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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.

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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 demon-

strated 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 pathogenesis 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 Schimmelmcultures, 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 ImmunoCAPTM 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'-GCCAATTCATGGTYGTYAAGGGTGTGAGT-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, fSPF (5'-CCTCAGCGCCAGATGGATTGCG-3') and fSPR (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 HisPurTM Ni-NTA (Ni²⁺-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

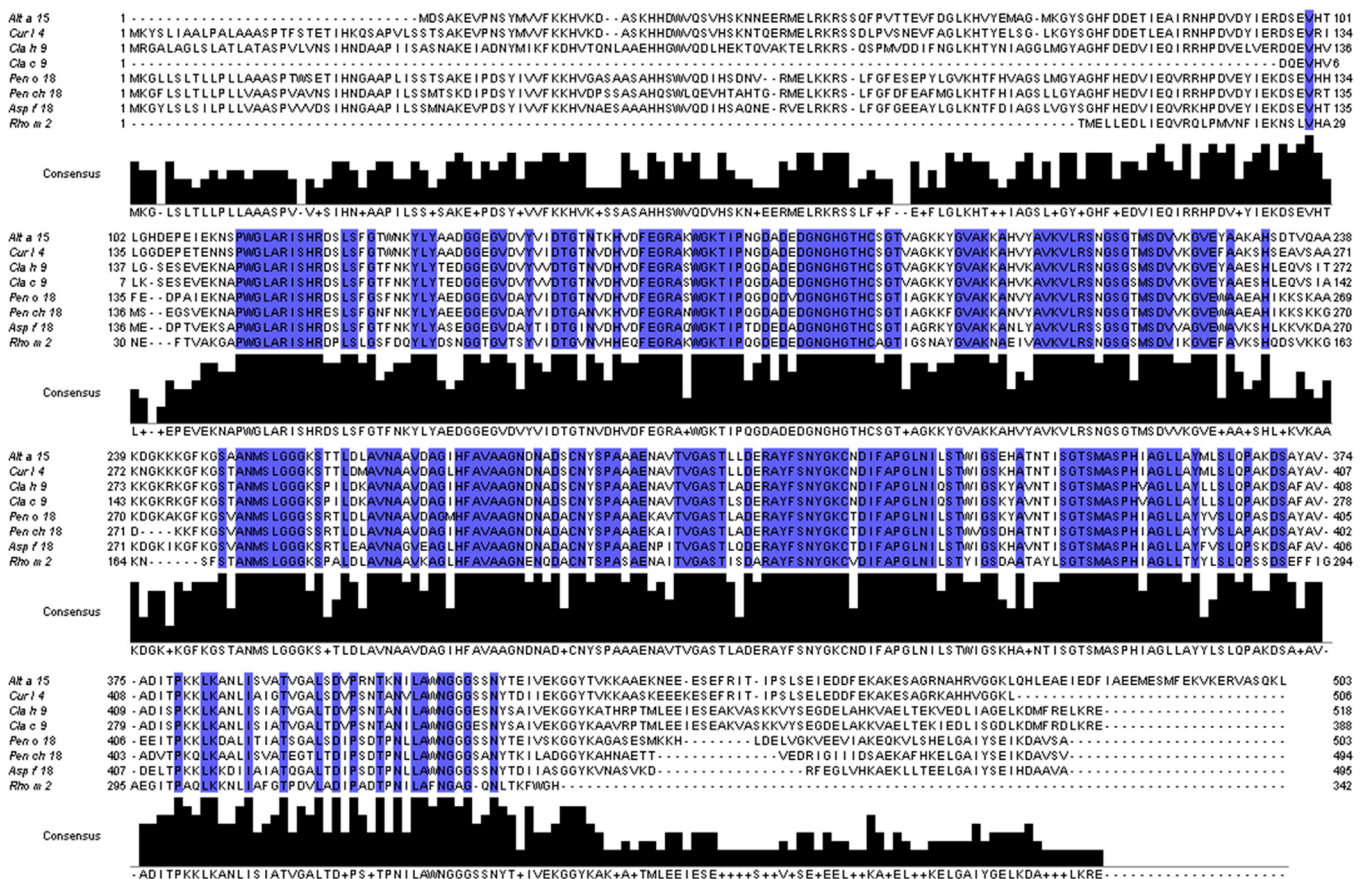


Fig. 1. Nucleotide identity found by multiple alignment of SDAP fungal vacuolar serine proteases protein sequences. The higher bar indicates higher homologies. Nucleotides that 100% identical (invariant) among all sequences are boxed.

antibody (Invitrogen) was also performed to detect the [His]₆-fusion protein.

