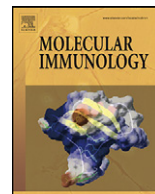




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Mutational analysis of amino acid residues involved in IgE-binding to the *Malassezia sympodialis* allergen Mala s 11

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ABSTRACT

The yeast *Malassezia sympodialis*, which is an integral part of the normal cutaneous flora, has been shown to elicit specific IgE- and T-cell reactivity in atopic eczema (AE) patients. The *M. sympodialis* allergen Mala s 11 has a high degree of amino acid sequence homology to manganese superoxide dismutase (MnSOD) from *Homo sapiens* (50%) and *Aspergillus fumigatus* (56%). Humoral and cell-mediated cross-reactivity between MnSOD from *H. sapiens* and *A. fumigatus* has been demonstrated. Taken together with the recent finding that human MnSOD (hMnSOD) can act as an autoallergen in AE patients sensitised to *M. sympodialis*, we hypothesized that cross-reactivity could also occur between hMnSOD and Mala s 11, endogenous hMnSOD thus being capable of stimulating an immune response through molecular mimicry. Herein we demonstrate that recombinant Mala s 11 (rMala s 11) is able to inhibit IgE-binding to recombinant hMnSOD and vice versa, indicating that these two homologues share common IgE-binding epitopes and providing an explanation at a molecular level for the autoreactivity to hMnSOD observed in AE patients sensitised to Mala s 11. Using molecular modelling and mapping of identical amino acids exposed on the surface of both Mala s 11 and hMnSOD we identified four regions each composed of 4–5 residues which are potentially involved in IgE-mediated cross-reactivity. Mutated rMala s 11 molecules were produced in which these residues were altered. Native-like folding was verified by enzymatic activity tests and circular dichroism. The rMala s 11 mutants displayed lower IgE-binding in comparison to wild-type rMala s 11 using plasma from AE patients. In particular, mutation of the residues E29, P30, E122 and K125 lowered the IgE-binding to Mala s 11. The results of this study provide new insights in the molecular basis underlying the cross-reactivity between Mala s 11 and hMnSOD.

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1. Introduction

Atopic eczema (AE) is a chronic inflammatory skin disease characterised by pruritic inflammatory skin lesions (Bieber, 2008). Although the pathogenic mechanisms underlying AE remain largely unknown several factors such as genetic predisposition, exposure to environmental allergens and skin colonization with microorganisms appear to be of importance (Akdis et al., 2006). The

opportunistic yeast *Malassezia sympodialis* is a member of the normal cutaneous flora (Scheynius et al., 2002). Approximately 50% of adult AE patients have serum IgE specific for *M. sympodialis* allergens or show immediate-type skin reactions against crude extracts of this yeast (Schmid-Grendelmeier et al., 2006) while such reactivity is very rare in other allergic diseases. (Casagrande et al., 2006) suggesting an association between *M. sympodialis* sensitization and AE. One contributing factor to the special host–microbe interaction between AE and *M. sympodialis* is likely to be the dysfunctional skin barrier characteristic for AE as a result of dryness and scratching (Leung et al., 2004) which facilitates the interaction between the yeast and antigen presenting cells in the skin. In addition, the higher pH associated with AE skin induces an increased release of allergens

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from *M. sympodialis* (Selander et al., 2006). Ten allergens from *M. sympodialis* have been cloned to date, six exhibit sequence homology to known proteins whereas four do not (Schmid-Grendelmeier et al., 2006). Studies of the three-dimensional structures and/or cellular interactions of some of these allergens have been performed in order to investigate their implications in AE (Vilhelmsson et al., 2007a,b; Limacher et al., 2007; Glaser et al., 2006). However, to further elucidate the role of the yeast allergens in the skin disease, detailed knowledge of their different structures is needed.

One allergen of particular interest is Mala s 11, which has a high degree of sequence identity to manganese superoxide dismutase (MnSOD) from various species including *Homo sapiens* (50%) and *Aspergillus fumigatus* (56%) (Andersson et al., 2004). MnSOD protects mitochondrial DNA against oxygen related radicals by dismutation of superoxide to oxygen and hydrogen peroxide (Hwang et al., 2003). MnSOD from *A. fumigatus*, denoted Asp f 6, is associated with allergic bronchopulmonary aspergillosis (ABPA) (Cramer et al., 1996, 1998). Humoral and cell-mediated cross-reactivity between MnSOD from *H. sapiens*, *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* has been demonstrated (Flückiger et al., 2002c). A comparative study of the crystal structures of the *H. sapiens*-derived MnSOD (hMnSOD) and its homologous *A. fumigatus*-derived MnSOD revealed patches of identical amino acids displayed on the surface of both enzymes that constitute potential cross-reactive IgE-binding epitopes (Flückiger et al., 2002b). Residues P19, P30, K43 and N50 were suggested as suitable targets for substitution by site-directed mutagenesis (Flückiger et al., 2002b). Based on these studies, on the high degree of sequence homology and on the recent finding that hMnSOD is sufficient to induce eczematous reactions in healthy skin areas of AE patients sensitised to *M. sympodialis* (Schmid-Grendelmeier et al., 2005) it can be assumed that cross-reactivity also occurs between Mala s 11 and hMnSOD. Under such circumstances endogenous hMnSOD may maintain activation of the immune response in the absence of environmental allergen exposure through molecular mimicry.

The aims of the current study were to (i) show the existence of such cross-reactivity and (ii) identify amino acid residues that are involved in IgE-binding to Mala s 11. Based on comparative analysis of amino acid sequences and on molecular modelling, four regions each comprising 4–5 phylogenetically conserved residues and all exposed on the surface of Mala s 11 were identified. Mutated Mala s 11 variants were tested for their ability to inhibit IgE-binding to wild-type (WT) Mala s 11. We thus demonstrate the existence of IgE-mediated cross-reactivity between Mala s 11 and hMnSOD and our mutation analysis study suggests that a specific region in Mala s 11, composed by residues E29, P30, E122 and K125, is important for IgE binding. The identification of shared key residues contributing to IgE binding of homologous structures provides a more detailed understanding of cross-reactivity at molecular level.

