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Characterisation of *Alternaria alternata* manganese-dependent superoxide dismutase, a cross-reactive allergen homologue to Asp f 6

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ABSTRACT

It is well known that *Alternaria alternata* presents a significant level of allergenic cross-reactivity with several other phylogenetically related and non-related allergenic moulds. To improve the molecular diagnosis, the identification and characterisation of all clinically relevant allergens, including both species-specific and cross-reacting proteins, is required. In this study we report the molecular and immunological characterisation of the *A. alternata* manganese-dependent superoxide dismutase (Alt a MnSOD) and its cross-reactivity with Asp f 6, a diagnostic marker allergen in allergic bronchopulmonary aspergillosis (ABPA). The cDNA coding for Alt a MnSOD sequence was isolated by RACE and PCR. Alt a MnSOD is a protein of 191 amino acids that presented significant homology and potential cross-reactive epitopes with Asp f 6. The recombinant protein was produced in *Escherichia coli* and the immunoreactivity was evaluated in patient sera. Immunoblotting analyses showed that seven of sixty-one *A. alternata*-sensitised patient sera and two ABPA patient sera reacted with the recombinant Alt a MnSOD. The native counterpart contained in both *A. alternata* and *Aspergillus fumigatus* extracts inhibited IgE binding to the recombinant molecule. The allergen was named Alt a 14 by the official Allergen nomenclature subcommittee. Thus, Alt a 14 is a relevant allergen in *A. alternata* sensitisation that may be used to improve diagnostic procedures. Evidence of cross-reactivity between Asp f 6 and Alt a 14-recognition by ABPA patient sera suggest the existence of an Alt a 14-mediated mechanism that, similar to Asp f 6, may be related to the pathogenesis of ABPA.

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Introduction

Fungi are recognised as important sensitisers and triggers of respiratory allergies (Simon-Nobbe et al., 2008). Among the common airborne fungal species, *Alternaria alternata* is one of the principal species associated with allergic disease (Simon-Nobbe et al., 2008; Bush and Portnoy, 2001; Bousquet et al., 2009; Mari et al., 2003). Exposure and sensitisation to this mould have been increasingly recognised as risk factors for the development and

persistence of asthma, asthma severity, and potentially fatal asthma exacerbations (Salo et al., 2006; Black et al., 2000; Kobayashi et al., 2009; Brito et al., 2012).

Among several allergens described in *Alternaria*, Alt a 1 has been demonstrated to be the most important, being recognised by approximately 80% of *A. alternata*-sensitised patients (Unger et al., 1999; Morin et al., 2012; Asturias et al., 2005). Other fungi from the Pleosporaceae family (*Stemphylium*, *Ulocladium*, *Nimbya* and *Embellisia*) have been described to possess Alt a 1 gene homologues (Hong et al., 2005). However, to date, evidence of allergenicity and cross-reactivity with Alt a 1 from *A. alternata* has only been reported for *Stemphylium botryosum* and *Ulocladium botrytis* Alt a 1 homologues (Gutiérrez-Rodríguez et al., 2011). Although in addition to Alt a 1, 12 other proteins have been accepted by the International Union of Immunological Societies (IUIS) as official *Alternaria* allergens (<http://www.allergen.org>), the clinical observations do not appear to be completely explained by the previously identified allergens. In fact, the *A. alternata* sensitisation data are

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; Alt a MnSOD, *A. alternata* manganese-dependent superoxide dismutase; sIgE, specific immunoglobulin E.

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quite complex, showing a significant level of cross-reactivity and poly-sensitisation with several other phylogenetically related and non-related moulds (Cramer, 2011; Katotomichelakis et al., 2012; Postigo et al., 2011).

Recently, Postigo et al. (2011) identified a new 24 kDa IgE-reactive protein from *A. alternata*, included in the manganese-dependent superoxide dismutase (MnSOD) protein family. Other fungal MnSODs have also been identified as important allergens in *Aspergillus fumigatus* (Asp f 6) and *Malassezia sympodialis* (Mala s 11) (Lambou et al., 2010; Andersson et al., 2004). In particular, Asp f 6 has been widely described as an allergen that is strongly associated with allergic bronchopulmonary aspergillosis (ABPA) and frequently used as a tool to distinguish ABPA from fungal sensitisation (Fricker-Hidalgo et al., 2010; Schwienbacher et al., 2005).

In the present study, we report the cloning, expression, immunologic characterisation of the *A. alternata* MnSOD and its cross-reactivity with Asp f 6.