2.7. Mass spectrometry

The purified 65-kDa band revealed by the anti-His-HRP antibody was sent to the Proteomic Unit of the Spanish National Centre for Biotechnology (Madrid, Spain) for identification using MALDI-TOF/TOF. The MALDI result was obtained in the form of a MASCOT search chart.

2.8. IgE immunoblotting and ELISA inhibition assays

The serum of an Alt a 15-sensitized patient (25 μ L) was separately incubated overnight at 4 °C with different total protein concentrations (0.2–2000 ng) of purified recombinant protein and *A. alternata* extract. IgE quantification was performed as described by Tawde (Tawde et al., 2006). Briefly, microtiter plates were pre-coated with 50 ng of purified rAlt a 15 diluted in 50 mM carbonate-bicarbonate buffer, pH 9.6. The binding of IgE was detected using a mouse anti-human IgE-HRP, and the reaction was developed with Sigma-Fast OPD (o-phenylenediamine dihydrochloride). The OD was measured in a LabSystems Multiskan Bichromatic plate reader (LabSystems, Helsinki, Finland) at 495 nm. The percent inhibition was calculated as $(1 - OD_{495nm} \text{ with inhibitor} / OD_{495nm} \text{ without inhibitor}) \times 100$.

A serum sample from a patient sensitized to *A. alternata* with positive immunoblotting to rAlt a 15 was preincubated at 4 °C overnight with *A. alternata* and *C. lunata* crude extracts (5 mg protein/mL) or PBS for the immunoblotting inhibition assays. The

inhibited sample was tested for binding to rAlt a 15 blotted onto a PVDF membrane, and bound IgE was detected using the procedure described above.

3. Results

3.1. Cloning and sequencing of the alternata vacuolar serine protease

The complete cDNA sequence of Alt a 15 obtained using RACE, PCR and sequencing assays was submitted to the National Center for Biotechnology Information (NCBI) GenBank database under accession number KJ558435. The open reading frame encodes a deduced 503-amino acid polypeptide presenting a theoretical molecular weight and an isoelectric point of 54.4 kDa and 5.8, respectively.

3.2. Bioinformatic analyses of deduced protein sequence

A motif search of the coding sequence of Alt a 15 using Gene Runner software detected the presence of two conserved serine protease motifs of the subtilase family, HGTHCSGTVAG (182–192 residues) and GTSMASPHIAG (346–356 residues). The active-site residues for serine proteases (Asp150, His182 and Ser348) and three potential N-glycosylation sites that are conserved in several vacuolar serine proteases (Shen et al., 2003) were also noted.

Comparisons of the Alt a 15 sequence with the NCBI database revealed a high similarity to a family of subtilisin-like serine proteases from other filamentous fungi. The protein sequence showed significant homology with fourteen recognized fungal allergens, namely, Cur l 4, Cla h 9, Pen o 18, Asp f 18, Pen ch 18, Cla c 9,

Table 1
Homology of Alt a 15 protein sequence with allergens in SDAP database.

	Allergen	Source	Sequence accession number Swissprot/NCBI/PIR	Sequence length	(%) Identity	E score
Vacuolar Serine proteases	Cur l 4	<i>Curvularia lunata</i>	ACF19589	506	90	4.3e–152
	Cla h 9	<i>Cladosporium herbarum</i>	AAX14379	518	70	5.3e–119
	Pen o 18	<i>Penicillium oxalicum</i>	AAG44478	503	68	2.4e–114
	Asp f 18	<i>Aspergillus fumigatus</i>	P87184	495	71	4.8e–111
	Pen ch 18	<i>Penicillium chrysogenum</i>	AAF71379	494	71	7.9e–111
	Cla c 9	<i>Cladosporium cladosporioides</i>	ABQ59329	388	76	1.2e–99
	Rho m 2	<i>Rhodotorula mucilaginosa</i>	Q32ZM1	342	64	1.6e–73
Alkaline Serine proteases	Tri r 2	<i>Trichophyton rubrum</i>	AAD52013	412	42	1.6e–37
	Pen ch 13	<i>Penicillium chrysogenum</i>	AAF23726	397	40	1.1e–23
	Asp v 13	<i>Aspergillus versicolor</i>	ADE74975	403	42	1.5e–20
	Asp f 13	<i>Aspergillus fumigatus</i>	P28296	403	40	2.8e–20
	Asp o 13	<i>Aspergillus oryzae</i>	CAA35594	403	41	9.8e–19
	Asp fl 13	<i>Aspergillus flavus</i>	AAD47202	403	40	9.8e–19
	Pen c 13	<i>Penicillium citrinum</i>	AAD25926	397	39	5.3e–15

