

ORIGINAL PAPER

Alternaria alternata NADP^{*}-dependent mannitol dehydrogenase is an important fungal allergen

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Clinical and Experimental Allergy

Summary

Background *Alternaria alternata* is one of the most important allergenic fungi worldwide. Mannitol dehydrogenase (MtDH) has previously been shown to be a major allergen of *Cladosporium herbarum* and cross-reactivity has been demonstrated for several fungal allergens.

Objective The present study's objective was to clone the MtDH from an *A. alternata* cDNA library, express and purify the recombinant non-fusion protein and test its IgE-binding properties.

Methods A cDNA library prepared from *A. alternata* hyphae and spores was screened for mannitol dehydrogenase by DNA hybridization with the radioactively labelled *C. herbarum* homologue as a probe. The resulting clone was sequenced and heterologously expressed in *Escherichia coli* as a recombinant non-fusion protein, which was purified to homogeneity and analysed for its IgE-binding capacity.

Results The coding sequence of the full-length cDNA clone comprises 798 bp encoding a protein with a molecular mass of 28.6 kDa and a predicted pI of 5.88. Protein sequence analysis revealed an identity of 75% and a homology of 86% between the MtDHs of *A. alternata* and *C. herbarum*. The functional mannitol dehydrogenase was expressed in the *E. coli* strain BL21(DE3) transformed with the vector pMW172 and purified to homogeneity. The enzyme catalyses the NADPH-dependent conversion of D-fructose to D-mannitol. In IgE-ELISA and immunoblots, MtDH is recognized by 41% of *A. alternata*-allergic patients. *In vivo* immunoreactivity of the recombinant MtDH was verified by skin prick testing. Finally, inhibition-ELISA experiments confirmed cross-reactivity between the MtDHs of *A. alternata* and *C. herbarum*.

Conclusion Mannitol dehydrogenase (Alt a 8) represents an important new allergen of the ascomycete *A. alternata* that might be suitable for improving diagnostic and therapeutic procedures.

Keywords allergen, allergy, Alt a 8, *Alternaria alternata*, cross-reactivity, mannitol dehydrogenase, mould, MtDH

Submitted 16 May 2006; revised 19 July 2006; accepted 21 August 2006

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Introduction

The ascomycete *Alternaria alternata* worldwide represents an important cause of inhalant allergic reactions, with sensitization rates ranging from 2% to 30% among the allergic population [1]. *A. alternata* represents the

most important fungal allergen source in the United States. In a study with more than 17 000 US citizens, 3.6% showed a positive skin prick testing (SPT) with *Alternaria* and sensitization to *Alternaria* was observed among 70% of the patients with fungal allergies [2]. In European multi-centre study conducted in 30 centres throughout Europe, Australia, New Zealand and Portland (USA) with 1132 asthmatic patients, sensitization to

*nicotine amide adenine dinucleotide.

Alternaria was the lowest in southern Europe, with a prevalence of 4.7%, and the highest in Portland (28.2%), the United Kingdom and the Republic of Ireland (17.6%) [3]. Although sensitization to two or more moulds is common among the allergic population, *Alternaria* is also one of the main mono-sensitizers along with *Candida* and *Trichophyton* [4]. In addition, several studies have shown, that sensitization to this mould is a risk factor for allergic involvement of the lower respiratory tract and asthma in both children and adults [3, 5–7], which is especially true in the case of severe asthmatics [8–11].

Fungal allergen extracts are complex mixtures of allergenic and various non-allergenic substances and the latter might interfere with diagnostic results. Immunotherapy with these extracts might even induce new sensitization patterns in immunotherapy-treated patients, emphasizing the need for defined recombinant allergens suitable for molecule-based patient-tailored diagnostic and therapeutic approaches. So far, at least nine *A. alternata* allergens have been cloned and characterized, but this number is still not sufficient to explain the complex IgE-reactivity patterns obtained in immunoblots with the sera of *A. alternata* allergic patients [12]. The major allergen Alt a 1 is recognized by the sera of more than 80% of *A. alternata*-sensitized patients. It forms a dimer in non-reducing SDS-PAGE with an apparent molecular mass of 28 kDa and was cloned by Unger et al. [13] and De Vouge et al. [14]. About 22% of *A. alternata* allergics react with enolase (Alt a 6) [15, 16]. Heat shock protein 70 (Alt a 3) [17], protein disulphide isomerase (Alt a 4), the two acidic ribosomal proteins P1 (Alt a 12) and P2 (Alt a 5), a yeast CP4-homologous protein with unknown function (Alt a 7), aldehyde dehydrogenase (Alt a 10) [18] and nuclear transport factor 2 [19] are minor *A. alternata* allergens recognized by less than 20% of patients. Bush et al. [20] reported a 25 kDa protein with homology to a common transposable region and mouse RNA-dependent eukaryotic initiation factor-2 α -kinase to be IgE-reactive, but its clinical relevance still has to be verified [21].

