

Identification of allergens homologous to Alt a 1 from *Stemphylium botryosum* and *Ulocladium botrytis*

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Several studies have demonstrated that proteins homologous to the Alt a 1 major allergen of *Alternaria alternata* are expressed in other members of the Pleosporaceae family. However, since no direct biochemical data have been reported concerning the presence of Alt a 1 allergen homologues capable of binding IgE in the excretion-secretion products of *Stemphylium* and *Ulocladium*, our objective was to explore their presence in *Stemphylium botryosum* and *Ulocladium botrytis*. *S. botryosum* and *U. botrytis* culture filtrate extracts were analyzed by two-dimensional (2D)-electrophoresis and 2D-immunoblotting using polyclonal rabbit antibodies raised against recombinant Alt a 1, as well as five human sera from patients allergic to *Alternaria*. Cross-reactivity immunoassays were performed by ImmunoCAP inhibition and 2D-immunoblotting inhibition. IgE-binding proteins recognized by the rabbit antiserum raised against Alt a 1, with apparent molecular weights of 17–18 kDa and isoelectric points of 4, were identified as Alt a 1-like proteins. Alt a 1 inhibited IgE-specific binding to the Alt a 1 homologues from *S. botryosum* and *U. botrytis*. In conclusion, it was demonstrated that allergens which are homologous to Alt a 1 are expressed in the excretory-secretory materials of the phylogenetically-related species *S. botryosum* and *U. botrytis*.

Keywords *Stemphylium botryosum*, *Ulocladium botrytis*, Alt a 1, allergens, homologues

Introduction

Allergic diseases affect approximately one-third of the general population [1], and exposure to allergenic moulds can lead to IgE-mediated rhinitis, asthma and atopic dermatitis [2]. Fungal allergens cause sensitization in 80% of asthmatic patients in the USA [3] and in 20% of Spanish patients with allergies [4]. *Alternaria alternata* is considered to be one of the most important agents of fungal allergens in the world and is associated with severe asthma and respiratory arrest [2]. First described more than 10 years ago, the allergen Alt a 1 is known to be the main allergen of *A. alternata* [5], with more than 80% of patients allergic to *Alternaria* being sensitive to Alt a 1 [5,6].

It was previously demonstrated that other members of the Pleosporaceae family express Alt a 1-like proteins that are capable of reacting with anti-Alt a 1 experimental serum [7]. Furthermore, Hong *et al.* [8] amplified Alt a 1 chromosomal gene homologues from several species of *Alternaria* and related taxa. However, this study did not examine the IgE binding of the isolated homologues, and to date, no direct evidence has been reported concerning the identification of IgE-binding proteins homologous to Alt a 1 in other species of Pleosporaceae. The aims of the study were to identify and characterise Alt a 1 allergen homologues in the excretion-secretion products of *Stemphylium botryosum* and *Ulocladium botrytis*.

Materials and methods

Extracts

Stemphylium botryosum strain FMR 3952 and *Ulocladium botrytis* strain FMR 5819 (Dr J. Guarro Artigas, Laboratory

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of Mycology, Department of Medical Sciences, Rovira Virgili University, Tarragona, Spain) were used for the production of allergenic culture filtrate extracts as previously described by Martínez *et al.* [9].

Sera

Polyclonal rabbit serum raised against recombinant Alt a 1 (rAlt a 1) was obtained from Bial Laboratories (BIAL, Aristegui, Bilbao, Spain). Additionally, we used sera from five patients allergic to *Alternaria* who had high levels of IgE against Alt a 1. The patients were diagnosed by clinical history, cutaneous tests and allergen-specific IgE (ImmunoCAP System, Phadia, Uppsala, Sweden). Informed oral consent for participation in this study was obtained from the patients [10].

Two-dimensional (2D)-electrophoresis and 2D-immunoblotting

Fungal extracts were analysed by 2D-electrophoresis and 2D-immunoblotting using rabbit anti-rAlt a 1 serum and the five human sera, according to the methods presented by Postigo *et al.* [11]. The IgE-binding proteins recognised by the rabbit antiserum raised against Alt a 1 were analyzed by mass spectrometry using MALDI-TOF-MS [11].