Materials and methods

Strains and fungal extracts

A. alternata strain CBS 104.26 (Centraalbureau von Schimmelcultures, Utrecht, the Netherlands) and *A. fumigatus* strain AF54 (Municipal Institute for Medical Research, Barcelona, Spain) were used. Fungal extracts were prepared as previously described by Martinez et al. (2006).

Human sera

Sera from sixty-one individuals sensitised to *A. alternata* were selected according to a positive skin prick test (ALK-Abelló *A. alternata* extract, >3 mm wheal diameter) and positive *A. alternata*-specific IgE (sIgE) levels (>0.35 kU/L). sIgE to rAlt a 1 and rAsp f 6 was quantitatively determined using the ImmunoCAP™ System (Phadia AB, Uppsala, Sweden). Additionally, one *Dermatophagoides pteronyssinus*-sensitised patient serum sample that was negative for *A. alternata*- and *A. fumigatus*-specific IgE was used as a negative control. Two sera from patients with ABPA were also used. The diagnostic features for both ABPA subjects were intradermal test reactivity to *A. fumigatus* extract, an *A. fumigatus* sIgE level higher than 10 kU/L, positive precipitins to *A. fumigatus* extract, central bronchiectasis, positive sputum culture to *A. fumigatus* and positive Asp f 6 sIgE (>3 kU/L). All sera were acquired from our sera collection (C.0002774, registered in the Institute of Health Carlos III, Ministry of Economy and Competitiveness, Government of Spain). Written informed consent to participate in the study was obtained from all individuals. The study was approved by the local ethics committee and was conducted in accordance with the Declaration of Helsinki.

cDNA cloning and sequencing

Total RNA was extracted from the fungal mat of 5-day-old *A. alternata* using TRIzol (Invitrogen, Carlsbad, CA), and reverse transcription was performed using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturers' instructions. Degenerated primers iSODF (5'-GTCKCCGTGCGAGCARGCSATCAAG-3') and iSODR (5'-CTCCACATRTCSAYRCCAAAGAC-3') were designed based on the highly conserved nucleotides of the several available fungal MnSOD sequences in GenBank and used to amplify an internal fragment of the Alt a MnSOD. The PCR product was purified, subcloned and sequenced. Then, full-length cDNA was obtained using 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, fSODF (5'-ATCATGGAGCTGCATCACAGCAAG-3') and fSODR (5'-TTGAGAGTGAATGCGTGTCTTTG-3'), were designed for the full-length sequence, and cDNA was amplified, cloned and sequenced.

Computational analysis and homology search

The Alt a MnSOD gene and deduced protein sequences were analysed and compared with those in the GenBank database and the Structural Database of Allergenic Proteins (SDAP). Sequence alignments were performed using the ClustalW Analysis Programme (<http://searchlauncher.bcm.tmc.edu>).

Theoretical protein parameters were calculated with the ProtParam programme (<http://web.expasy.org/protparam/>). The sequences were also analysed by Gene Runner software (<http://www.generunner.net/>). The three-dimensional structure of the MnSOD from *A. fumigatus* (Asp f 6, Protein Database Bank (PDB) code 1KKC) was used as a template to build the homology model of the Alt a MnSOD. Monomeric and tetrameric molecular models were generated using the programme ESyPred3D (<http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/>) (Lambert et al., 2002). Visualisation of the models was performed with the programme Swiss-Pdb Viewer (<http://spdbv.vital-it.ch>).

Expression of the recombinant protein

The open reading frame encoding Alt a MnSOD was amplified using gene-specific primers, i.e., a forward primer with an Nco I (underlined) restriction site (5'-CCATGGATGAGCTGCATCACAGCA-3') and a 3' reverse primer introducing a Hind III (underlined) restriction site (5'-AAGCTTGATGGAAGCCTTGAGCTC-3'). Then, it was cloned into the plasmid expression vector pBAD-TOPO (Invitrogen) and transformed into One Shot® TOP10 Chemically Competent *Escherichia coli* cells (Invitrogen). The cells were grown at 37 °C in Luria–Bertani broth medium containing 100 µg/ml ampicillin in an OD_{600nm} of 0.5 and induced by treatment with 0.02% (w/v) L-arabinose for 4 h. Then, induced cells were pelleted at 10,000 × g for 10 min and lysed with B-PER bacterial protein extraction reagent (Thermo Scientific, Rockford, IL, USA). After inclusion bodies were solubilised using an inclusion body solubilisation reagent, recombinant protein was purified by affinity chromatography using His Pur™ Ni-NTA (Ni²⁺-nitrilotriacetate) resin (Thermo Scientific). Eluted protein was refolded by dialysis against decreasing concentrations of urea and, finally, against Tris buffer (25 mM Tris–HCl, 150 mM NaCl, pH 7.5).