Recently, we have shown that MtDH is a major allergen of the ascomycete *C. herbarum* recognized by about 57% of *C. herbarum*-allergic individuals in IgE-immunoblots [22]. As cross-reactivity has been observed for several fungal allergens, we sought to investigate whether mannitol dehydrogenase is also an allergen in the closely related mould *A. alternata*. Here we report the nucleotide and the deduced protein sequence of *A. alternata* mannitol dehydrogenase (Alt a 8). Its enzymatic and molecular properties were determined by SDS-PAGE and mass spectrometry using the purified recombinant non-fusion protein. We further show that it is recognized by *A. alternata*-allergic patients' IgE and demonstrate IgE cross-reactivity between *A. alternata* and *C. herbarum* MtDH.

Materials and methods

Materials

Unless otherwise stated, all chemicals were purchased from Applichem, Darmstadt, Germany, enzymes were obtained from Promega (Madison, WI, USA) and columns and chromatography media from GE Healthcare, Uppsala, Sweden. Nucleotide primers were synthesized by VBC-Genomics, Vienna, Austria.

Patients and sera

Sera were collected at the Allergy Unit of the National Health Service (Rome, Italy) and the Allergieambulatorium Reumannplatz (Vienna, Austria). Patients were selected according to an appropriate clinical case history suggesting *A. alternata* allergy as well as a positive SPT and/or a positive radioallergosorbent test with commercial *A. alternata* extract. Sensitization to *A. alternata* was confirmed by IgE-immunoblots with *A. alternata* crude extract. Serum of a birch-allergic patient and normal human serum, i.e. serum of a non-atopic person, were used as negative controls in IgE-immunoblots and ELISA experiments. Sera were stored at -20°C .

Preparation of *Alternaria alternata* crude extract

Alternaria alternata strain 08–0203 from the Research and Teaching Institute for Brewing at the Technical University of Berlin (Berlin, Germany) was grown for five days at 23°C on YPD plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar]. Mycelium and spores were then ground to a fine powder in liquid nitrogen and extracted at 4°C in 10 mM potassium phosphate, pH 7.0, 2 mM EDTA, 3 mM NaN_3 containing 10 $\mu\text{L}/\text{mL}$ of a protease inhibitor cocktail (0.1 M benzamidine, 0.2 mg/mL aprotinin, 0.2 mg/mL leupeptin, 10 mg/mL bacitracin, 0.1 mg/mL antipain, 70 $\mu\text{g}/\text{mL}$ pepstatin A).

Hybridization screening of the λ -ZAP cDNA library

Alternaria alternata MtDH was isolated from an *in vivo* excised cDNA library prepared from *A. alternata* mycelium and spores in the phage vector Uni-ZAP XR (Stratagene, La Jolla, CA, USA) as described earlier [18]. In order to make a probe for hybridization screening, the homologous *C. herbarum* MtDH cDNA (GenBank accession number AY191816) was PCR-amplified using the primers 5'-ATG CCT GGC CAG CAA GCA AC-3' and 5'-TCT GGT GGT GTA ACC ACC C-3'. The product was gel-purified (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany), randomly labelled with [α - ^{32}P]dATP using the Prime-a-Gene Labelling System (Promega). Before hybridization, the probe was denatured at 95°C .

for 2 min and, after addition of 20 mM EDTA, stored at -20°C until use.

