

The major allergen of *Alternaria alternata* (Alt a 1) is expressed in other members of the Pleosporaceae family

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Summary

There is general consensus regarding the scarce cross-reactivity existing between *Alternaria alternata* and other allergenic moulds such as *Aspergillus fumigatus*, *Penicillium notatum* or *Cladosporium herbarum*. However, *A. alternata* has been shown to have a very significant level of allergenic cross-reactivity with other fungi belonging to the Pleosporaceae family. To date, no biological identity or homologies with other proteins have been described for Alt a 1, and it remains unclear whether the major allergen Alt a 1 contributes to the cross-reactivity shown for these moulds. Specific quantification of Alt a 1 in culture filtrates of *Stemphylium botryosum*, *Ulocladium botrytis*, *Curvularia lunata*, *Alternaria tenuissima*, *C. herbarum*, *Penicillium chrysogenum* and *Asp. fumigatus*, and immunoblotting using culture filtrate extracts from the above-mentioned moulds and rabbit serum anti-recombinant Alt a 1 have shown significant amounts of Alt a 1 in culture filtrates as well as antigenic components ranging from 14 to 20 kDa that strongly react with the specific serum for all taxonomically related species (Pleosporaceae family). No reactions were revealed in culture filtrates of *Cladosporium*, *Penicillium* and *Aspergillus*. These results restrict the cross-reactivity phenomenon due to Alt a 1 to the scope of the taxonomical term of family.

Key words: Alt a 1, *Alternaria alternata*, Pleosporaceae, cross-reactivity.

Introduction

It is well known that patients allergic to moulds are usually sensitised to several species. This could be caused by cross-reactivity due to shared epitopes of proteins belonging to different related and non-related species, or by multiple concomitant sensitisation to different fungal entities.

Although the reproducibility of mould extracts complicates the evaluation of results from studies of mould allergens and their inter-relationships,¹ several studies have examined cross-reactivity in allergenic mould

species and have contributed to our understanding of this phenomenon.²

In general, studies investigating immunological cross-reactions of *Alternaria alternata* have demonstrated high levels of immunoglobulin-E (IgE) cross-reactivity between this mould and phylogenetically related moulds such as *Stemphyllium*, *Ulocladium* or *Curvularia*,² and low or moderate IgE cross-reactivity between *Alternaria* and other allergenic moulds from different taxonomical families.^{2, 3}

The contribution of molecular cloning techniques to the identification of individual allergens and their antigenic structure has been crucial in the development of the cross-reactivity concept.

Several recombinant allergens from *A. alternata* have been produced, and a number of them have been characterised.^{1, 4}

To date, the biological activity of the major allergen of *A. alternata*, Alt a 1, which has been extensively

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produced by recombinant technology,⁵ remains unclear. This fact complicates the full characterisation of this protein and the search for homologies with other proteins.

The aim of this study was to identify proteins from non-*A. alternata* moulds sharing epitopes with Alt a 1 and to determine how this protein could contribute to the taxonomical relationship between *Alternaria* and other allergenic fungi.

The possibility of extending the range of fungal species expressing Alt a 1 could have very important implications in the sensitisation and developing of the 'allergy to *Alternaria*'.

Material and methods

Strains

Alternaria alternata strain IMIM 93039 (Dr J.M. Torres, Medical Institute for Research, Barcelona, Spain) was used for Alt a 1 cloning.

Aspergillus fumigatus strain AF54 and *Penicillium notatum* strain PC4 (Dr JM Torres), *Cladosporium herbarum* strain CBS 134.31 (Centraalbureau von Schimmelcultures, Utrecht, the Netherlands), *Ulocladium botrytis* strain FMR5819, *Stemphylium botryosum* strain FMR3952, *Curvularia lunata* strain FMR5790 and *Alternaria tenuissima* strain FMR5813 (Dr J. Guarro, Faculty of Medicine, Universitat Virgili Rovira, Reus, Spain) were used to produce allergenic extracts.

Extracts

Culture filtrate extracts were obtained from fungal cultures in Czapek broth medium. All strains were incubated for 3 weeks at 25 °C, except *Asp. fumigatus*, which was kept at 37 °C.