Rho m 2, Tri r 2, Pen ch 13, Asp v 13, Asp f 13, Asp o 13, Asp fl 13 and Pen c 13, in the SDAP database (Table 1).

Analysis of the deduced protein sequence of Alt a 15 using the ProP 1.0 program predicted the presence of a propeptide cleavage site that is typically located at the N-terminal end of fungal vacuolar serine proteases between Arg46 and Ser47.

Lai et al. (2004) demonstrated that two critical amino acids played a role in IgE recognition in patient sera, using site-directed mutagenesis mapping of these on immunodominant epitopes in Pen ch 13, and these amino acids are highly conserved in all fungal serine protease allergens. Alignment of the allergenic vacuolar SP sequences (Fig. 1) identified that the conservation of these two amino acids was also observed in Alt a 15 (His157 and Phe160). Moreover, the critical core amino acid residues recognized by the monoclonal antibody that was produced against different epitopes of the serine proteinase major allergens in prevalent *Penicillium* and *Aspergillus* species, which has been generally accepted as a monoclonal antibody against serine protease pan-fungal allergens (FUM20) (Lin et al., 2000; Lee et al., 2007), were also conserved in the Alt a 15 protein sequence (Lys111, Pro114 and Trp115).

3.3. Immunological characterization of the alternata vacuolar serine protease

The recombinant *A. alternata* vacuolar SP was successfully expressed in *E. coli* and detected in the inclusion bodies fraction. His-tagged Alt a 15 was purified using Ni-NTA affinity chromatography and subsequently visualized at approximately 65 kDa using SDS-PAGE and western blotting with an anti-histidine antibody (Fig. 2). The 65-kDa protein was subjected to MALDI-TOF analysis to confirm its function. The peptide sequences were searched in the matrix science database, showing that the subtilisin-like serine protease from *C. lunata* is the closest homologue of the produced 65-kDa recombinant protein. The expressed protein was purified by one-step affinity chromatography, which yielded approximately 0.2 mg of rAlt a 15 from 1 L of bacterial culture. Investigation of the IgE reactivity of purified rAlt a 15 using immunoblotting revealed that six (10.2%) of the tested sera from atopic patients with *A. alternata* sensitization demonstrated IgE reactivity to this recombinant molecule (patient nos. 10, 32, 35, 37, 44 and 47, Fig. 3 A and Table 2).

The levels of sIgE in study serum samples measured using ImmunoCAP (Table 2) revealed that 51 (86.4%) of the 59 *A. alternata*-sensitized patients (patients no. 1–59) presented sIgE to Alt a 1, 26 (44.1%) patients presented sIgE to *C. lunata* extract, and 15 (25.4%) patients presented sIgE to Alt a 6. Positive levels of sIgE to *C. lunata* in all rAlt a 15-immunoreactive sera were also revealed using ImmunoCAP. Only one serum from a patient who was not sensitized to Alt a 1 (patient no. 35) presented IgE reactivity to rAlt a 15 using