Escherichia coli DH10 α cells were electro-transformed with the *A. alternata* plasmid library and plated on LBamp agar plates (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agarose, 100 mg/L ampicillin). Approximately 1.7×10^5 colonies were transferred onto nitrocellulose filters (0.45 μm , PROTRAN BA 85, Schleicher & Schuell, Dassel, Germany) that were treated with 1.5 M NaCl/0.5 M NaOH for 5 min. Filters were neutralized with 1.0 M Tris/HCl, pH 7.4, containing 1.5 M NaCl for another 5 min and rinsed with $2 \times$ SSPE buffer (300 mM NaCl, 20 mM sodium phosphate, pH 7.0, 2 mM EDTA). Colonies were fixed by baking the filters at 80°C for 2 h. Before over-night hybridization with the denatured probe at 65°C , pre-hybridization with 100 $\mu\text{g}/\text{mL}$ herring sperm DNA was performed in 100 mM sodium phosphate buffer, pH 7.0, 850 mM NaCl, 2.5 mM EDTA, 0.1% (w/v) SDS, $10 \times$ Denhardt's solution (2 g/L BSA, 2 g/L Ficoll, 2 g/L polyvinylpyrrolidone). After 1 h, the denatured probe was added to the pre-hybridization buffer and allowed to hybridize overnight at 65°C . High-stringency washes were conducted at 65°C with pre-warmed $4 \times$ SSPE, $2 \times$ SSPE and $1 \times$ SSPE containing 0.1% (w/v) SDS for 30 min each. Filters were air-dried and exposed onto a high-performance autoradiography film (Hyperfilm MP, GE Healthcare, Uppsala, Sweden). Positive clones were propagated in LB medium (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl) containing 100 mg/L ampicillin and plated in order to obtain single colonies, which were rescreened as described above. Only single colonies were picked and screened by PCR for inserts of the expected size using the T3 and T7 promoter primers (5'-AAT TAA CCC TCA CTA AAG GG 3'-and 5'-GTA ATA CGA CTC ACT ATA GGG C-3', respectively) binding to vector-encoded sequences flanking the multiple cloning site. Plasmid DNA of positive clones was isolated and sequenced on both strands.

Subcloning of Alternaria alternata mannitol dehydrogenase as a non-fusion protein

The complete coding sequence of *A. alternata* MtDH was PCR-amplified using the proofreading Pfu DNA polymerase (Boehringer Ingelheim, Vienna, Austria) and directionally subcloned into the pMW172 expression vector [23] to produce a recombinant non-fusion (rnf)-protein. 5' Nde I and 3' Eco RI restriction sites were added to the coding sequence by using the primers 5' GAAATTC CATATG CCC ATC ACC GTT CCC-3' and 5'-AAT GAAATTC TTA CCT GAC GCA GTA ACC AC-3' (restriction sites are shown in italics). Both vector and PCR product were digested with the respective enzymes at 37°C for 2 h. Additionally, the cut vector DNA was dephosphorylated with 0.1 U of calf intestinal alkaline phosphatase per

microgram of DNA. Fragments were separated by means of electrophoresis on a 1% (w/v) agarose gel and purified with the QIAquick PCR Purification Kit (Qiagen) before ligation at 4°C overnight. The constructs were used to electro-transform *E. coli* BL21(DE3). Bacteria cells were selected on LBamp plates, and insert lengths were checked by colony PCR using vector-specific primers. Plasmid DNA of positive clones was isolated and sequenced to ensure sequence identity with the original clone isolated from the *A. alternata* cDNA library.

Isolation of genomic DNA coding for Alternaria alternata mannitol dehydrogenase

The open reading frame coding for MtDH was PCR-amplified from genomic DNA isolated from *A. alternata* using the primers used for subcloning the cDNA into the pMW172 vector and a proofreading DNA polymerase (Pfu Polymerase, Boehringer Ingelheim, Vienna, Austria). PCR products were digested and ligated into the pMW172 vector in order to transform *E. coli* BL21(DE3) cells.

Expression and purification of the Alternaria alternata recombinant non-fusion mannitol dehydrogenase