2. Materials and methods

2.1. Plasma from patients with AE and healthy individuals

Plasma from six AE patients (four women and two men, median 29 years, range 20–44 years) diagnosed according to the UK working party criteria (Williams et al., 1994) with positive Phadiatop® (IgE antibodies to 11 common airborne allergens, Phadia AB, Uppsala, Sweden, ≥ 0.35 kU/l), elevated total serum IgE levels, median 3 500 kU/l, range 2 000–10 600 kU/l (ImmunoCAP™, Phadia AB), positive IgE serology against *M. sympodialis*, median 32 kU_A/l, range 1.8–77 kU_A/l (m70, Phadia AB), and IgE reactivity to rMala s 11, median 136 EU/ml, range 70–304 EU/ml and rhMnSOD, median 144 EU/ml, range 91–247 EU/ml, determined by ELISA as described

below were included in the study. Five healthy individuals (four women and one man, median 41 years, range 20–54 years) with no history of eczema or allergic symptoms, low total serum IgE levels (median 13 kU/l, range 8–57 kU/l), negative Phadiatop® and with negative IgE serology against *M. sympodialis* were used as controls. These five plasma samples displayed a negative IgE serology against rMala s 11 (median 2.8 EU/ml, range 0.0–6.9 EU/ml) and rhMnSOD (median 2.1 EU/ml, range 0.0–38 EU/ml). In addition, plasma from 10 AE patients (five women and five men, median 29 years, range 18–65 years) with elevated total serum IgE levels (median 2 700 kU/l, range 1 700–12 835 kU/l), positive Phadiatop® and with positive IgE serology against *M. sympodialis* (median 12 kU_A/l, range 12–52 kU_A/l) were used as controls for non-specific IgE binding. These 10 samples had negative IgE serology against rMala s 11 (median 0.4 EU/ml, range 0.0–8.3 EU/ml) and rhMnSOD (median 33 EU/ml, range 12–52 EU/ml). The study was approved by the Regional Ethics Committee of the Karolinska University Hospital.

2.2. Immunoassays for IgE binding to rMala s 11 and rhMnSOD

IgE-binding to rMala s 11 and rhMnSOD was analysed by standard direct solid phase ELISA as described elsewhere (Moser et al., 1994; Flückiger et al., 2002a,c). Briefly, 96-well polystyrene microtiter plates (Maxisorb™, Nunc, Roskilde, Denmark) were coated overnight at 4 °C with rMala s 11 or rhMnSOD (5 µg/ml), washed and incubated with serial diluted 1:2 plasma overnight at 4 °C. Plates were then incubated with mouse anti-human IgE mAb TN-142 (Moser et al., 1994) followed by incubation with alkaline phosphate-conjugated goat anti-mouse IgG H+L chains (Pierce, Rockford, IL, USA). IgE binding was determined by measuring OD at 405 nm as described (Moser et al., 1994; Flückiger et al., 2002a,c). Results were expressed as ELISA Units (EU)/ml calibrated against an in-house reference standard arbitrarily defined as 100 EU/ml for both rMala s 11 and rhMnSOD. Patient samples were considered positive at a value above 3-fold the mean EU/ml of the healthy controls resulting in cut-off values of 9 EU/ml for rMala s 11 and 64 EU/ml for rhMnSOD. Plasma samples from 10 AE patients with high total serum IgE levels did not yield detectable IgE binding above the cut-off level to rMala s 11 or rhMnSOD, indicating that the positive results obtained in the solid phase ELISA are not due to non-specific IgE-binding.

Inhibition ELISAs were performed as previously described (Flückiger et al., 2002a,c). Briefly, 96-well polystyrene microtiter plates (Maxisorb™, Nunc) were coated overnight at 4 °C with rMala s 11 or rhMnSOD (5 µg/ml). On a separate plate, mutated versions of rMala s 11, WT rMala s 11, rhMnSOD or BSA (Sigma, St. Louis, MO, USA) as a negative control were serially diluted 1:3 and incubated for 1 h at 37 °C with 1:10 diluted plasma from AE patients sensitised to rMala s 11 and rhMnSOD. The pre-incubated plasma was then transferred to the plates pre-coated with rMala s 11 or rhMnSOD and further incubated at 4 °C overnight. IgE was detected using the same procedure as for solid phase ELISA described above. Percentage of inhibition was calculated from the OD value for 1:10 diluted plasma alone set to 0% inhibition.

2.3. Molecular modelling of Mala s 11

Conserved residues between MnSOD from *M. sympodialis* (accession number CAD68071), *A. fumigatus* (accession number AAB60779), *H. sapiens* (accession number P04179), *D. melanogaster* (accession number NP_476925) and *S. cerevisiae* (accession number CAA26092) were mapped with sequence alignment using the program MultAlign (Corpet, 1988). The monomeric and tetrameric molecular models of Mala s 11 were created on the basis of its