SDS-PAGE and immunoblotting

Proteins were separated by 12% SDS-PAGE gels. For immunoblotting, proteins 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. Then, bound IgE was detected by incubation with 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). To detect the [His]₆-fusion protein, western blotting using an anti-His-HRP antibody (Invitrogen) was also performed.

For the immunoblotting inhibition assays, a serum sample from a patient sensitised to *A. alternata* with positive immunoblotting to rAlt a MnSOD was preincubated at 4 °C overnight with *A. alternata* and *A. fumigatus* extracts (5 mg protein/mL) or PBS. Then, the inhibited sample was tested for binding to rAlt a MnSOD blotted onto a PVDF membrane.

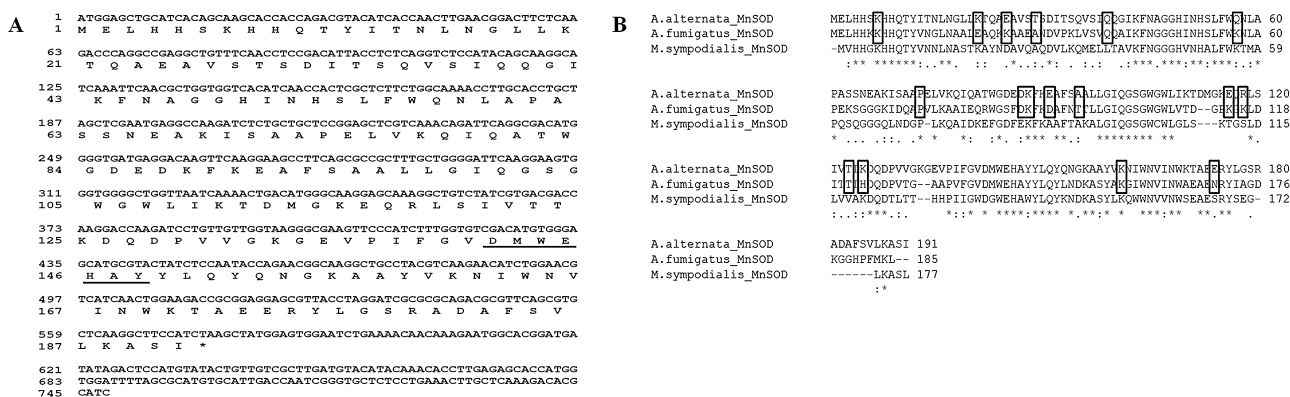


Fig. 1. (A) The nucleotide and deduced amino acid sequences of Alt a MnSOD (GenBank accession No. KC923297). The stop codon TAA is shown (*). The manganese and iron superoxide dismutase signature is underlined. (B) Sequence alignment of Alt a MnSOD protein with fungal MnSOD allergens, Asp f 6 from *Aspergillus fumigatus* and Mala s 11 from *Malassezia sympodialis*. Identical amino acids exposed to the solvent (FSASA > 0.3) in both *A. fumigatus* and Alt a MnSOD structures are boxed.

Mass spectrometry

The 26 kDa band, revealed by western blotting, was identified by MALDI-TOF/TOF. The MALDI result was obtained in the form of a MASCOT search chart.

Specific IgE ELISA inhibition

The serum of an *A. alternata*-positive patient (25 μ L) was incubated with different concentrations (0.2–2000 ng) of purified recombinant protein and *A. alternata* extract separately, overnight at 4 °C. Serum from a *D. pteronyssinus*-sensitised patient was used as a control. IgE quantification was performed as described by Tawde et al. (2006). Briefly, microtiter plates were pre-coated with 50 ng of the purified rAlt a MnSOD diluted in 50 mM pH 9.6 carbonate-bicarbonate buffer. The binding of IgE was detected by 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.

Results

Cloning of cDNA coding for *A. alternata* MnSOD

Full-length Alt a MnSOD cDNA clones were identified using RACE-PCR, standard cloning techniques and nucleotide sequencing. The complete cDNA sequence of 748 bp was submitted to the GenBank database under accession number KC923297. Alt a MnSOD-encoding cDNA consists of 576 bp, which corresponds to a deduced amino acid sequence of 191 residues (Fig. 1A). A conserved motif, DMWEHAY (142–148 residues), was detected in the

sequence of the deduced protein. A motif search using Gene Runner software identified this sequence as a manganese and iron superoxide dismutase signature. Its theoretical molecular weight was 21.3 kDa, with an isoelectric point of 6.6.