A stationary overnight culture of the *E. coli* BL21(DE3) cells transformed with the recombinant *A. alternata* MtDH in the pMW172 vector was diluted 1 : 100 in 500 mL fresh LB medium containing 100 mg/L ampicillin and grown at 37°C until an OD_{600} of 0.8 was reached. LacZ promoter-mediated expression of the recombinant MtDH was induced with 0.4 mM isopropyl β -D-1-thiogalactopyranoside and cells were shaken for 20 h at 16°C . Cells were harvested by centrifugation at $6000 \times g$ and resuspended in a tenth culture volume (50 mL) of 50 mM sodium phosphate, pH 7.5, 5 mM dithiothreitol, 4 mM MgCl_2 , 2 mM EDTA containing 1 tablet/100 mL of protease inhibitor (Complete EDTA-free protease inhibitor cocktail tablets; Roche Diagnostics, Mannheim, Germany). Bacteria were lysed with 1 mg/mL lysozyme for 30 min at room temperature and three cycles of freezing in liquid nitrogen and thawing at 37°C . Genomic DNA was sheared by ultrasonication. After the cell lysate had been cleared by centrifugation at $10\,000 \times g$ for 20 min, the supernatant was brought to 40% saturation with ammonium sulphate, equilibrated for at least 45 min and centrifuged at $20\,000 \times g$ for 20 min. The supernatant was filtered (0.45 μm ; Millex HPF PVDF membrane filters, Millipore, Bedford, MA, USA) before loading it onto a hydrophobic interaction chromatography column (source 15PHE medium, 20 mL column volume) equilibrated with chromatography buffer A (10 mM sodium phosphate, pH 6.5, 1.2 M ammonium sulphate, 5 mM dithiothreitol, 4 mM MgCl_2 , 2 mM EDTA). MtDH was eluted by a linear ammonium sulphate and pH gradient of buffer B (10 mM sodium

phosphate, pH 7.5, 5 mM dithiotreitol, 4 mM MgCl_2 , 2 mM EDTA) in buffer A declining by 4.8 mM ammonium sulphate per/mL buffer at a flow rate of 3 mL/min. Fractions containing MtDH as detected by SDS-PAGE were pooled and concentrated (Amicon Centriprep YM-10 Centrifugal Filter Devices, Millipore). The buffer was exchanged for buffer B (PD-10 Column Sephadex G-25M, GE Healthcare, Uppsala, Sweden). Subsequently, an anion exchange chromatography was performed (source 15Q medium, 8 mL column volume). The column equilibrated with buffer B was loaded at a flow rate of 1.5 mL/min, yielding the MtDH in the flowthrough. As a final step, Cibacron Blue F3G-A affinity chromatography (5 mL HiTrap Blue HP column equilibrated with buffer B) was performed. MtDH was eluted with a salt gradient sloping by 4 mM NaCl per/mL buffer at a flow rate of 3 mL/min, concentrated (Amicon Centriprep YM-10 Centrifugal Filter Devices, Millipore) and stored at -70°C with addition of 10% (v/v) glycerol.

Enzyme kinetics

MtDH activity was shown spectrophotometrically by monitoring the oxidation of NADPH at 340 nm using an extinction coefficient of 6.3 mM/cm. Enzyme assays were performed in 1 mL total volume at room temperature. For recording the Michaelis–Menten kinetics for D-fructose, the assay mixture consisted of 20 mM phosphate buffer, pH 7.0, 250 μM NADPH and D-fructose concentrations of 100 to 1 200 mM. The curve for NADPH was assayed in 20 mM sodium phosphate buffer containing 400 mM D-fructose and NADPH concentrations ranging from 50 to 500 μM . Assays were performed in triplicate with results expressed as specific activities in units per milligram of protein and are presented as the means with standard errors. Non-linear regression curves, Michaelis–Menten constants (K_M) and maximal reaction velocities were calculated with the GraphPad Prism Software (GraphPad, San Diego, CA, USA).

Low-Energy nano-ESI-QTOF mass spectrometry

Protein solutions were prepared for mass spectrometry by buffer exchange against pure water performed with a Sephadex G-25 column (PD-10 Column Sephadex G-25M, GE Healthcare, Uppsala, Sweden). Proteins were diluted to a final concentration of 300 fmol/ μL in an aqueous solution of 20% (v/v) acetonitrile containing 0.2% (v/v) formic acid. Low-energy nanoESI-QTOF mass spectra of positively charged ions of the fragmented proteins were recorded with a Micromass Global Ultima Q-TOF mass spectrometer (Waters, Milford, MA, USA) and deconvoluted with the MaxEnt software provided with the instrument.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoglobulin E-immunoblots