Table 1
List of mutations in Mala s 11

Region	Version	Amino acid changed	Primer 5'–3' sequence
Region 1	A1	K43 → A	(K43 → A and N50 → A)
		N50 → A	ggtgcaccacggtGCGcaccatcagacgtatgtgGCCaacctgaacgcgtccacg
		A77 → D	(A77 → D and K79 → A)
	B1	K79 → A	ggagctgctgaccGATgtcGCATtcaatgggtggg
		K43 → D	(K43 → D and N50 → R)
		N50 → R	ggtgcaccacggtGACcaccatcagacgtatgtgCGCaacctgaacgcgtccacg
		A77 → D	(A77 → D and K79 → D)
Region 2	A2	K79 → D	ggagctgctgaccGATgtcGCATtcaatgggtggg
		E29 → A	(E29 → A and P30 → A)
		P30 → A	gccttacgagtacaacgcctcttGCGGCAttcatttcggc
		E122 → A	(E122 → A and K125 → A)
	B2	K125 → A	ggaatttggcgactttGCGaagttcGCGccgctttcacggcc
		E29 → R	(E29 → R and P30 → A)
		P30 → A	gccttacgagtacaacgcctcttGCGGCAttcatttcggc
Region 3	A3	E122 → R	(E122 → R and K125 → D)
		K125 → D	ggaatttggcgactttGCGaagttcGACgccgctttcacggcc
		Q136 → A	(Q136 → A)
		L180 → A	ggcgcttggcatcGCCggctctggctgggtgc
		Q181 → A	(L180 → A, Q181 → A, Y182 → A and N184 → A)
	B3	Y182 → A	cggctgggagcagcctgtacGCGCGGCCaagGCCgacaaggcttcgt
		N184 → A	acctgaagcagtggtg
Region 4	A4	Q136 → D	(Q136 → D)
		L180 → D	ggcgcttggcatcGACggctctggctgggtgc
		Q181 → D	(L180 → D, Q181 → D, Y182 → D and N184 → D)
		Y182 → D	cggctgggagcagcctgtacGACGACGACaagGACgacaaggcttcgt
		N184 → D	acctgaagcagtggtg
	B4	P19 → A	(P19 → A and Y23 → A)
		Y23 → A	gaacacactcttGTCtctctgcctGACaggtacaacgcctttgagccg
	A4	P97 → A	(P97 → A and Q98 → A)
		Q98 → A	ggaagaccatggctGACCGagccaggggtgggtggcc
		P19 → D	(P19 → D and Y23 → D)
	B4	Y23 → D	gaacacactcttGATcctctgcctGACgagtacaacgcctttgagccg
		P97 → D	(P97 → D and Q98 → D)
		Q98 → D	ggaagaccatggctGATGACagccaggggtgggtggcc

sequence homology to human MnSOD using the SWISS-MODEL Protein Modelling Server (Schwede et al., 2003) using the crystal structure of hMnSOD (PDB code 1JA8) (Borgstahl et al., 1992) as a template.

2.4. Mutagenesis of Mala s 11

Site-directed mutagenesis was performed using a QuikChange mutagenesis kit from Stratagene (La Jolla, CA, USA) and the primers listed in Table 1. A pET17b vector (Novagen, Inc., Madison, WI, USA) carrying the Mala s 11 gene with a C-terminal His-tag was used as template DNA. Introduction of specific mutations into the Mala s 11 gene was performed according to manufacturer's instructions (Stratagene). In brief, PCR reactions were carried out using 50 ng of double stranded DNA template, 2.5 µl 10× QuickChange Multi reaction buffer, 1 µl dNTP mix, 100 ng 5'-phosphorylated mutagenic primers, 1 µl QuickChange multi enzyme blend, and distilled water to a final volume of 25 µl. PCR amplifications were performed in a hot-top Eppendorf Mastercycler gradient (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) under the following conditions: an initial denaturation step at 95 °C for 1 min, followed by 30 cycles each of 1 min at 95 °C, 1 min at 55 °C, and 8 min at 65 °C. Thereafter, reaction mixtures were placed on ice for 2 min, and the parental double-stranded DNA was digested with 1 µl of 10 U/µl Dpn I at 37 °C for 2 h before being transformed into competent *E. coli* XL2 Blue cells (Stratagene). Mutations were verified by DNA sequencing. Plasmids with correct insert sequences of the WT or mutated version of the enzyme were transformed into *E. coli* BL21 Star (DE3)/pLysS (Invitrogen,

CA, USA) chemically competent cells according to the supplier's instructions.

2.5. Expression and purification of mutated rMala s 11 and rhMnSOD

E. coli BL21 Star (DE3)/pLysS cells transfected with pET17b containing the gene for WT or mutated forms of Mala s 11 or WT hMnSOD were grown at 37 °C in Luria–Bertani (LB) medium containing ampicillin (100 µg/ml) and chloramphenicol (30 µg/ml) to a D_{600} of 0.6. Expression was induced by adding 1 mM isopropyl thio-β-D-galactoside (Sigma) and incubation was continued for 4 h. Cells were harvested by centrifugation and pellets resuspended in lysis buffer (6 M guanidine hydrochloride, 100 mM Na₂H₂PO₄ and 10 mM Tris pH 8). The lysate was centrifuged and purified by Ni–NTA (Ni²⁺–nitrilotriacetate)-affinity chromatography using a 5 ml HiTrap chelating HP column (Amersham Biosciences, Buckinghamshire, UK) under denaturing conditions according to the manufacturer's recommendations (Qiagen, Hilden, Germany). The purified proteins were dialysed against distilled water containing 0.1 mM MnCl₂. The concentration was determined by measuring the absorbance at 280 nm and the purity was analysed with SDS-PAGE.

2.6. Enzymatic activity

Enzymatic activity of the purified proteins was determined using a specific enzymatic assay calibrated with bovine erythrocyte SOD (Roche, Basel, Switzerland) as previ-