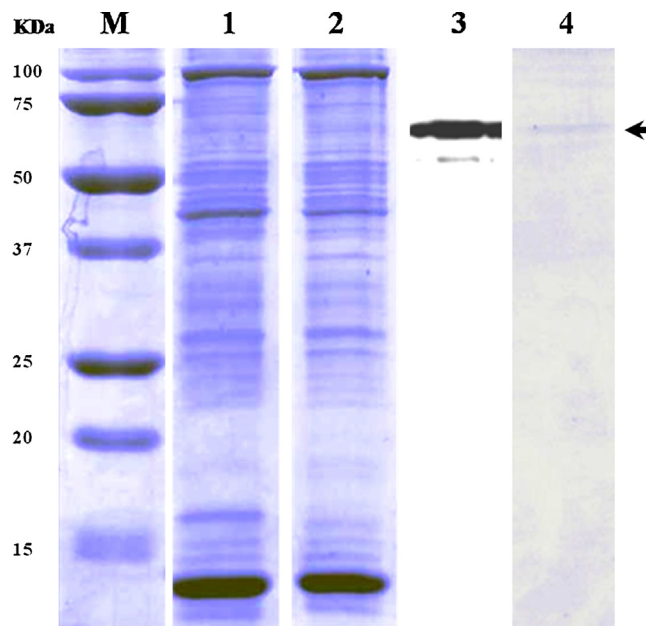


Fig. 2. Expression of Alt a 15. Lane M- Molecular mass marker, Lane 1-Non-induced *E. coli* inclusion bodies fraction, Lane 2- L-arabinose-induced *E. coli* inclusion bodies fraction, Lane 3- anti-His Western Blotting, Lane 4- rAlt a 15 purified by Ni-NTA resin. Arrow indicates position of recombinant Alt a 15 band identified by MALDI.

immunoblotting. This patient exhibited negative IgE responses to Alt a 1 and Alt a 6 but high IgE responses to *C. lunata*. Fifteen of the 26 patients who were sensitized to *C. lunata* exhibited sIgE to Alt a 6. Only one of the six *C. lunata* and rAlt a 15-reactive subjects exhibited IgE reactivity to fungal enolase (patient no. 44).

Inhibition ELISA assays were performed using serum from patient no. 37 to assess the relative reactivity of recombinant and native Alt a 15. The preincubation of this patient's serum with increasing amounts of *A. alternata* mycelial extract yielded an evident dose-dependent inhibition of sIgE-binding to purified rAlt a 15 immobilized in the solid phase (Fig. 4). One nanogram of purified recombinant protein was required for 50 percent inhibition (IC₅₀) of the solid phase-bound rAlt a 15, but 51 ng of total protein content in the *A. alternata* extract was required for the same outcome.

The IgE cross-reactivity between *A. alternata* SP and *C. lunata* SP was demonstrated using the immunoblotting inhibition assay and serum from patient no. 35. *C. lunata* and *A. alternata* extracts completely inhibited the binding of serum IgE to rAlt a 15 (Fig. 3 B).

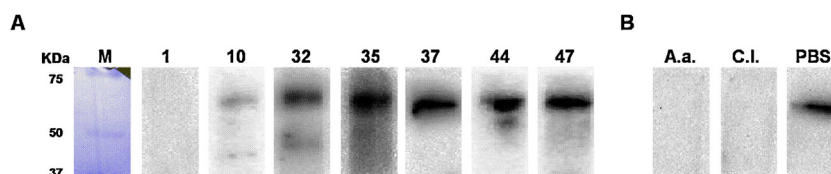


Fig. 3. A. IgE-immunoblotting with representative sera from 59 *A. alternata*-sensitized patients showing IgE reactivity to rAlt a 15 (patient/lane 10, 32, 35, 37, 44 and 47) and one example of an Alt a 15-sIgE negative sera (patient/lane 1). B. Inhibition of the IgE-immunoblotting reactivity of a serum sample no 35 that has been mixed and pre-incubated with: (A.a.) *A. alternata* extract, (C.I.) *C. lunata* extract and (PBS) phosphate-buffered saline.