Cross-reactivity immunoassays: ImmunoCAP/immunoblotting inhibition

For the ImmunoCAP inhibition assay, three patient sera were pre-incubated with rAlt a 1 (Biomay AG, Vienna, Austria) or with *A. alternata*, *S. botryosum*, *U. botrytis* or *Dermatophagoides pteronyssinus* extracts. Specific IgE responses to rAlt a 1 of *A. alternata*, *S. botryosum*, *U. botrytis* and *D. pteronyssinus* were then measured by ImmunoCAP 100 (Phadia), from which the percentage of inhibition was calculated [12]. For the 2D-immunoblotting inhibition assay, a patient's serum was pre-incubated with rAlt a 1 (Biomay) before use [12].

Results

Figure 1 shows the 2D-immunoblotting and the 2D-immunoblotting inhibition results. The polyclonal rabbit antiserum recognized one protein with an apparent molecular weight (Mw) of 17–18 kDa and an isoelectric point (pI) of 4 in the *S. botryosum* and *U. botrytis* extracts (Fig. 1a). Proteins with identical Mw and pI (arrows in Fig. 1b–f) were also recognized by the five patient sera used in the study, and these proteins were analysed by MALDI-TOF-MS. Fig. 1g shows the 2D-immunoblotting inhibition results. No spot was detected at the same Mw and pI using rAlt a 1 in the inhibitory phase and each fungal extract (*S. botryosum* and *U. botrytis*) in the solid phase.

Table 1 shows the identification by mass spectrometry analysis of the IgE-immunoreactive proteins recognized by the rabbit antiserum raised against Alt a 1. The IgE-binding proteins of *S. botryosum* and *U. botrytis* had sequences that match 41% and 21%, respectively, with the major allergen of *A. alternata* Alt a 1. These two new protein homologues of Alt a 1 were registered in the UniProtKB/Swiss-Prot database with the accession numbers Q5EZC5 for the *S. botryosum* protein and Q5EZ82 for the *U. botrytis* protein.

Allergen cross-reactivity was measured by specific IgE-ImmunoCAP inhibition (Table 2). When rAlt a 1 or *A. alternata*, *U. botrytis* or *S. botryosum* extracts were used as competitive inhibitors and rAlt a 1 was used in the solid phase, the inhibition of solid-phase allergen-specific IgE reactions was equal to or greater than 90%. Variable percentages of inhibition were observed when using *A. alternata*, *U. botrytis* or *S. botryosum* in the solid-phase and rAlt a 1 as the inhibitor. When *D. pteronyssinus* was used as the inhibitor (negative control), no inhibition was demonstrated.

Discussion

The applications of genomics and proteomics have contributed to the characterization and identification of the individual proteins that cause allergic diseases and to the elucidation of the possible biological and immunological relationships [13].

It is well known that while *A. alternata* shows limited cross-reactivity with allergenic moulds such as *Aspergillus fumigatus*, *Penicillium chrysogenum* or *Cladosporium herbarum*, it has significant levels of allergenic cross-reactivity with other fungi in the Pleosporaceae family [14]. However, it remains unclear whether Alt a 1 contributes to the cross-reactivity shown for these moulds and whether Alt a 1-like proteins from other species contribute to the airborne presence of fungal sensitizers.

Sáenz de Santamaría *et al.* [7] demonstrated that culture filtrate extracts from *S. botryosum* and *U. botrytis* contain antigenic proteins that react with experimental serum containing IgG antibodies specific to rAlt a 1. Hong *et al.* [8] amplified chromosomal genes homologous to Alt a 1 in *Alternaria* spp., *Stemphylium* spp., *Ulocladium* spp., *Pleospora* spp., *Nimbya* spp. and *Embellisia* spp. However, these studies did not demonstrate whether the Alt a 1 homologues were also homologous allergens by assessing their cross-reactivity to human IgE Alt a 1-specific antibodies. Consistent with these authors, our results also revealed the presence of two Alt a 1 protein homologues with apparent molecular weights of 17–18 kDa and pI values of 4 from *S. botryosum* and *U. botrytis*.