Bioinformatic analysis of the deduced protein sequence

Comparisons of the Alt a MnSOD cDNA sequence with the NCBI database showed homology with manganese-dependent superoxide dismutases from various sources at both the DNA and protein levels. At the protein level, it was similar to the MnSODs from *Pyrenophora tritici-repentis* (94%) XP_001937231.1, *Leptosphaeria maculans* (90%) XP_003842884.1, and *Curvularia lunata* (89%) AAQ87929.1. Further, it also showed significant homology with two fungal allergens in the SDAP database, namely Asp f 6 from *A. fumigatus* (59%, e-score: 7e-80) AAB60779 and Mala s 11 from *M. sympodialis* (48%, e-score: 2e-57) CAD68071, and two non-fungal allergens, Hev b 10 from *Hevea brasiliensis* (45%, e-score: 8e-34) CAB53458 and Pis v 4 from *Pistacia vera* (45%, e-score: 4e-33) EF470980.

Molecular modelling of Alt a MnSOD and identification of putative cross-reactive IgE-binding epitopes

Sequence alignment revealed that Alt a MnSOD shares significant sequence homology with MnSODs from *A. fumigatus* and *M. sympodialis*, two important fungal allergens (Fig. 1B). In all known MnSOD structures, the metal ion is bound by four invariant protein ligands: one aspartate and three histidines. In fact, the conserved amino acids include the three histidines at positions 4, 52 and 146 and the aspartate at position 142 in the Alt a MnSOD sequence, which are involved in the binding of Mn²⁺ metal.



Fig. 2. Structural models. (A) Ribbon representation of the Alt a MnSOD monomer. α -helices are shown in red, β sheets in green and turns in yellow. (B) Ribbon diagram of the Alt a MnSOD tetramer. (C) Alt a MnSOD molecular surface. Identical amino acids that are solvent-exposed (FSASA > 0.3) in both Asp f 6 and Alt a MnSOD models are shown in blue.

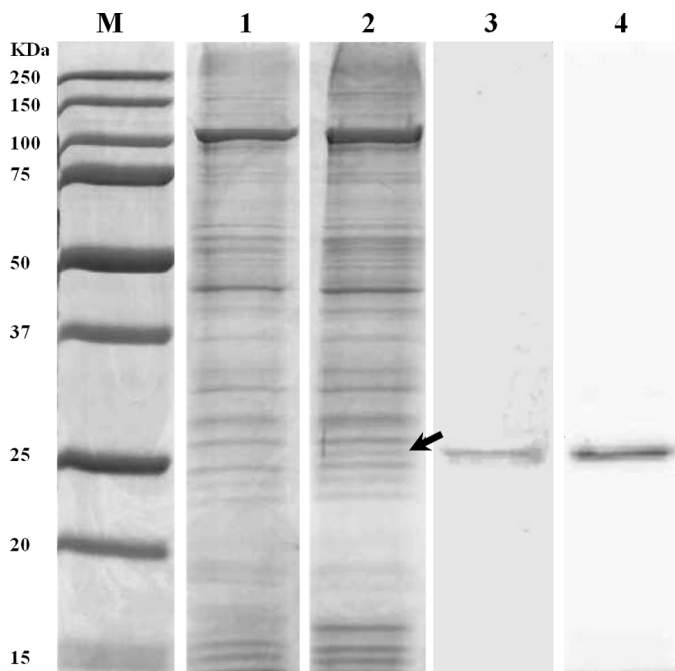


Fig. 3. Expression of Alt a MnSOD. Lane M – molecular mass marker, Lane 1 – non-induced *E. coli* inclusion bodies fraction, Lane 2 – L-arabinose-induced *E. coli* inclusion bodies fraction. Arrow indicates position of recombinant Alt a MnSOD band identified by MALDI, Lane 3 – rAlt a MnSOD purified by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

A three-dimensional structural model of Alt a MnSOD was generated using the previously solved crystal structure of Asp f 6 (Fluckiger et al., 2002). Model data are available in the PMDB database under the accession number PM0079554. The monomer exhibits an α/β fold (Fig. 2A) in accordance with the 3D structure of Asp f 6. Based on the oligomeric state of the template, the tetrameric state of the target molecule was created (Fig. 2B).