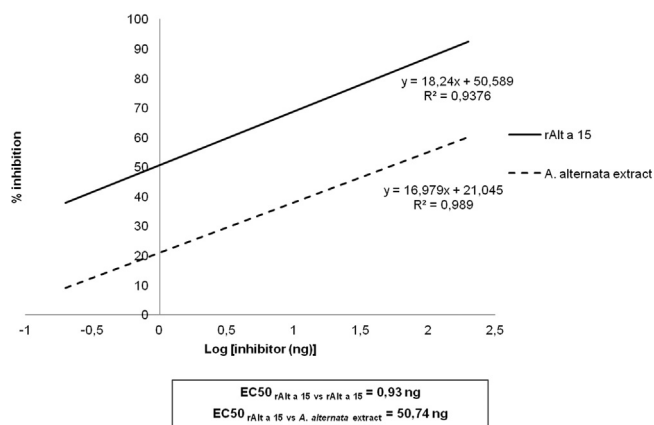


Fig. 4. ELISA-inhibition of IgE-binding to recombinant Alt a 15 coated on the solid phase. Serum from an Alt a 15-sensitized patient was preincubated with increasing amounts of recombinant Alt a 15 and *A. alternata* extract.

4. Discussion

Several fungal allergens have been produced as recombinant proteins and applied in molecular-based allergy diagnostic approaches in the past few decades, which allows for the definition of individual reactivity profiles for each patient (Canonica et al., 2013; Sastre, 2010). An understanding of the complete panel of allergens contained within an allergenic source and the identification of markers of sensitization are key issues for achieving a correct diagnosis and subsequent prescription of allergen-specific immunotherapy (Mothes et al., 2006; Valenta and Niederberger, 2007).

This study cloned, expressed and assessed the allergenic relevance of a vacuolar serine protease from the most important fungal specie that produces airborne allergens, *A. alternata*. The similarity of different fungal SP nucleotide sequences in the GenBank database was analyzed and used in the design of degenerate oligonucleotides, which allowed the isolation and cloning of the Alt a 15-encoding cDNA using RACE experiments. Sequence comparisons of the deduced Alt a 15 protein sequence and allergenic members of the SP protein family from a variety of molds, including Cur l 4, Cla h 9, Pen o18, Asp f 18, Pen ch 18, Cla c 9 and Rhom 2, detected significant identities higher than 50%. This sequence conservation may reflect the high extent of IgE cross-reactivity that has been described within members of this protein family.

The expression of Alt a 15 as a recombinant protein in *E. coli* yielded low quantities of soluble protein. SDS-PAGE revealed that the purified protein had an apparent molecular mass that was higher than expected, and this difference may be associated with the irregular migration patterns of histidine-tagged proteins (Pico de Coaña et al., 2010). The immunological relevance of the recombinant *A. alternata* vacuolar SP was analyzed in an *A. alternata*-sensitized population using immunoblotting. Alt a 15 was found to be a minor allergen of *A. alternata* with the IgE-reactivity to rAlt a 15 demonstrated in 10.2% of study population. The ELISA

inhibition assays using purified rAlt a 15 in the solid phase and an Alt a 15-sensitized patient serum preincubated with *A. alternata* crude extract and rAlt a 15 demonstrated similar slope values for the log-transformed dose-dependent inhibition curves. This outcome indicated that the produced recombinant polypeptide and the natural Alt a 15 contained in the *A. alternata* crude extract exhibited similar immunochemical characteristics for Alt a 15-specific IgE antibodies. This similarity was also reflected in immunoblotting inhibition results, in which a complete inhibition of IgE binding to rAlt a 15 was obtained using the same *A. alternata* extract.

An increasing number of authors suggest that it would be prudent to search for sensitization to other allergenic sources when *A. alternata* sensitization is diagnosed because a significant level of cross-reactivity and poly-sensitization with several other phylogenetically related and non-related molds is reported for *A. alternata* (Katotomichelakis et al., 2012; Cramer, 2011; Postigo et al., 2011; Gutierrez-Rodriguez et al., 2011). Accordingly, our recorded skin prick test results revealed that 67.8% of selected *A. alternata*-sensitized individuals were apparently multiple fungi sensitized to at least *Curvularia*. Curiously, measurement of sIgE to *C. lunata* extract revealed that this rate was much lower (44.1%). In general, patients with multiple fungi sensitization present a trend of IgEs that react to several protein components in *Alternaria* extracts, which suggests that a complete panel of cross-reacting molecules is needed to completely define poly-sensitization patterns (Mari et al., 2003). Previously, Postigo et al. (2011) examined 30 subjects who were allergic to *A. alternata* and demonstrated via molecular diagnosis that most of the apparently poly-sensitized patients were sensitized to enolase Alt a 6 or other *Alternaria* allergenic proteins, which present cross-reactive homologues in other fungal species. Our sIgE investigations verified that the sensitization to Alt a 6 justified the majority (57.6%) of poly-sensitization cases ($n=26$). However, sIgE levels to *C. lunata* extract and immunoreactivity to rAlt a 15 clearly highlighted that all Alt a 15-sensitized patients exhibited in vitro IgE reaction to *C. lunata* extract, which suggests that 23.1% of poly-sensitization cases in this group of patients are likely mediated by a cross-reactivity to vacuolar SP. Moreover, since only one poly-sensitized patient presented sensitization to both Alt a 6 and Alt a 15 allergens, the sensitization of the 23.1% of poly-sensitized patients to other *A. alternata* cross-reacting molecules must be studied. Our research group recently characterized a manganese-dependent superoxide dismutase, Alt a 14, as a minor *A. alternata* allergen that likely participates in cross-reactivity, particularly with *A. fumigatus* (Gabriel et al., 2015). This allergen and the other *A. alternata* cross-reacting molecules identified to date, such as the enolase Alt a 6, helped clarify the diagnostic problems that are associated with cross-reactivity and multiple sensitization phenomena.