The fungal extracts were obtained as described by Martinez *et al.* [6]. Briefly, culture filtrate was obtained by filtration in Whatman no. 1 (Whatman Int. Ltd, Maidstone, England), glass-fibre prefilter (Millipore, Bedford, MA, USA) and sterilising filter, successively. The filtrate was dialysed by tangential ultrafiltration with a 10 000 Da cut-off point. The retained material was finally freeze-dried.

Cloning of Alt a 1 encoding cDNA and nucleotide sequencing

Cloning of Alt a 1 encoding cDNA and nucleotide sequencing were performed as previously described by Asturias *et al.* [7].

Briefly, poly(A⁺)-enriched mRNA was reverse transcribed using a first-strand cDNA synthesis kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and random hexadeoxynucleotides as primers. Alt a 1 encoding cDNA was amplified by polymerase chain reaction using the described degenerate primers.^{7, 8} The predominant band of 400 bp was isolated from 2% agarose gels (GeneClean, Bio101, La Jolla, CA, USA) and after cutting with *Bam*HI and *Hind*III, cloned into pBluescript II SKJ. Recombinant DNA techniques were performed following standard methods.⁹

Nucleotide sequences were determined using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and analysed on an ABI 373A DNA Sequencer (Perkin-Elmer, Foster City, CA, USA). The analysis of the sequences was done using the gCG Program Package (Genetic Computer Inc., Madison, WI, USA) and the Blast Program.¹⁰

Production and purification of recombinant Alt a 1

Production of rec Alt a 1 was performed according to Asturias *et al.* [7]. Briefly, sequenced cDNA clone Alt a 1 was subcloned into the *Eco*RI/*Xho*I sites of a pGEX-4T-1 expression vector (Amersham Biosciences). Production of the recombinant protein was performed after transforming *Escherichia coli* BL21 competent cells with resulting plasmid. Overnight cultures of *E. coli* BL21 cells containing GST-Alt a 1 encoding plasmid were diluted with LB medium containing 0.1 mg ml⁻¹ ampicillin. Cells were grown with IPTG. After incubation for 3 h, cells were harvested and the pellet was resuspended. Lysozyme and DTT were added and incubated. After mild sonication, Triton X100 was added and incubated at room temperature for 30 min, and the lysate was centrifuged. The precipitated inclusion bodies were resuspended in 0.1 mol l⁻¹ glycine/NaOH pH 9.0 containing 8 mol l⁻¹ urea, and chromatographed through a Sephadex G-25 column (Amersham Biosciences). The eluted protein was stored overnight at 4 °C for refolding. The fusion protein was bound to glutathione-sepharose 4B (Amersham Biosciences) and later cleaved in the column with bovine thrombin. Recombinant Alt a 1 was eluted from the column.

Rabbit serum anti-rec Alt a 1

Experimental immunosera were raised in adult male white New Zealand rabbits according to Gallart *et al.* [11], by subcutaneous injections with 200 µg of rec Alt a 1 in Freund's complete adjuvant. Immunoglobulin fraction containing specific antibodies was obtained by

affinity chromatography in Protein A (*Hi-trap* columns, Amersham Biosciences, Uppsala, Sweden) following the manufacturer's instructions.

Control serum also was obtained using the same protocol, injecting sterile phosphate-buffered saline (PBS) in Freund's complete adjuvant.

SDS-PAGE immunoblotting

Electrophoresis of proteins was performed by SDS-PAGE according to Laemmli [12], using 12.5% polyacrylamide separating gels.

Separated proteins were electrophoretically transferred to PVDF membranes (Immobilon-P, Millipore) according to Towbin *et al.* [13].

Detection of immune complex was made according to Shen *et al.* [14] with modifications during development using chemoluminescent reagents (ECL+, Western Blotting Detection Reagents, Amersham Biosciences).

Peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins (Dako, Glostrup, Denmark) were used to detect bound-specific rabbit antibodies.

Quantification of Alt a 1 in culture filtrates

Enzyme-linked immunosorbent assay (ELISA) using 1D6 monoclonal antibody anti-Alt a 1 in solid phase and biotinylated polyclonal anti-Alt a 1 antibodies as a second antibody reagent (Bial Laboratorios, Bilbao, Spain) was used to measure Alt a 1 concentrations in fungal culture filtrates, as previously described by Asturias *et al.* [7]. Each sample was processed in duplicate and the culture filtrate samples were used at two different concentrations: undiluted and 10-times concentrated.