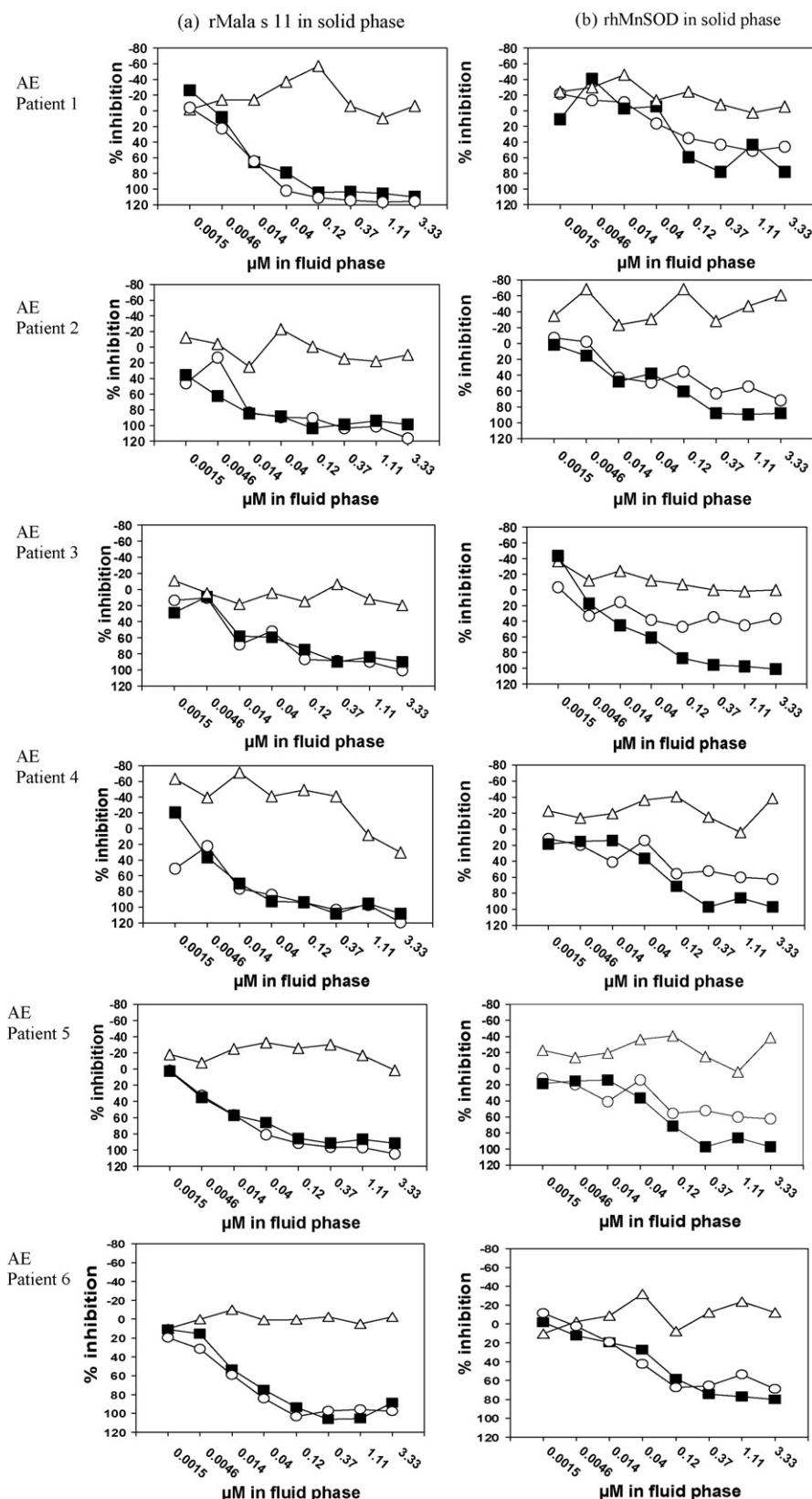


Fig. 1. Inhibition of IgE-binding to rMnSODs in the solid phase. In each assay plasma from an AE patient sensitised to rMala s 11 and rhMnSOD was pre-incubated with increasing amounts of rMala s 11 (○), rhMnSOD (■) or BSA (△). Pre-incubated plasma was transferred to plates pre-coated with rMala s 11 (a) or rhMnSOD (b) and IgE binding was analysed by ELISA.

ously described (Flückiger et al., 2002a,c; Henning and Lynen, 1963).

2.7. Circular dichroism (CD) spectroscopy

Far UV circular dichroism spectra were recorded with a JASCO J-810 spectropolarimeter (JASCO Corporation, Tokyo, Japan) at 25 °C, using a demountable 0.02 cm path length cuvette (Hellma, Mühlheim, Germany). Spectra were recorded in 0.1 mM MnCl₂ at 1 nm intervals using a spectral bandwidth of 1.5 nm and averaging times of 8 or 16 s.

3. Results

3.1. IgE-mediated cross-reactivity between Mala s 11 and hMnSOD

Plasma samples from six AE patients sensitised to *M. sympodialis* with specific IgE-binding to both recombinant Mala s 11 (rMala s 11) and recombinant hMnSOD (rhMnSOD) were used to study the ability of rMala s 11 and rhMnSOD to inhibit IgE-binding to each other in inhibition ELISAs. In contrast to BSA used as a negative control, rMala s 11 and rhMnSOD in the fluid phase exhibited comparable inhibition of IgE-binding to solid phase-coated rMala s 11 for the six

plasma samples (Fig. 1a). IgE binding to solid-phase coated rhMnSOD was also inhibited by rMala s 11 added in fluid phase, although for some patients to a lower extent than with rhMnSOD, for all six plasma samples tested (Fig. 1b), demonstrating that both proteins share IgE-binding epitopes.

3.2. Molecular modelling of Mala s 11 and identification of potential cross-reactive IgE binding epitopes

Sequence alignment reveals that Mala s 11 shares sequence homology with MnSOD from *A. fumigatus*, *H. sapiens*, *D. melanogaster* and *S. cerevisiae* (Fig. 2a). A molecular model of Mala s 11 was created using the previously solved crystal structure of hMnSOD (Borgstahl et al., 1992) as a template (Fig. 2b). As expected from the high sequence homology, the predicted three-dimensional structure of Mala s 11 is similar to the crystal structure of hMnSOD (Borgstahl et al., 1992). The monomer exhibits an α/β -fold in accordance with crystal structures of MnSODs (Flückiger et al., 2002b; Schmid-Grendelmeier et al., 2005; Borgstahl et al., 1992) (Fig. 2b). Since the crystal structures of *A. fumigatus* and *H. sapiens* MnSOD indicate that both enzymes form tetramers, we hypothesized that Mala s 11 may also form a tetramer. A hypothetical tetrameric molecular model of Mala s 11 was thus created (Fig. 3). Analysis of the domains of interaction between Mala s 11