Data from sIgE investigations also revealed that 86.4% of the patients who were clinically labeled as sensitized to *A. alternata* exhibited IgE responses to the *A. alternata* major allergen, Alt a 1. Among the eight *A. alternata* sensitized non-reactive to Alt a 1 and Alt a 6 allergens, only one subject presented positive anti-rAlt a 15 IgE immunoblotting. Interestingly, this patient exhibited high IgE responses to *C. lunata* extract (patient no. 35). The complete

Table 2Results of allergen-specific IgE detection in serum samples from *A. alternata*-sensitized patients (1–59), a subject mono-sensitized to *D. pteronyssinus* (60).

ID	Skin Prick test	Specific IgE (KU/L)				Immunoblotting sIgE
	A.a.	Alt a 1	C. l.	Alt a 6	CCD	Alt a 15
1	+	>100	2.3	16.00	<0.35	–
2	+	>100	2.48	7.60	<0.35	–
3	+	<0.35	<0.35	<0.35	<0.35	–
4	+	6.39	<0.35	<0.35	<0.35	–
5	+	15.20	<0.35	<0.35	<0.35	–
6	+	11.10	<0.35	<0.35	<0.35	–
7	+	44.20	2.74	<0.35	<0.35	–
8	+	64.30	<0.35	<0.35	<0.35	–
9	+	4.29	1.80	<0.35	<0.35	–
10	+	1.90	5.10	<0.35	<0.35	+
11	+	<0.35	<0.35	<0.35	<0.35	–
12	+	19.00	<0.35	<0.35	<0.35	–
13	+	4.32	<0.35	<0.35	<0.35	–
14	+	4.40	<0.35	<0.35	<0.35	–
15	+	3.42	<0.35	<0.35	<0.35	–
16	+	<0.35	<0.35	<0.35	<0.35	–
17	+	10.10	<0.35	<0.35	<0.35	–
18	+	13.60	3.71	5.05	<0.35	–
19	+	9.21	<0.35	<0.35	<0.35	–
20	+	3.84	<0.35	<0.35	<0.35	–
21	+	1.97	<0.35	<0.35	<0.35	–
22	+	1.58	<0.35	<0.35	<0.35	–
23	+	<0.35	<0.35	<0.35	<0.35	–
24	+	18.50	0.94	<0.35	<0.35	–
25	+	1.58	<0.35	<0.35	<0.35	–
26	+	20.10	3.69	<0.35	<0.35	–
27	+	8.75	4.20	4.30	<0.35	–
28	+	>100	4.21	11.82	<0.35	–
29	+	34.50	25.31	6.35	<0.35	–
30	+	3.03	<0.35	<0.35	<0.35	–
31	+	9.68	<0.35	<0.35	<0.35	–
32	+	15.70	10.84	<0.35	<0.35	+
33	+	19.00	<0.35	<0.35	<0.35	–
34	+	36.50	3.57	<0.35	<0.35	–
35	+	<0.35	23.21	<0.35	<0.35	+
36	+	24.20	3.23	5.91	<0.35	–
37	+	17.00	8.62	<0.35	<0.35	+
38	+	1.55	<0.35	<0.35	<0.35	–
39	+	96.00	5.70	<0.35	<0.35	–
40	+	13.10	6.00	8.53	<0.35	–
41	+	<0.35	<0.35	<0.35	<0.35	–
42	+	<0.35	<0.35	<0.35	<0.35	–
43	+	10.40	4.25	3.35	<0.35	–
44	+	4.10	2.43	4.28	<0.35	+
45	+	73.40	19.71	7.35	<0.35	–
46	+	30.00	<0.35	<0.35	<0.35	–
47	+	8.70	9.55	<0.35	<0.35	+
48	+	13.70	1.80	2.25	<0.35	–
49	+	<0.35	<0.35	<0.35	<0.35	–
50	+	11.30	<0.35	<0.35	<0.35	–
51	+	3.62	<0.35	<0.35	<0.35	–
52	+	4.66	<0.35	<0.35	<0.35	–
53	+	97.10	4.28	10.10	<0.35	–
54	+	66.40	3.51	4.35	<0.35	–
55	+	7.24	<0.35	<0.35	<0.35	–
56	+	7.37	<0.35	<0.35	<0.35	–
57	+	>100	5.80	2.80	<0.35	–
58	+	1.70	<0.35	<0.35	<0.35	–
59	+	1.90	<0.35	<0.35	<0.35	–
60 ^a	–	<0.35	<0.35	<0.35	<0.35	–