In accord with the FAO/WHO Allergenicity Rules based on sequence homology [15], the cross-reactivity between a query protein and a known allergen must be considered when there is more than 35% identity in the

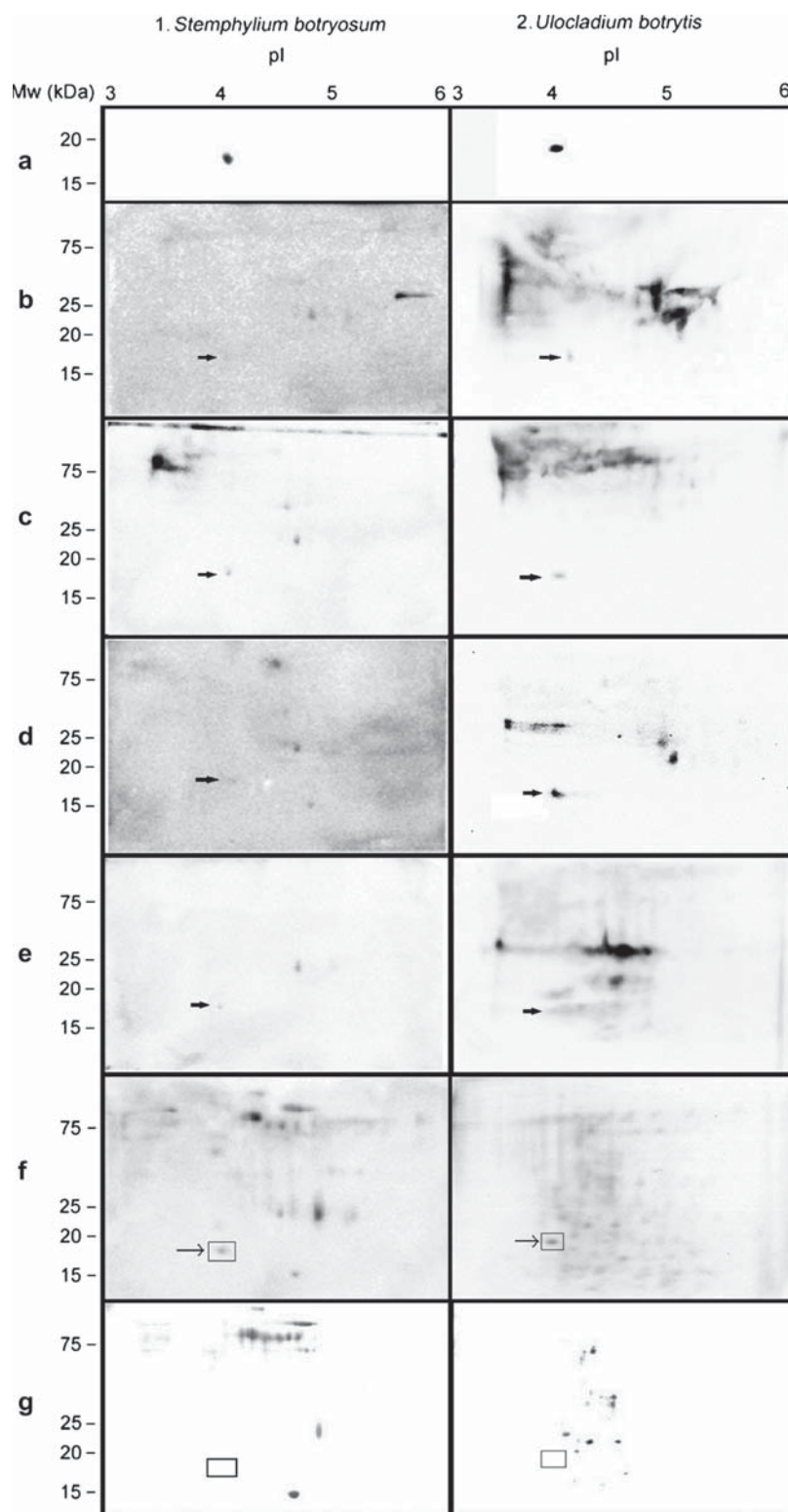


Fig. 1 Two-dimensional-immunoblotting and 2D-immunoblotting inhibition of the *Stemphylium botryosum* (1) and *Ulocladium botrytis* (2) culture filtrate extracts. 2D-immunoblotting with polyclonal rabbit serum anti-rAlt a 1 (a). 2D-immunoblotting with five sera from patients allergic to *Alternaria*, (b–f). 2D-immunoblotting inhibition with a patient's serum inhibited with rAlt a 1 (g). The arrows indicate the position of the proteins homologous to Alt a 1. Mw, molecular weight; pI, isoelectric point.