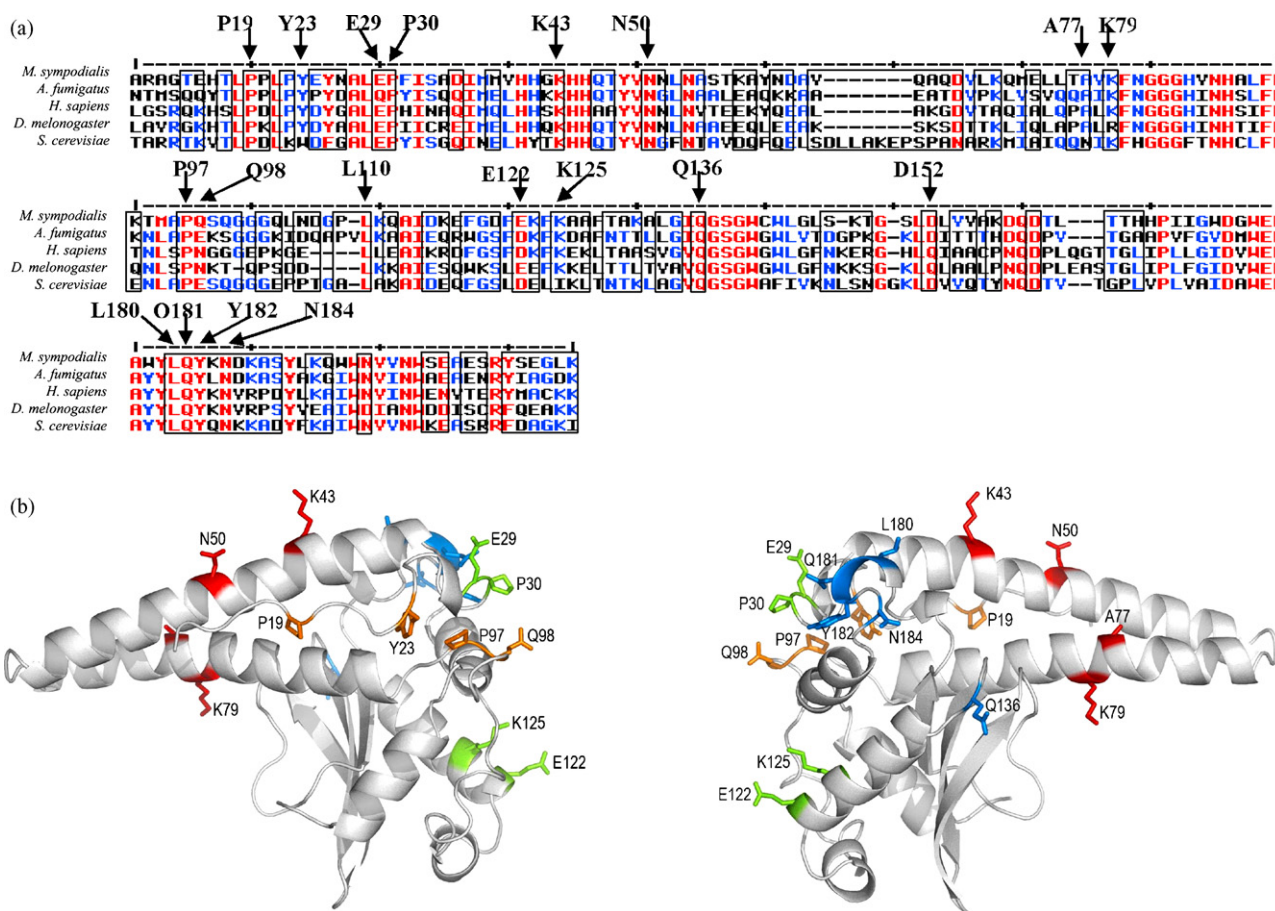


Fig. 2. (a) Sequence alignment. Alignment of the amino acid sequences of conserved residues between MnSOD from *M. sympodialis* (accession number CAD68071), *A. fumigatus* (accession number AAB60779), *H. sapiens* (accession number P04179), *D. melanogaster* (accession number NP.476925) and *S. cerevisiae* (accession number CAA26092) using the program MultAlign (Schwede et al., 2003). Highly conserved residues are marked in red and weakly conserved residues are marked in blue. Additional residues are marked in black. Residues accessible to the solvent are indicated with boxes. Mutated residues are marked with arrows. (b) Molecular model of monomeric Mala s 11 and localization of conserved residues exposed to the solvent. Conserved residues that may potentially be involved in cross-reactivity between Mala s 11 and hMnSOD were grouped in four independent regions. Regions 1, 2, 3 and 4 were coloured red, green, blue and orange, respectively. The figure was created using the program pymol (<http://www.pymol.org>). The right and left images are rotated 180° in relation to each other. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

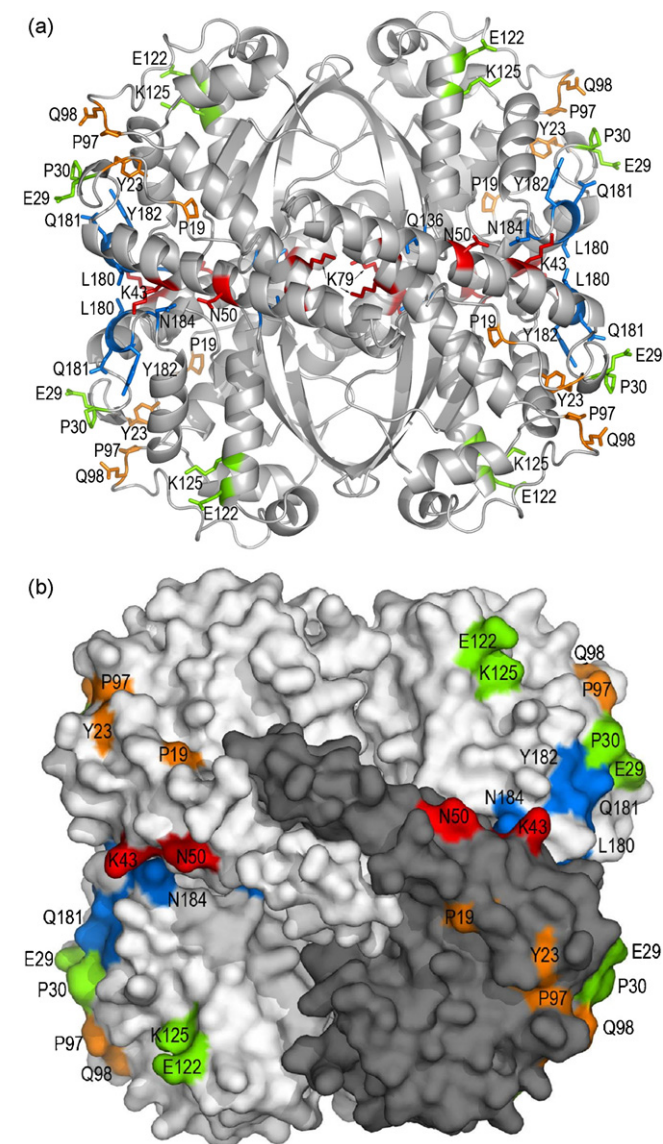


Fig. 3. Molecular model of the Mala s 11 tetramer presented as a ribbon diagram (a) and surface diagram (b) with the residues potentially involved in cross-reactivity between Mala s 11 and hMnSOD marked in different colours; red (region 1), green (region 2), blue (region 3) and orange (region 4). The pictures were created using the program pymol (<http://www.pymol.org>). To depict the relative positioning of the monomers, one monomer is coloured dark grey whereas the remaining three are coloured light grey.