To determine which of the residues shared by Asp f 6 and Alt a MnSOD could be critical for cross-reactivity, conserved residues that are exposed to the solvent on the surface of the molecular models were identified. It was observed that although Asp f 6 and Alt a MnSOD share 59% of the primary amino acid sequence, only 17 of the pairwise conserved residues, shown in boxes in Fig. 1B, were at least 30% exposed to the solvent in both models. As shown in Fig. 2C, these critical residues are scattered over the entire surface of the Alt a MnSOD molecule.

Expression of recombinant *A. alternata* MnSOD in *E. coli*

The coding region of the isolated cDNA was fused with a short DNA sequence encoding an extra peptide containing a V5 epitope and a [His]₆-tag at the C-terminus of the protein. Alt a MnSOD recombinant protein was expressed in *E. coli* TOP 10, and it was detected, recovered and purified from the inclusion body fraction. It presented an apparent molecular mass of approximately 26 kDa, as estimated by SDS-PAGE and western blotting with an anti-histidine antibody (Fig. 3). The MALDI results showed that MnSODs from *Pyrenophora teres f. teres* and *P. tritici-repentis* are the closest homologues of the recombinant protein produced in this study.

Human IgE recognition of the recombinant protein

The levels of sIgE to Alt a 1 and Asp f 6 in the 64 study serum samples measured by ImmunoCAP are shown in Table 1. Among the 61 serum samples from *A. alternata*-sensitised patients (patients no.

Table 1

Results of allergen-specific IgE detection in serum samples from *A. alternata*-sensitised patients (1–61), a subject mono-sensitised to *D. pteronyssinus* (62) and patients with diagnosed ABPA (63–64).

Serum I.d.	ImmunoCap sIgE (kU/L)		Immunoblotting sIgE
	Asp f 6	Alt a 1	Alt a 14
1	0	>100	–
2	0	>100	–
3	0.55	78.2	+
4	0	0	–
5	0	6.39	–
6	0	15.2	–
7	0	11.1	–
8	0	44.2	–
9	0	64.3	–
10	0	4.29	–
11	0	66.4	–
12	0	0	–
13	0	19	–
14	0	4.32	–
15	0	7.24	–
16	0	3.42	–
17	0	3.85	–
18	0	0	–
19	0	10.1	–
20	0	13.6	–
21	0.44	33.7	+
22	0	9.21	–
23	0.46	36	+
24	0	3.84	–
25	0	1.97	+
26	0.37	1.58	+
27	0	0	–
28	0	18.5	–
29	0	1.58	–
30	0	20.1	–
31	0	8.75	–
32	0	>100	–
33	0	34.5	–
34	0	3.03	–
35	0	9.68	–
36	0	15.7	–
37	0	19	–
38	0	36.5	–
39	0	0	–
40	0	24.2	–
41	0	17	–
42	0	1.55	–
43	0	96	–
44	0	13.1	–
45	0	0	–
46	0	0	–
47	0	10.4	–
48	0	7.37	–
49	0	73.4	–
50	0	30	–
51	0	>100	–
52	0	13.7	–
53	0	0	–
54	0	11.3	–
55	0	3.62	–
56	0	4.66	–
57	0	97.1	–
58	0	35	–
59	12.8	0.76	+
60	1.04	0.59	+
61	0	7.89	–
62	0	0	–
63	5.11	0	+
64	3.20	0	+

1–61), 54 had Alt a 1 sIgE, and only 6 presented detectable rAsp f 6 sIgE.

Investigation of the IgE reactivity of rAlt a MnSOD by immunoblotting revealed that 7 out of 61 *A. alternata*-sensitised patient sera reacted with the recombinant molecule (Fig. 4). Thus,