SDS-PAGE was performed according to Laemmli [24] using 13.5% (w/v) polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, CA, USA). For immunoblots, proteins were electro-transferred (1.5 h, 300 mA) onto a polyvinylidene fluoride (PVDF) membrane (ESI-QTOF Immobilon-P, 0.45 μm pore size; Millipore). Membranes were cut into 3 to 4 mm wide strips and incubated with patients' sera diluted 1 : 10 in gold buffer (50 mM sodium phosphate, pH 7.5, 0.5% (w/v) BSA, 0.5% (v/v) Tween-20, 0.05% (w/v) NaN_3) for 6 h at room temperature or at 4°C overnight. Sera were removed by washing with gold buffer three times for 10 min each and membrane strips were incubated with ^{125}I -labelled rabbit anti-human IgE antibody (Medpro, Vienna, Austria). After three washes to remove unbound secondary antibodies, membrane strips were exposed to a Fujifilm BAS-MS 2325 Imaging Plate for 24 to 48 h. Imaging Plates were scanned with a Fujifilm BAS-1800 II instrument using the BASReader software for Windows version 2.26 (Raytest, Straubenhardt, Germany).

Immunoglobulin E enzyme linked immunosorbant assay

Flat-bottom 96 well plates (Nunc Maxisorp, Nunc, Roskilde, Denmark) were coated overnight with 500 ng purified *A. alternata* rnfMtDH in 100 μL phosphate buffered saline (PBS) (1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 140 mM NaCl, 3 mM KCl). Wells were briefly washed with PBS+0.05% (v/v) Tween-20 and blocked with 250 μL 1% (w/v) BSA in PBS+0.05% (v/v) Tween-20 for 1 h at room temperature before overnight incubation at 4°C with 100 μL patients' sera diluted 1 : 5 in PBS+0.05% (v/v) Tween-20 containing 0.5% (w/v) BSA. Following extensive washing with PBS+0.05% (v/v) Tween-20, bound IgE was detected with 100 μL horseradish peroxidase-conjugated goat anti-human IgE (Bethyl Laboratories, Montgomery, TX, USA) diluted 1 : 500 with PBS+0.05% (v/v) Tween-20 containing 0.5% (w/v) BSA at room temperature. After four washes to remove unbound antibodies and rinsing with PBS, the colour reaction was performed with 100 μL 0.22 mg/mL 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt in 50 mM phosphate-citrate buffer, pH 4.2, with addition of 0.05% H_2O_2 . The absorption was measured after 30 min at 405 nm with a standard ELISA reader.

For the cross-inhibition experiments, the diluted sera were pre-incubated with 1 or 10 $\mu\text{g/mL}$ of purified *A. alternata* or *C. herbarum* rnfMtDH overnight at 4°C before applying them onto the coated wells. In case of positive controls used as references for cross-inhibition experiments, sera were mock-pre-incubated with PBS.

Skin prick tests

SPT were performed at the Department of Dermatology at the University Hospital Zürich (Zürich, Switzerland) with approval of the ethical committee of the University of Zürich. Tests were conducted with *A. alternata*-allergic patients and healthy controls using purified recombinant *A. alternata* MtDH as well as commercial *A. alternata* SPT extract (ALK, Copenhagen, Denmark) in duplicate in inverse order on the volar surface of the forearm. For the recombinant MtDH, a dilution of 100 µg/mL was chosen. The commercial extract was used in the provided dilution of 100 000 SQE/mL. Tests were considered positive if after 15 min, the mean weal diameter was at least 3 mm in the absence of a reaction with the saline control but with a positive reaction to histamine dihydrochloride [25]. Patients gave their written consent for these tests, after they had been provided with a detailed oral and written explanation of the procedure.

Results

Cloning of *Alternaria alternata* mannitol dehydrogenase

Using the *C. herbarum* MtDH cDNA sequence as a probe, *A. alternata* MtDH was cloned via hybridization screening of an *A. alternata* *in vivo*-excised cDNA library in the phage vector Uni-ZAP XR (Stratagene). Approximately 1.7×10^5 clones were screened with the ^{32}P -labelled probe and several clones coding for MtDH were isolated, of which five were sequenced on both strands with identical results (Fig. 1).

The full-length cDNA clone comprises an 801 bp open reading frame flanked by an 86 bp 5' untranslated region and a 124 bp 3' untranslated region, followed by a poly(A) tail. A putative polyadenylation signal (AATAAA) frequently occurring in fungi was identified 27–22 bp upstream the poly(A) sequence at position 985–990 in the 3' untranslated region. No signalling peptide was found downstream the starting methionine as predicted by the SignalP programme [26], confirming the expected intracellular localization.