monomers within the hypothetical tetramer suggested that neither steric hindrance nor any major electrostatic repulsion may disturb the formation of such a tetramer. Furthermore, sequence alignment allowed the identification of amino acids residues that are conserved between MnSOD from *M. sympodialis*, *A. fumigatus*, *H. sapiens*, *D. melanogaster* and *S. cerevisiae* (Fig. 2a). To determine which of the residues shared between Mala s 11 and hMnSOD could be of importance for IgE-binding we identified conserved residues that are exposed to the solvent on the surface of the molecular model of Mala s 11. Four independent regions including 17 conserved residues were identified as potential cross-reacting areas (Figs. 2b and 3). Regions 1, 2, 3 and 4 (coloured in red, green, blue and orange, respectively, in Figs. 2b and 3) include residues K43, N50, A77 and K79, residues E29, P30, E122 and K125, residues Q136, L180, Q181, Y182 and N184, and residues P19, Y23, P97 and Q98, respectively.

Two of the residues in region 1 (K43 and N50) point towards the solvent in both the monomer and the tetramer, whereas accessibility of residues A77 and K79 to the solvent is relatively reduced in the tetramer compared to in the monomer (Figs. 2b and 3). It is therefore likely that residues K43 and N50 are the most important for IgE-binding in region 1 and have also been suggested as being involved in IgE-mediated cross-reactivity between *A. fumigatus* and *H. sapiens* MnSOD (Flückiger et al., 2002b). All four residues in region 2 protrude into the solvent both in the monomer and in the tetramer model of Mala s 11, indicating their accessibility

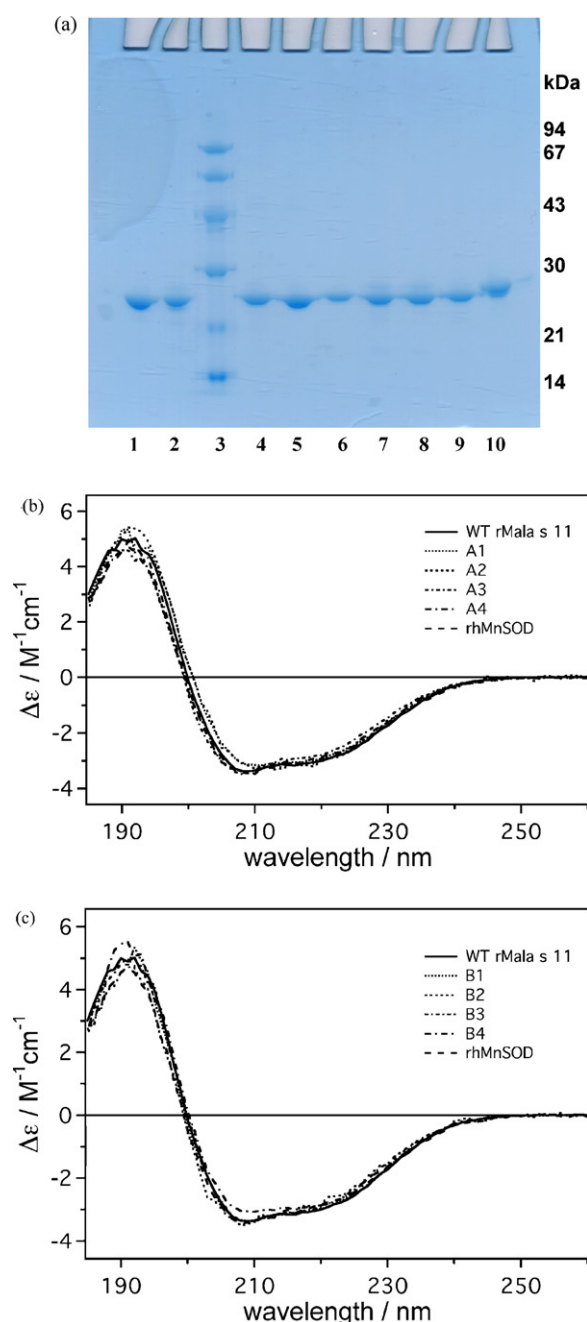


Fig. 4. (a) rMala s 11 and mutated rMala s 11 versions (Table 1) separated by SDS-PAGE under denaturing conditions and stained with Coomassie blue dye. Lane 1; A1, lane 2; B1, lane 3; molecular mass marker, lane 4; wild-type (WT) rMala s 11, lane 5; A2, lane 6; B2, lane 7; A3, lane 8; B3, lane 9; A4, lane 10 B4. (b and c) Far UV circular dichroism spectra of mutated rMala s 11 A and B versions (Table 1). Spectra of WT rMala s 11 and rhMnSOD are included for reference.