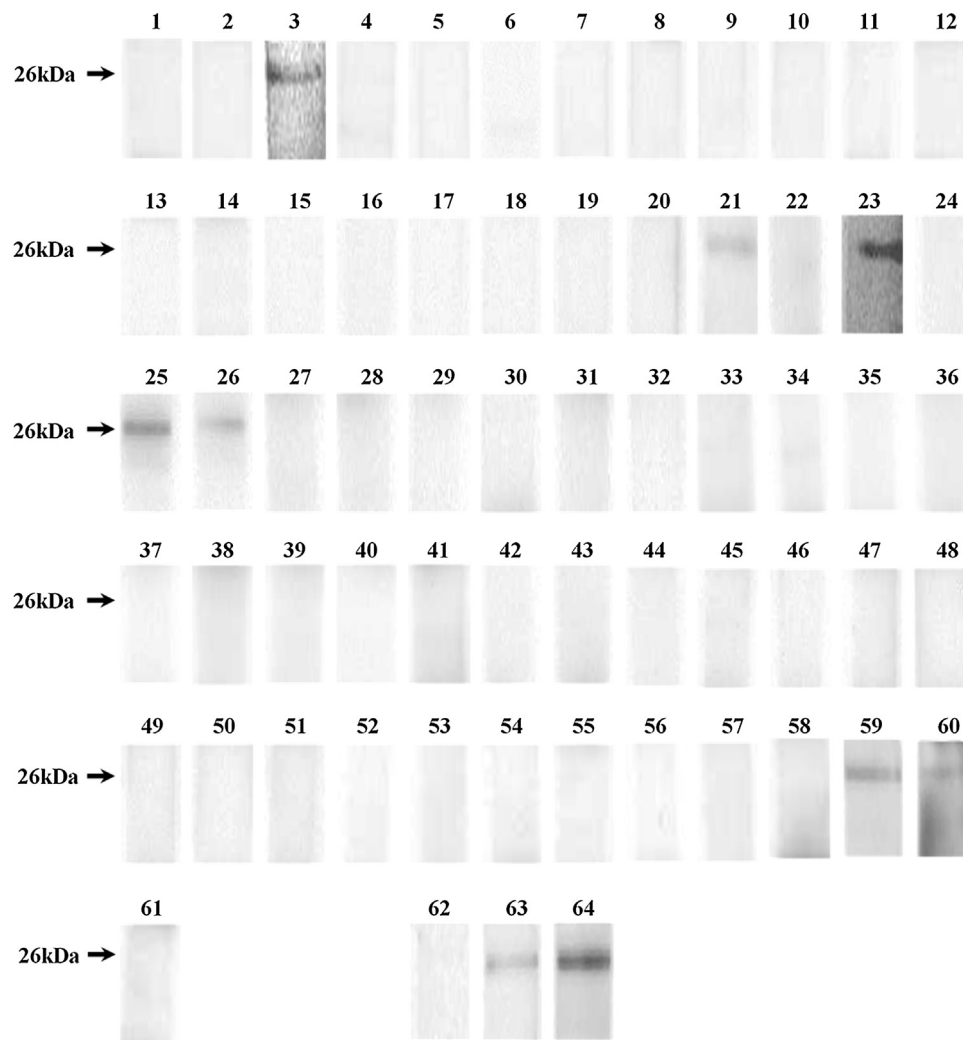


Fig. 4. IgE-immunoblot of rAlt a MnSOD using 64 selected serum samples. Blotted rAlt a MnSOD probed with: Lanes 1–61 – individual *A. alternata*-sensitised patients' sera, Lane 62 – serum from a subject sensitised to *D. pteronyssinus*, Lanes 63–64 – probed with sera from patients with ABPA.

all sera from *A. alternata*-sensitised patients containing sIgE to rAsp f 6 (Table 1) labelled the rAlt a MnSOD protein (Fig. 4, patients/lanes 3, 21, 23, 26, 59, and 60). Additionally, rAlt a MnSOD was able to bind IgE from ABPA individuals (patients 63 and 64) who presented rAsp f 6 sIgE.

Immunoblotting inhibition was assayed using serum from patient no. 59. The inhibition of sera with *A. alternata* and *A. fumigatus* extracts resulted in an evident inhibition of the protein band at 26 kDa (Fig. 5A). Furthermore, the ELISA results (Fig. 5B) showed that IgE binding of patient number 59's serum to Alt a MnSOD could be inhibited in a dose-dependent manner by preincubation with increasing amounts of the purified rAlt a MnSOD or the *A. alternata* mycelial extract.

Alt a MnSOD was submitted to the International Union of Immunological Societies Allergen Nomenclature Sub-Committee (<http://www.allergen.org/Allergen.aspx>) and was officially named Alt a 14.

Discussion

Apparent sensitisation to multiple fungi, caused by co-sensitisation and/or cross-reactivity, represents a frequent clinical observation in patients with mould allergy that makes an accurate diagnosis of mould allergy difficult (Cramer et al., 2009). In recent years, component-resolved diagnosis has been presented as

a valuable tool to discriminate between cases of co-sensitisation and/or cross-reactivity (Mothes et al., 2006; Vieira et al., 2012). To improve the molecular diagnosis, the identification and characterisation of all clinically relevant allergens, including both species-specific and cross-reacting proteins, is required. With this in mind, in the present work, we aimed to characterise the recently identified *A. alternata* Asp f 6 homologue (Alt a 14) and to demonstrate that it is an *A. alternata* clinically relevant allergen showing cross-reactivity with Asp f 6.

