the 16,000–18,000 M_r region. These appear to be the two low molecular mass bands seen in reduced Alt I (Fig. 5), the larger of the pair being the Alt a Bd29K sub-unit.

The apparently low yield in the purification of Alt a Bd29K is consistent with reports of a low content of Alt I in other *Alternaria* extracts. Nyholm *et al.* (1983) found Alt I to be present at 0.7% (w/w) in a whole extract.

In describing the allergen Alt a Bd29K, we have used the IUIS recommended nomenclature (Marsh *et al.*, 1987). This allergen was found to be a significant component of the Alt I allergen group. One of the major *Alternaria* allergens, Alt a I, has been defined as being identical to Alt I. Because Alt I is a mixture, Alt a I remains ill defined. We suggest that Alt a I could be better defined by equating it to Alt a Bd29K.

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