A genomic PCR-clone of the open reading frame of *A. alternata* MtDH was isolated by amplifying the MtDH-encoding DNA from genomic cDNA of *A. alternata* as described in the methods section. Comparison with the cDNA coding for *A. alternata* MtDH showed that the coding sequence is not interrupted by any introns on the chromosome. The complete cDNA sequence was submitted to the National Center for Biotechnology Information (NCBI) GenBank database under the accession number AY191815.

Translation is initiated with the first translation initiation codon (ATG) available in the sequence. Without the initiator methionine, the cDNA sequence codes for a

protein of 265 amino acid residues with a predicted molecular mass of 28.6 kDa and a calculated isoelectric point of 5.88.

On the amino acid level, *A. alternata* MtDH shares 75% identity and 86% homology with the homologous *C. herbarum* gene. Similar identities and homologies were observed with *Cladosporium fulvum* MtDH (74% and 86%, respectively) [27] (GenBank accession number AF387300) and a hypothetical mannitol dehydrogenase from *Aspergillus nidulans* (71% identity and 82% homology; NCBI Protein database accession number EAA62170; Fig. 2).

Purification of the recombinant *Alternaria alternata* mannitol dehydrogenase

A. alternata MtDH was expressed as an rnf-protein in the heterologous host *E. coli* BL21(DE3). The expressed protein was purified to apparent homogeneity as described in the methods section. MtDH was recovered from the hydrophobic column with an ammonium sulphate concentration of about 0.5 M. Although under the conditions used MtDH did not bind to the anion exchange material, purification to near homogeneity could be achieved with this step. Minor impurities within the preparation at molecular masses of about 28 and 34 kDa were removed in a final affinity chromatography step performed with blue sepharose exploiting the high affinity of NAD(P)^+ -binding enzymes to the structurally related chromophore Cibacron Blue F3G-A, where peak concentrations of MtDH were eluted with about 360 mM NaCl.

MtDH with an expected molecular mass of 28.6 kDa was successively enriched until a single band was attained as analysed by SDS-PAGE and Coomassie Brilliant Blue staining (Fig. 3), yielding 9 mg rnfMtDH per litre of bacterial culture.

Mass spectrometrical analysis

The measured molecular mass of the recombinant protein of 28 618 Da corresponded well to the calculated value of 28 617.3 Da derived from the *A. alternata* MtDH cDNA sequence. This provides strong evidence for the correct amino acid composition of the recombinant protein. The starting methionine is cleaved off after translation.

Enzyme activity of recombinant non-fusion mannitol dehydrogenase

Pattern and profile searches in the InterPro database [28] with InterPro Scan [29] revealed the presence of a short-chain dehydrogenase family signal (PROSITE database [30] accession number PDOC00060 and Protein families database (Pfam) [31] accession number PF00106). Based on homology to *Agaricus bisporus* MtDH with a known three-dimensional structure [32], two functionally