The strategy used in this work takes advantage of the similarity of the different fungal MnSOD nucleotide sequences submitted to the GenBank database, which allowed the design of degenerate oligonucleotides and the cloning of Alt a 14-encoding cDNA by RACE experiments. A sequence similarity search revealed that Alt a 14 presents high homology with MnSODs from other members of Pleosporales (*P. tritici-repentis* (94%), *L. maculans* (90%), and *C. lunata* (89%)). The percent identity decreased when Alt a 14 and homologous molecules from fungi belonging to other orders were compared. Significant homology with the allergens contained in the official list, Asp f 6 (59%), Mala s 11 (48%), Hev b 10 (45%) and Pis v 4 (45%), was also detected. Additionally, comparison between the multiple sequence alignments and the surface accessibility of conserved amino acids in the 3D models of Alt a 14 and Asp f 6 indicated that although the majority of the residues are identical, only seventeen are at least 30% solvent exposed in both

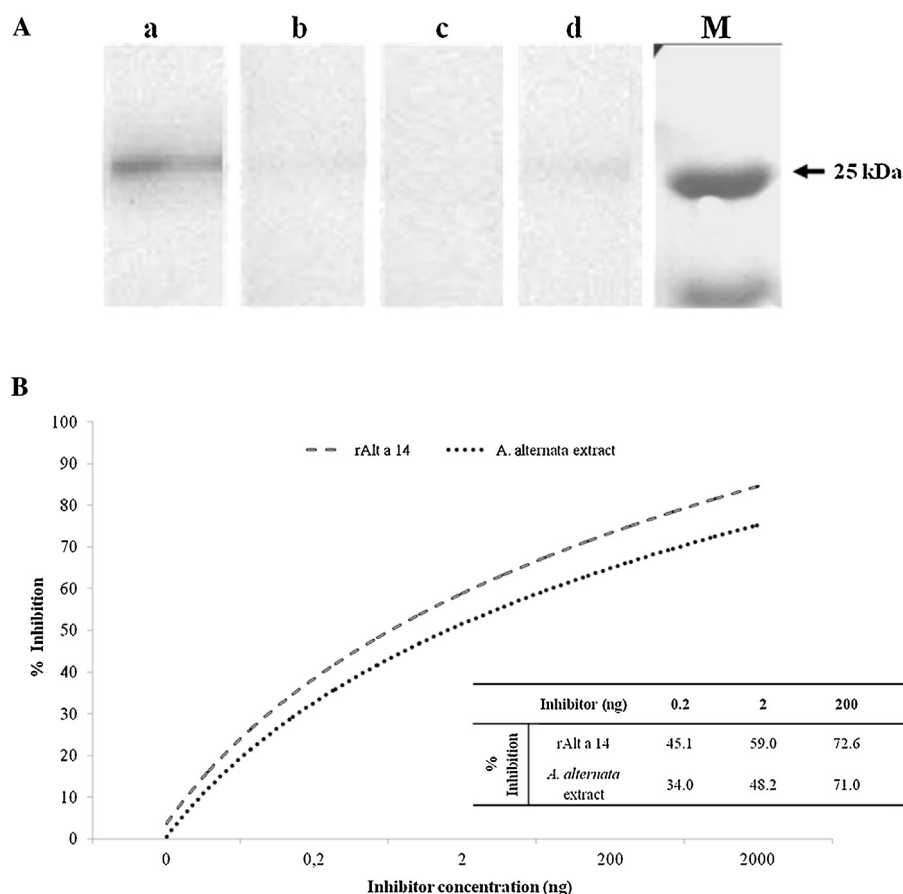


Fig. 5. (A) Inhibition of the IgE-immunoblot reactivity of a serum sample that has shown positive reactivity to rAlt a 14. Strips represent results of immunoblot obtained by incubating the PVDF blots with the same serum sample that has been mixed and pre-incubated with: (a) PBS, (b) *A. alternata* metabolic extract, (c) *A. alternata* cellular extract and (d) *A. fumigatus* cellular extract. (B) Inhibition of IgE-binding to recombinant Alt a 14 coated on the solid phase. Serum from an Alt a 14-sensitised patient was preincubated with increasing amounts of recombinant Alt a 14 and *A. alternata* extract.

structures and therefore likely to be accessible for interactions with cross-reactive antibodies. These residues appear scattered over the entire surface of the Alt a 14 molecule, suggesting that the entire surface is potentially cross-reactive with Asp f 6. The exposed amino acids, either alone or together with surrounding residues, are likely to define cross-reactive conformational B cell epitopes shared between Alt a 14 and Asp f 6 and possibly among MnSODs from other species. In this regard, based on the solvent-exposed amino acid distribution profile in the 3D structure, two regions including the residues K21, E25, T29 and Q57 and the residues P73, K88, E91 and K161 could be defined as potential cross-reactive areas. Both areas are present in at least two different sections of the tetramer surface, satisfying the requirement needed to induce the cross-linking of allergen-specific IgE bound to the surface of effector cells.