Results

Figure 1 shows the electrophoretic patterns of all moulds included in this study [anamorphs of the Pleosporales (*A. alternata*, *S. botryosum*, *U. botrytis*, *C. lunata*, *A. tenuissima*), anamorphs of the Dothideales (*C. herbarum*) and anamorphs of the Eurotiales (*Penicillium chrysogenum*, *Asp. fumigatus*)]. The species included in this study showed a variable number of bands, ranging from 100 to 14 kDa: 16 components revealed by *A. alternata*, 22 by *A. tenuissima*, 26 by *U. botrytis*, 17 by *S. botryosum*, 16 by *C. lunata*, 26 by *Asp. fumigatus*, 34 by *C. herbarum* and 18 by *P. chrysogenum*.

Figure 2 shows the results of immunoblotting of the antigens contained in culture filtrate extracts from *A. alternata*, *S. botryosum*, *U. botrytis*, *C. lunata*,

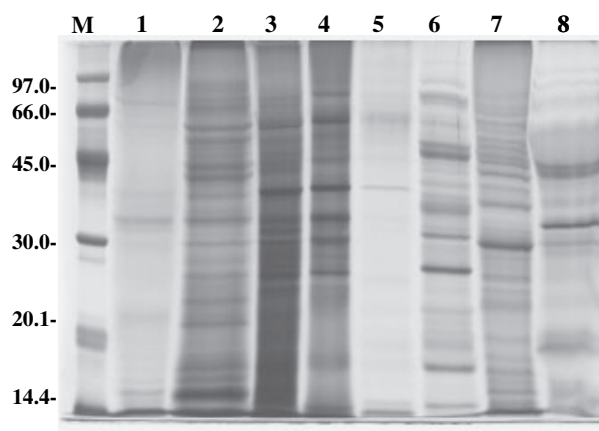


Figure 1 SDS-PAGE of culture filtrate extracts from different fungal species. The components were revealed by silver staining procedure. *Alternaria alternata* (1), *Alternaria tenuissima* (2), *Ulocladium botrytis* (3), *Stemphylium botryosum* (4), *Curvularia lunata* (5), *Aspergillus fumigatus* (6), *Cladosporium herbarum* (7) and *Penicillium chrysogenum* (8).

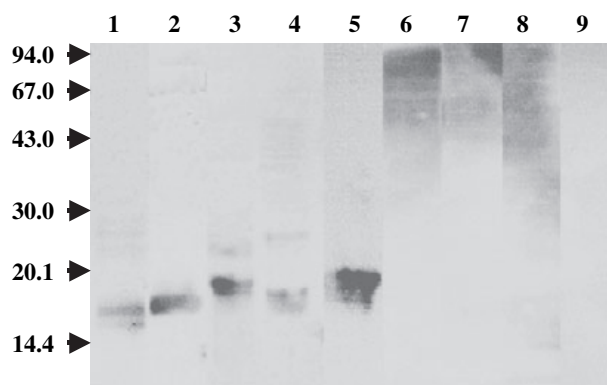


Figure 2 SDS-PAGE immunoblotting of culture filtrate extracts from different fungal species incubated with rabbit serum anti-recombinant Alt a 1. *Alternaria alternata* (1), *Alternaria tenuissima* (2), *Stemphylium botryosum* (3), *Curvularia lunata* (4), *Ulocladium botrytis* (5), *Aspergillus fumigatus* (6) *Cladosporium herbarum* (7), *Penicillium chrysogenum* (8) and *Alternaria alternata* incubated with rabbit sera immunised with PBS in Freund's complete adjuvant (9).

A. tenuissima, *C. herbarum*, *P. chrysogenum* and *Asp. fumigatus*, revealed by rabbit serum anti-rec Alt a 1.

The species corresponding to *A. alternata*, *S. botryosum*, *U. botrytis*, *C. lunata* and *A. tenuissima* showed antigenic components ranging between 14 and 20 kDa, and strongly reacted with specific serum anti-rec Alt a 1.

Cladosporium herbarum, *P. chrysogenum* and *Asp. fumigatus* showed no reaction caused by the experimental serum.

