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Alt a 15 is a new cross-reactive minor allergen of *Alternaria alternata*

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ABSTRACT

Alternaria alternata is one of the most common saprophytes worldwide that is clinically and epidemiologically associated with severe asthma. Therefore, the identification and characterization of all *A. alternata* allergens are of major clinical importance. This study describes a new cross-reactive *A. alternata* allergen that was officially named Alt a 15 by the official Allergen Nomenclature Subcommittee. The complete coding region for Alt a 15 was amplified using 5′ and 3′ rapid amplification of cDNA ends and PCR. The recombinant protein was produced in *Escherichia coli* as a 65-kDa fusion protein, and the protein sequence exhibits high homology with several important fungal allergens. Immunoblotting analyses revealed that IgE antibodies from *A. alternata*-sensitized patients ($n = 59$) bound to rAlt a 15 with a prevalence of 10.2%. All patients who presented sIgE to rAlt a 15 were apparently poly-sensitized to *A. alternata* and *C. lunata*. The extensive cross-reactivity between *A. alternata* and *C. lunata* serine proteases was confirmed using immunoblotting inhibition assays. Overall, Alt a 15 is an important new cross-reactive allergen of *A. alternata* that explains some allergies to *A. alternata* without Alt a 1 sensitization and initial diagnostic errors for allergies to *Alternaria.* This molecule may improve the accuracy of the diagnosis, the understanding, and the management of IgE-mediated fungal diseases.

1. Introduction

Exposure to ubiquitously present fungal spores, primarily from *Alternaria*, *Cladosporium* and *Aspergillus* species, may significantly impact the human immune system, which makes fungal allergy a well-known worldwide problem (Simon-Nobbe et al., 2008; Pant et al., 2009). Several reports found links between sensitization to common airborne fungi and the risk for the development of severe IgE-mediated respiratory symptoms as well as between spore prevalence and asthma (Denning et al., 2006; Delfino et al., 1997). Although a predominant role for fungi has been demonstrated in airway disease, fungal allergens are largely neglected in molecular allergology.

Compelling evidence shows that apparent sensitization to multiple fungi is a frequent clinical observation in patients with mold allergy, which may be a consequence of the cross-reactivity between fungal proteins (Gupta et al., 2002; Crameri et al., 2009). Serine proteases (SPs), particularly subtilisin-like SPs, belong to one of the most important families of allergenic proteins (Radauer et al., 2008). Many fungal SPs exhibit significant cross-reactivity, likely because of conserved protein sequences and shared IgE-binding epitopes, which prompted the designation of SPs as a cross-reactive allergen group of prevalent airborne fungal species (Shen et al., 2007; Yike, 2011), including major allergens in the *Aspergillus*, *Cladosporium*, *Curvularia*, *Penicillium*, *Rhodotorula* and *Trichophyton* species (Pöll et al., 2009). In addition to the phenomenon of cross-reactivity, several lines of evidence suggest that proteases appear to play an important role in the mechanisms underlying allergenicity and to be essential in the eliciting of Th2 responses, which exert a greater impact on the pathogenesis of respiratory allergies (Yike et al., 2011; Wills-Karp et al., 2010 Wills-Karp et al., 2010).

Alternaria alternata is one of the most common environmental fungal species that has been clinically and epidemiologically

Abbreviations: CCD, cross-reactive carbohydrate determinants; RACE, rapid amplification of cDNA ends; SP, serine protease; sIgE, specific IgE; SDAP, structural database of allergenic proteins; PVDF, polyvinylidene difluoride.

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associated with severe asthma and life-threatening acute exacerbations of asthma (Neukirch et al., 1999; Zureik et al., 2002; Black et al., 2000). Alt a 1 is the most important specific allergen of the *A. alternata* allergens and is used to identify genuine IgE-mediated allergy to *Alternaria* (Unger et al., 1999; Asturias et al., 2005). In addition to Alt a 1, however, ten other *A. alternata* allergens have been identified and characterized thus far. The biological role of *Alternaria* allergens in the development of allergy and asthma is poorly understood. Therefore, a more comprehensive definition of the *A. alternata* allergen repertoire appears crucial for the accurate understanding, diagnosis and management of allergic disease.

Several studies recently demonstrated that intrinsic SP-specific activity of *A. alternata* extracts plays an important role in the physiopathogenesis of asthma via the elicitation of an increase in the permeability of bronchial epithelial cells (Leino et al., 2013) and the promotion of a rapid and robust release of early innate mediators and prolonged Th2 inflammation (Kouzaki et al., 2009; Boitano et al., 2011; Snelgrove et al., 2014). The above-mentioned data support the need to further investigate the allergenic properties of *A. alternata* SPs.

The present study describes a new cross-reactive minor allergen of *A. alternata*, which is included in the vacuolar serine protease protein family. The IUIS allergen nomenclature subcommittee approved this allergen, which is officially named Alt a 15.

2. Methods

2.1. Strains and fungal extracts

The *A. alternata* strain CBS 104.26 (Centraalbureau von Schimmelcultures, Utrecht, the Netherlands) and *Curvularia lunata* strain FMR5790 (Laboratory of Mycology, Rovira Virgili University, Tarragona, Spain) were used. Cellular extracts were prepared as previously described by Sáenz-de-Santamaría et al. (2006).