The recombinant production of Alt a 14 was performed in *E. coli* and revealed an apparent molecular mass of 26 kDa. Assessment of the allergenic significance of Alt a 14 in *A. alternata*-sensitised individuals by immunoblotting showed that 11.5% had sIgE to the 26 kDa band, suggesting that Alt a 14 is an *A. alternata* minor allergen. The results of immunoblotting and ELISA inhibition showed that native Alt a 14 contained in the *A. alternata* extracts strongly inhibited the reaction between rAlt a 14 and the sIgE of an Alt a 14-sensitised patient's serum, suggesting that the allergenic ability of the native Alt a 14 is also present in the recombinant polypeptide.

Data obtained from the investigation of sIgE to single fungal allergens, including Alt a 1, Alt a 14 and Asp f 6, revealed that

among the *A. alternata*-sensitised individuals ($n=61$), 86.9% were sensitised to Alt a 1, 11.5% to Alt a 14 and 9.8% to Asp f 6. Thus, only one individual who was sensitised to Alt a 14 was not sensitised to Asp f 6 (Table 1). To explore the potential cross-reactivity between Alt a 14 and the recognised clinically relevant *A. fumigatus* allergen Asp f 6, two sera from patients with diagnosed ABPA and detectable Asp f 6-sIgE were tested by immunoblotting. This assay demonstrated that both sera recognised rAlt a 14. This finding, together with the results obtained from immunoblotting-inhibition assays using *A. fumigatus* extract suggest that both allergens possess allergen-cross-reactive IgE epitopes.

The evidence of cross-reactivity between Alt a 14 and Asp f 6, demonstrated for the first time in this study, raises some questions because of the recognised importance of Asp f 6 in allergic diseases to moulds. In fact, Asp f 6 is regarded, together with Asp f 4, as a specific marker for ABPA (Fricker-Hidalgo et al., 2010; Hemmann et al., 1999). Currently, ABPA is associated with an array of immune responses to *A. fumigatus* and has been reported to occur primarily in asthmatic individuals and in patients suffering from cystic fibrosis (Knutson and Slavin, 2011; Baxter et al., 2013). In this regard, and based on the recognised fact that most *A. alternata*-sensitised patients are asthmatic and the recent revelation that *A. alternata* contains an allergen that is homologous and cross-reactive to Asp f 6, it will be important to determine whether Alt a 14-sensitised individuals are at risk of ABPA. Interestingly, this is in line with the findings of Jubin et al. (2010), who demonstrated that ABPA was significantly associated with a prior or concomitant sensitisation to *Alternaria* species and defined *Alternaria* sensitisation as a

risk factor for ABPA. The existence of cross-reactive proteins from *Alternaria* and *Aspergillus* species, such as the MnSOD described in this study, could explain a similar mechanism of sensitisation that may contribute to the pathogenesis of ABPA. Considering the results reported by Agarwal et al. (2012, 2013) the possibility that the individuals who presented positive levels of anti-Asp f 6 and/or Alt a 14 IgE, are in an asymptomatic phase of ABPA with controlled asthma, stage 0 of ABPA, must not be excluded. For this reason, they should be evaluated for ABPA to aid in the early diagnosis of the disorder before the onset of bronchiectasis. Furthermore, in the clinical practice, it is not unusual to find patients who present negative skin-pick test results to an *A. fumigatus* extract but a positive Asp f 6-specific IgE serum levels. The sensitisation to the Asp f 6-like from *A. alternata*, Alt a 14, may explain this observation.

In conclusion, this study characterised Alt a 14 as a minor allergen of *A. alternata* that can trigger a cross-reactive IgE response to Asp f 6. The data disclosed by this study suggest that the Asp f 6-like allergen found in *A. alternata* may be related to ABPA and should greatly contribute to improving the accuracy of the diagnosis and, subsequently, the understanding and management of IgE-mediated fungal diseases.

Databases

GenBank: nucleotide sequence data are available in the GenBank database under the accession number KC923297.

PMDB: model data are available in the PMDB database under the accession number PM0079554.

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

Conflict of interest

The authors declared no conflict of interest.

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