Table 1 Alt a 1 concentrations in culture filtrates of different allergenic mould species.

Species	Strain	Alt a 1 concentration in culture filtrate (ng mL ⁻¹)
<i>Alternaria alternata</i>	IMIM 93039	0.45
<i>Alternaria tenuissima</i>	FMR5813	1.93
<i>Stemphylium botryosum</i>	FMR3952	3.22
<i>Ulocladium botrytis</i>	FMR5819	11.26
<i>Curvularia lunata</i>	FMR5790	0.2
<i>Aspergillus fumigatus</i>	AF54	ND
<i>Cladosporium herbarum</i>	CBS 134.31	ND
<i>Penicillium chrysogenum</i>	PC4	ND

ND, non-detectable (<0.05 ng mL⁻¹).

Control serum raised in rabbits immunised with PBS in Freund's complete adjuvant did not show any reaction.

Table 1 shows Alt a 1 concentrations in filtrates of the different cultured moulds. The results showed significant amounts in species belonging to Pleosporaceae (*A. alternata*, *S. botryosum*, *U. botrytis*, *C. lunata* and *A. tenuissima*). No significant Alt a 1 concentrations could be detected for species belonging to Dothideales (*C. herbarum*) or Eurotiales (*P. chrysogenum* and *Asp. fumigatus*).

Discussion

The application of recombinant DNA and proteomics to the knowledge of allergens has contributed to the characterisation and identification of the individual components causing allergic diseases and their possible biological and immunological relationships.¹⁵

The impact of allergen structure on basic science, taxonomy and phylogeny as well as on the standardisation, prevention, diagnosis and therapy of allergic diseases is an established fact.¹⁵ In this sense, it has been shown for some species including *Alternaria* spp. that the use of a limited number of recombinant allergens can replace the total allergenic extract.^{16–18} In addition, the phylogenetic relationships and taxonomical affiliation of several proteins applied to the concept of immunological and allergenic cross-reactivity has contributed to the definition of terms such as 'panallergen'.^{19, 20}

In relation to obtaining recombinant allergens, the first principal issue to be taken into account is the antigenic/allergenic character of the resulting recombinant proteins and the ability of the epitopes contained in these proteins to bind specific serum antibodies.

To date, no biological identity or homologies with other proteins have been described for Alt a 1.

General consensus exists regarding the scarce level of cross-reactivity existing between *A. alternata* and other allergenic moulds such as *Asp. fumigatus*, *P. notatum* or *C. herbarum*.^{3, 21} However, *A. alternata* has been shown to have very significant levels of allergenic cross-reactivity with other fungi belonging to the Pleosporaceae family (*Stemphylium*, *Ulocladium*, *Curvularia*).²¹

It remains unclear whether the major allergen Alt a 1 contributes to the cross-reactivity shown for these moulds.

The present study demonstrated that the antigens contained in culture filtrate extracts from *S. botryosum*, *U. botrytis*, *C. lunata* and *A. tenuissima* reveal proteins reacting with specific antibodies from rabbit serum anti-rec Alt a 1, and that these proteins possess molecular weights within the range of 10–25 kDa. By means of ELISA, significant concentrations of Alt a 1 also could be shown in filtrates from cultures of species belonging to Pleosporaceae and some of them showed higher Alt a 1 concentrations than *A. alternata*. Taking into account the intraspecific variability existing in the contents and potency of *A. alternata*-secreted allergens,⁶ the study of more strains of *A. alternata* might make it possible to obtain others capable of producing higher amounts of Alt a 1.

In the same way as Alt a 1, these antigenic proteins are also secreted and could be regarded as exoantigens.^{6, 22} *Cladosporium herbarum*, *P. chrysogenum* and *Asp. fumigatus* did not reveal any reaction indicating the presence of proteins with antigenic homology to Alt a 1.

These results restrict the cross-reactivity phenomenon due to Alt a 1 to the scope of the taxonomical term of family and would make it possible to rule out protein Alt a 1 as a panallergen.

However, all data herein obtained suggest that the components from species other than *A. alternata* reacting with antibodies against rec Alt a 1 are proteins very similar and homologous to Alt a 1. Further studies to elucidate the structure of these proteins will therefore be necessary to confirm this.

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