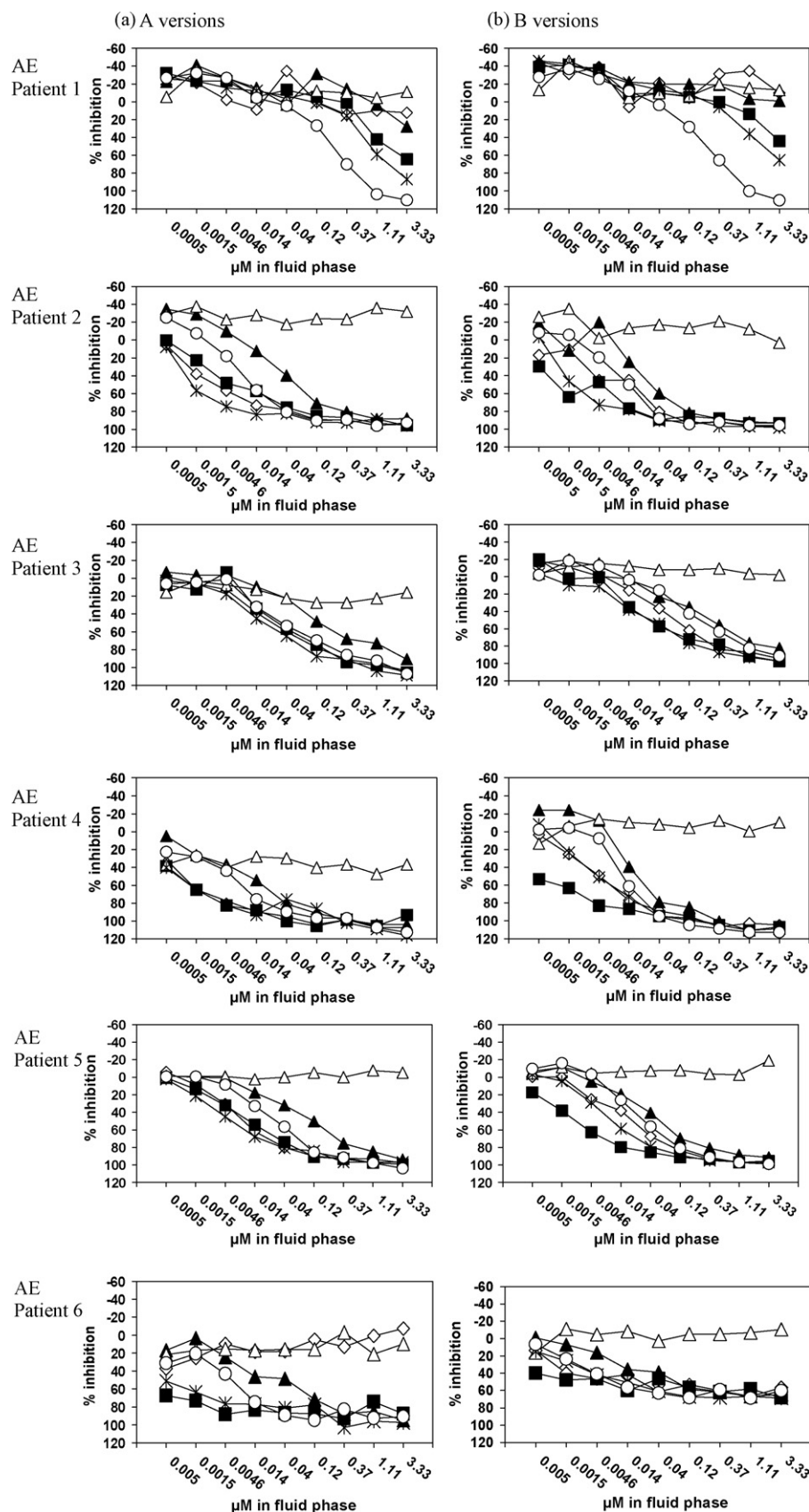


Fig. 5. Inhibition of IgE binding to WT rMala s 11 in solid phase. In each assay plasma from an AE patient sensitised to rMala s 11 and rhMnSOD was pre-incubated with increasing amounts of WT rMala s 11 (○) or the A (a) or B (b) versions of rMala s 11 (Table 1) mutated in region 1 (*), region 2 (▲), region 3 (■), region 4 (◊) or BSA (△) as control. Pre-incubated plasma was transferred to plates pre-coated with rMala s 11 and IgE-binding was analysed with ELISA.

for antigen–antibody interactions. In region 3, residues L180, Q181, Y182 and N184 are exposed to the solvent in both the monomer and the tetramer (Figs. 2b and 3). However, upon the formation of a hypothetical tetramer, our model suggests that the side chain of residue L180 may interact with the side chain of residue L180 in the adjacent monomer, and may thus not be as exposed to the solvent when compared to the four other residues of region 3. Similarly, our molecular model suggests that antibody access to residue Q136 could be sterically hindered in the tetramer. Three residues, P19, P97 and Q98, are accessible to the solvent in region 4 within the tetramer model of Mala s 11. Similarly to residue Q136 in region 3, the side chain of residue Y23 is buried within the tetrameric form of Mala s 11.

3.3. Expression and purification of mutated and WT MnSOD

All identified residues were altered using site-directed mutagenesis. For each of the four regions (1–4 in Table 1), two mutated versions were produced. In the first version (A1–A4) all residues except A77 were modified to alanine. In the second version (B1–B4) all residues were mutated to aspartate or to residues with opposing polarity (Table 1). WT rMala s 11, rhMnSOD and mutated forms of rMala s 11 were produced using an inducible bacterial expression system. Following refolding and dialysis, recombinant proteins were purified and concentrated to 1–2 mg/ml. All purified proteins migrated as a single band in agreement with the predicted size when analysed by SDS-PAGE (Fig. 4a).

3.4. Characterisation of mutated and WT MnSOD

To assess the relative impact of the introduced mutations in rMala s 11, all mutated and WT rMala s 11 were evaluated for similar folding using circular dichroism (CD). Far UV CD spectra obtained for mutated rMala s 11 proteins were similar to that obtained with WT rMala s 11, indicating a close secondary structure similarity. We interpret these data as evidence that all rMala s 11 mutants used in the present study are structurally similar to the WT rMala s 11. Importantly, it should also be noted that both native and mutated forms of the proteins were enzymatically active, strongly suggesting native-like folding (Table 2).