ID, patient identification number; A.a., *Alternaria alternata*; C.l., *Curvularia lunata*; CCD, cross-reactive carbohydrate, +, positive result; –, negative result. The cut offs for positive results were 3 mm for SPT and 0.35 KU/L for detection of specific IgE by ImmunoCAP.

^a Negative control.

inhibition of IgE binding to rAlt a 15 by the *C. lunata* extract using the serum from this patient strongly suggests that his IgE reactivity to *A. alternata* was the result of cross-reactivity between highly homologous SPs, and it is possible that his antibodies were primarily elicited by SPs from other allergenic sources, likely *C. lunata*. The extensive cross-reactivity between Alt a 15 and Cur l 4 is not surprising, given the high level of overall sequence identity (90%). In

fact, some patients who are primarily sensitized by a cross-reactive allergen from one fungal species may develop allergic symptoms by exposure to other environmental fungi because of cross-reacting IgE antibodies that often cause diagnostic errors.

The stimulating/allergenic features of proteases from *A. alternata* and correlations between the severity of the allergic disease and proteolytic activity of *Alternaria* extracts were clearly demon-

strated in various models (Leino et al., 2013; Kouzaki et al., 2009; Boitano et al., 2011; Snelgrove et al., 2014). The inherent allergenic properties of proteases appear to be not only structural but also biochemical in nature, though this hypothesis has not been unequivocally proven and remains a topic of discussion. Therefore, the percentage of the clinical relevance of Alt a 15 that was found using immunoblotting may not represent the real influence of this protein in the development of allergic disease. Based on sequence homology to other serine proteases and on the knowledge that serine proteases are expressed as large precursor molecules, which are post-translationally cleaved to form the mature enzymes, it will be a challenge to produce enzymatically active rAlt a 15 by removal of the prosequence.

In conclusion, the *A. alternata* vacuolar serine protease Alt a 15 behaves as a cross-reactive minor allergen in an *A. alternata*-sensitized population. Alt a 15 (or the highly homologous protein Cur l 4) may also be considered a marker for *C. lunata* sensitization in the absence of Alt a 1 sensitization, which may underlie the initial diagnostic errors that occur for allergies to *Alternaria*. Thus, this molecule can be added to the panel of cross-reacting components needed to completely define polysensitization patterns and solve diagnostic problems associated with cross-reactivity and/or co-sensitization phenomena.

Therefore, the recombinant protein and immunological information provided in this study are useful to improve immunodiagnoses and understanding of IgE-mediated fungal diseases for the development of therapeutic strategies.

Conflict of interest

The authors declared no conflict of interest.

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Databases

Genbank nucleotide sequence data are available in the GenBank database under the accession number KJ558435.

Allergen database: this new allergen has been submitted, accepted and assigned with an official name by the International World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (<http://www.allergen.org>).

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