2.2. Human Sera

Sera from fifty-nine respiratory allergic adults sensitized to *A. alternata* were selected from our serum collection (C.0002774, registered in the Institute of Health Carlos III, Ministry of Economy and Competitiveness, Government of Spain) based on a positive skin prick test (ALK-Abelló *A. alternata* extract, >3 mm wheal diameter), positive *A. alternata*-specific IgE (sIgE) levels (>0.35 kU/L) and data availability of skin prick test reactivity to a panel of other fungal extracts. The results of skin prick tests with mold extracts from the database of samples from the serum collection included in this study revealed that 67.8% of the patients were sensitized to *Curvularia*, 54.2% were sensitized to *Aspergillus*, and 66.1% were sensitized to *Cladosporium*. Selected sera origins were the Medical Mycology Laboratory from Municipal Institute for Research, Barcelona, Spain (54 sera) and the Allergy department of University General Hospital of Alicante, Alicante, Spain (6 sera).

sIgEs to rAlt a 1, *C. lunata* extract, rAlt a 6 and cross-reactive carbohydrate determinants (CCDs) were quantitatively determined using the ImmunoCAP™ System (Phadia AB, Uppsala, Sweden). One *Dermatophagoides pteronyssinus*-sensitized patient serum sample that was negative for fungal extracts was also included as a negative control.

2.3. cDNA cloning and sequencing

Total RNA was extracted from the fungal mat of 5-day-old *A. alternata* using Trizol (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturers' instructions. The following degenerate primers were designed based on the highly conserved nucleotides of the several available fungal SP sequences in GenBank and used to amplify an internal fragment of the Alt a 15 cDNA using PCR: iSPF (5′-GCGAATTCATGGTYGTYAAGGGTGTCGAGT-3′) and iSPR (5′-GCCTCGAGCAGAGTCCTTRGCRGGCTGG-3′). The PCR products were purified, subcloned into pJET1.2 (Fermentas) and transformed into *E. coli* DH5 alpha competent cells (Invitrogen). Plasmid DNA was purified, and the nucleotide sequence of the insert was determined. Full-length cDNA was obtained using 5′- and 3′-end Rapid Amplification of cDNA ends (RACE), as previously described by Tripathi et al. (2011). Products of the RACE reactions were purified, subcloned and sequenced. New primers, flSPF (5′-CCTCAGCGCCAGATGGATTCG-3′) and flSPR (5′-TTAGGCGGGAGGAAAGGTTAA-3′), were designed for the full-length sequence, and cDNA was amplified, cloned and sequenced.

2.4. Computational analyses and homology search

The Alt a 15 cDNA and deduced protein sequences were analyzed and compared with sequences in the GenBank database and the Structural Database of Allergenic Proteins (SDAP). Sequence alignments were performed using the ClustalW Analysis Program (http://searchlauncher.bcm.tmc.edu).

Theoretical protein parameters were calculated using the ProtParam program (http://web.expasy.org/protparam/). The presence of putative signal peptides and propeptide cleavage sites was predicted using the SignalP program (http://www.cbs.dtu.dk/services/SignalP/) and ProP 1.0 Server (http://www.cbs.dtu.dk/services/ProP/). The sequences were also analyzed by Gene Runner software (http://www.generunner.net/).

2.5. Expression of the recombinant protein

The open reading frame encoding Alt a 15 was amplified using gene-specific primers: a forward (5′-ATGGATACGGCCAAGGAGGTGCC-3′) and a 3′ reverse primer (5′-AAGCTTCTGGCTAGCGACGCG-3′). The open reading frame was cloned into the plasmid expression vector pBAD-TOPO (Invitrogen) and transformed into One Shot® TOP10Chemically Competent *E. coli* cells (Invitrogen). The cells were grown at 37 °C in Luria–Bertani broth medium containing 100 μg/ml ampicillin to an OD_{600nm} of 0.5 and induced by treatment with 0.02% (w/v) L-arabinose for 4 h. Induced cells were pelleted at 10 000 × *g* for 10 min and lysed using B-PER bacterial protein extraction reagent (Thermo Scientific, Rockford, IL, USA). Inclusion bodies were solubilized using an inclusion body solubilization reagent, and recombinant protein was purified by affinity chromatography using HisPur™ Ni-NTA (Ni^{2+}-nitrilotriacetate) resin (Thermo Scientific). Eluted proteins were refolded using dialysis against decreasing concentrations of urea and Tris buffer (25 mM Tris–HCl, 150 mM NaCl, pH 7.5).

2.6. SDS-PAGE and immunoblotting

Proteins were separated using 12% SDS-PAGE according to Laemmli (1970). Proteins for immunoblotting were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA), as described by Towbin et al. (1979). Briefly, the membrane was cut into strips and incubated overnight with patient sera at 4 °C. Bound IgE was detected via incubation with a mouse anti-human IgE horseradish peroxidase (HRP) conjugate (Southern Biotech, Birmingham, AL, USA). The blots were developed by the addition of chemiluminescent reagents (ECL+, Amersham Biosciences, Bucks, UK). Western blotting using an anti-His-HRP