3.5. IgE binding capacity of mutated rMala s 11

The IgE binding capacity of mutated rMala s 11 versions was compared to that of WT rMala s 11 in inhibition ELISA assays. Six ELISA assays, each using a different patient sample, were performed using plasma from AE patients sensitised to both rMala s 11 and rhMnSOD. rMala s 11 inhibited IgE-binding to itself in a dose-dependent manner in all assays, whereas the negative con-

trol protein BSA did not inhibit IgE-binding to rMala s 11. For the assay performed with plasma from AE patient No. 1, lower IgE-binding was observed for all mutated rMala s 11 versions compared to the binding capacity of WT rMala s 11 (Fig. 5). Furthermore, substitutions introduced in regions 2 and 4 (Fig. 2b) resulted in the most significant loss of IgE-binding (Fig. 5). In the remaining five patients, only the use of rMala s 11 versions A2 and B2, in which mutations were introduced in region 2 (Fig. 2b), resulted in lower IgE-binding when compared to WT rMala s 11, with the exception of patient no. 6 where also rMala s 11 version A4 resulted in lower IgE-binding in comparison to WT rMala s 11 (Fig. 5). Thus our results indicate that region 2, comprising residues E29, P30, E122 and K125, is of importance for IgE-binding to Mala s 11.

4. Discussion

In the present study, we investigated a possible IgE-mediated cross-reactivity between the *M. sympodialis*-derived allergen Mala s 11 and its human homologue MnSOD. Using inhibition ELISA assays we demonstrate that rMala s 11 is able to inhibit IgE-binding to rhMnSOD and *vice versa*, indicating that these two homologous structures share common IgE epitopes. These results suggest that the mechanisms underlying autoreactive responses to hMnSOD in AE are due to molecular mimicry. In addition, the lower effect of rMala s 11 on IgE inhibition to rhMnSOD, in comparison to that of rhMnSOD (Fig. 1b), could indicate that hMnSOD is able to elicit additional IgE binding epitopes not present on the environmental Mala s 11 allergen. This observation raises questions about the ability of self-antigens to elicit autoreactive IgE epitopes, a topic which is still a controversial issue (Zeller et al., 2008).

IgE-mediated reactivity to other self-antigens has been suggested to be of importance in the pathogenic mechanisms of AE (Valenta et al., 1998, 1999; Ochs et al., 2000; Zeller et al., 2008). MnSOD is a stress-induced mitochondrial enzyme and hence is upregulated by factors such as oxidative stress and mechanical trauma (Hirose et al., 1993). Scratching associated with AE (Akdis et al., 2006) might cause an increased release of endogenous MnSOD and it has been demonstrated that higher levels of MnSOD are present in lesional skin areas of AE patients than in unaffected skin, or in the skin of healthy individuals (Schmid-Grendelmeier et al., 2005). Thus it is likely that autoreactivity to endogenous MnSOD in AE could enhance local inflammatory skin processes.

MnSODs from various species have been demonstrated to cross-react at B- and T-cell levels (Cramer et al., 1996; Flückiger et al., 2002a,c). Based on the results presented herein Mala s 11 can be added to the group of cross-reacting MnSODs, providing a plausible explanation for the autoreactivity observed in a subgroup of AE patients. A study based on a motif peptide approach indicates the importance of three-dimensional studies to accurately map B cell epitopes for multimeric proteins (Marti et al., 2007). Using molecular modelling and sequential comparisons we identified residues that are conserved between Mala s 11 and hMnSOD and which protrude towards the solvent, representing possible cross-reactive regions. All identified residues were grouped into four regions and were substituted using site-directed mutagenesis. When the IgE-binding capacity of mutated rMala s 11 molecules was compared to WT rMala s 11 in inhibition ELISA assays, a variation between AE patient No. 1 and the other five AE patients was observed (Fig. 5). For AE patient No. 1, all mutated rMala s 11 versions displayed lower IgE-binding when compared to WT rMala s 11, whereas region 2 seemed to be of importance for the observed

Table 2
Enzymatic activity of WT and mutated rMala s 11 proteins

rMala s 11 version ^a	Enzymatic activity ^b (U/μg)
WT rMala s 11	0.2
A1	0.6
B1	0.3
A2	0.6
B2	0.4
A3	0.2
B3	0.2
A4	0.4
B4	0.2

^a As defined in Table 1.

^b Enzymatic activity expressed as arbitrary units/μg (U/μg) in relation to bovine SOD used as a standard.

lower IgE-binding in the five additional patients. These results are in good agreement with previous reports defining that some of the IgE recognition patterns are patient-specific (Mittag et al., 2006; Cocco et al., 2007).

The reduction of IgE binding is unlikely to be due to misfolding since all mutated rMala s 11 versions were enzymatically active and displayed UV CD spectra comparable to those of WT rMala s 11. Region 2 (comprising residues E29, P30, E122 and K125, Figs. 2b and 3) overlaps with a region in hMnSOD comprising residues P19, P30, K43 and N50, previously suggested as being important for IgE-mediated cross-reactivity between hMnSOD and MnSOD derived from *A. fumigatus* (Flückiger et al., 2002b). It is noteworthy that with exception of patient No. 1 and 6, the mutations of Mala s 11 residues K43 and N50 in region 1, and of P19 in region 4, respectively, did not affect IgE binding (Fig. 5). Thus one may speculate that residue P30, either alone or together with other surrounding residues such as E29 may be of importance for IgE cross-reactivity among MnSOD proteins from different species.

Assuming that Mala s 11 forms a tetramer, as observed in the crystal structures of *A. fumigatus* MnSOD (Flückiger et al., 2002b) and *H. sapiens* (Borgstahl et al., 1992) one must consider that while some residues are solvent exposed in the monomer they are not accessible to antigen-antibody interactions in the tetramer due to steric hindrance. All the four regions examined in this study contain residues exposed to the solvent in the tetramer and could therefore be significant for IgE-binding *in vivo*. Although our results indicate that region f IgE-binding.

In conclusion, we have demonstrated that Mala s 11 and hMnSOD share common IgE binding epitopes, providing an explanation for the observed autoreactivity to hMnSOD in AE patients sensitised to *M. sympodialis* and for the ability of hMnSOD to induce eczematous reactions in AE patients sensitised to Mala s 11 (Schmid-Grendelmeier et al., 2005). In addition, we identified a set of residues that are involved in IgE binding to Mala s 11. This provides a more detailed understanding of cross-reactivity at the molecular level.

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