Table 1 Protein identification: mass spectrometry results of the IgE-binding proteins of *Stemphylium botryosum* (1) and *Ulocladium botrytis* (2) recognized by the rabbit antiserum anti-Alt a 1.

Spot ID	Observed pI/Mw	Theoretical pI/Mw	Protein identity	Matched peptide sequence	Sequence coverage %	MASCOT score	Expected value	NCBI accession number	UniProtKB registered number
1	4.0/17.0	4.6/15.1	Major allergen Alt a 1	K.ISEFYGRKPEGT YYNSLGFNIKATN GGTLDFTCSAQAD KLEDHKWYSCGE NSFMSFSFSDSR.S	41	364	3.70E-30	gi 209363467	Q5EZC5
2	4.0/18.0	4.6/11.6	Major allergen Alt a 1	K.ISEFYGRKPEGT YYNSLGFNIK.A	21	208	1.00E-14	gi 52060758	Q5EZ82

ID, identification; Mw, molecular weight; NCBI, National Center for Biotechnology Information; pI, isoelectric point.

amino acid sequence of the expressed protein or an identity of six contiguous amino acids. The identified Alt a 1 homologues presented 41% and 21% identity with the allergen Alt a 1, and both contained more than six contiguous identical amino acids. These results, along with these proteins' ability to bind specific IgE, prove that they are allergens.

In recent years, proteomics has proven to be an excellent approach to identify novel allergens [16]. Currently, the combination of this technology with immunochemical methods allows the inclusion of a protein into a formal protein family as a first step in identifying the significance of proteins as allergens.

These results confirm that Alt a 1 is not a species-specific allergen from *A. alternata* but a family-specific allergen from Pleosporaceae. This concept causes us to consider substituting the terminology 'allergy to *Alternaria*' with 'allergy to Pleosporaceae' or 'allergy to group 1 Pleosporaceae allergens'.

As *Alternaria* is considered to be the main allergen causing fungal sensitization in Europe [4,10,17], the fact that Alt a 1 could be defined as a family-specific allergen highlights the limitation of the use of this protein in diagnosis due its high cross-reactivity with homologues.

These results also challenge the classical concept of an airborne stockpile of *Alternaria* allergens. Alt a 1-like proteins can also be expressed by other potentially allergenic sources included in the panel of airborne fungal species.

In conclusion, we identified and partially characterized two Alt a 1 homologues from *S. botryosum* and *U. botrytis*. We also demonstrated that these identified proteins are allergens. Furthermore, we found that the allergen Alt a 1 can inhibit the homologues from *S. botryosum* and *U. botrytis* in 2D-immunoblotting inhibition assays. Using the ImmunoCAP inhibition method, we demonstrated that Alt a 1 is fully inhibited by *A. alternata*, *S. botryosum* or *U. botrytis*. However, Alt a 1 is not able to induce full inhibition when *A. alternata*, *S. botryosum* or *U. botrytis* extracts were used in the solid phases. This result can be explained by the variation in the specific IgE response to one or more allergens among patients. Therefore, we suggest that Alt a 1 homologues represent a distinct group of allergens within the family Pleosporaceae or among species phylogenetically related to *Alternaria*.

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Table 2 Specific IgE-ImmunoCap inhibition results.

Solid phase antigen (ImmunoCap™)	Inhibitor antigen	Percentage of inhibition (%)		
		Serum 1	Serum 2	Serum 3
rAlt a 1	rAlt a 1	100	99	100
	<i>Alternaria alternata</i>	95	98	90
	<i>Stemphylium botryosum</i>	90	97	98
	<i>Ulocladium botrytis</i>	91	90	100
	<i>Dermatophagoides pteronyssinus</i>	0	0	0
<i>Alternaria alternata</i>	rAlt a 1	87	59	75
<i>Stemphylium botryosum</i>		100	15	49
<i>Ulocladium botrytis</i>		100	61	31
<i>Dermatophagoides pteronyssinus</i>		0	0	0

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