5' -CTTCATATCACATCACACT																				9
TCAACTCAATTCCCATTATATACCCCAAACCTTCTTTACTCTTCATAAACCCACATAATCGCCACA																				86
ATG	CCC	ATC	ACC	GTT	CCC	CAA	GCT	ACC	GAG	CTC	AAG	GAC	CTC	TTC	AGC	CTT				137
M	P	I	T	V	P	Q	A	T	E	L	K	D	L	F	S	L				17
AAG	GGC	AAG	GTC	GTC	ATC	GTC	ACC	GGT	GCC	TCC	GGC	CCC	ACC	GGT	ATT	GGC				188
K	G	K	V	V	I	V	<u>T</u>	<u>G</u>	A	S	G	P	T	<u>G</u>	I	<u>G</u>				34
ACA	GAG	GCT	GCC	CGA	GGA	TGC	GCT	GAG	TAC	GGT	GCC	GAC	CTC	GCC	ATC	ACC				239
T	E	A	A	R	G	C	A	E	Y	G	A	D	L	A	I	T				51
TAC	AAC	TCT	CGC	GCC	GAG	GGT	GCC	GAG	AAG	AAC	GCA	AAG	GAG	ATG	AGC	GAG				290
Y	N	S	R	A	E	G	A	E	K	N	A	K	E	M	S	E				68
AAG	TAC	GGC	GTC	AAG	GTC	AAG	GCC	TAC	AAG	TGC	CAG	GTC	AAC	GAG	TAC	GCT				341
K	Y	G	V	K	V	K	A	Y	K	C	Q	V	N	E	Y	A				85
CAG	TGC	GAG	AAG	CTC	GTC	CAG	GAC	GTC	ATC	AAG	GAC	TTC	GGC	AAG	GTC	GAT				392
Q	C	E	K	L	V	Q	D	V	I	K	D	F	G	K	V	D				102
GTC	TTC	ATC	GCC	AAC	GCC	GGA	AAG	ACT	GCC	GAC	AAC	GGT	ATC	CTC	GAC	GCT				443
V	F	I	A	N	A	G	K	T	A	D	N	G	I	L	D	A				119
ACC	GTT	GAG	CAG	TGG	AAC	GAG	GTC	ATC	CAG	ACC	GAC	TTG	ACC	GGT	ACC	TTC				494
T	V	E	Q	W	N	E	V	I	Q	T	D	L	T	G	T	F				136
AAC	TGC	GCC	CGT	GCC	GTT	GGT	CTC	CAC	TTC	CGC	GAG	CGC	AAG	ACT	GGC	TCT				545
N	C	A	R	A	V	G	L	H	F	R	E	R	K	T	G	S				153
CTC	GTC	ATC	ACC	TCC	TCC	ATG	TCC	GGC	CAC	ATT	GCC	AAC	TTC	CCC	CAG	GAG				596
L	V	I	T	S	S	M	S	G	H	I	A	N	F	P	Q	E				170
CAG	GCC	TCC	TAC	AAC	GTT	GCT	AAG	GCT	GGC	TGC	ATT	CAC	CTC	GCC	AAG	TCG				647
Q	A	S	T	N	V	A	T	A	G	C	I	H	L	A	K	S				187
CTC	GCC	AAC	GAG	TGG	AGG	GAC	TTT	GCC	CGT	GTC	AAC	TCC	ATC	TCC	CCT	GGA				698
L	A	N	E	W	R	D	F	A	R	V	N	S	I	S	P	G				204
TAC	ATT	GAC	ACT	GGT	CTC	TCC	GAC	TTC	GTT	CCC	CAG	GAC	ATC	CAG	AAG	CTG				749
Y	I	D	T	G	L	S	D	F	V	P	Q	D	I	Q	K	L				221
TGG	CAC	TCC	ATG	ATC	CCC	ATG	GGC	CGT	GAC	GCC	AAG	GCT	ACT	GAG	CTC	AAG				800
W	H	S	M	I	P	M	G	R	D	A	K	A	T	E	L	K				238
GGT	GCC	TAC	GTC	TAC	TTC	GCA	TCG	GAT	GCC	TCA	TCC	TAC	TGC	ACT	GGT	TCC				851
G	A	Y	V	Y	F	A	S	D	A	S	S	Y	C	T	G	S				255
GAT	CTC	CTC	ATC	GAC	GGT	GGT	TAC	TGC	GTC	AGG	TAA									887
D	L	L	I	D	G	G	Y	C	V	R	*									267
ACGTGTCATTCCGGAAGGAAGATGCGAGTGGAGGAATATAATAATGGACGACGTCTTGCCGGAAGTC																				954
TTGTGTCCATGTAAATAGCATCGAGACATCAATAAAGCTTCGCGAGTTTCACATCACAAAAA																				1021
AAAAA-3'																				1026

Fig 1. Nucleotide and deduced amino acid sequence of the isolated *Alternaria alternata* mannitol dehydrogenase cDNA clones. Amino acids forming the catalytic triad are highlighted in black boxes. The two underlined sequence motives are the putative coenzyme-binding motif (bold) and the polyadenylation signal (in italics). The translation termination site TAA is depicted as *. Numbers on the right denote nucleotide and amino acid positions.

related conserved regions could be identified. Firstly, an N-terminal glycine-rich putative nucleotide coenzyme binding motif at position 25–34 and secondly, the catalytic triad composed of Ser-159, Tyr-174 and Lys-178 typical for short-chain dehydrogenases (Fig. 1) [33].

To determine whether the purified protein exhibits enzymatic activity of a mannitol dehydrogenase, its enzyme kinetics were studied. As expected, the enzyme catalysed the NADPH-dependent reduction of D-fructose to D-mannitol according to the following equation:



Initial velocities were determined in the standard assay mixtures at an optimal pH of 7.0 using the linear decrease of NADPH concentrations for calculation of enzyme activ-

ity values. Testing of increasing substrate and coenzyme concentrations both resulted in typical Michaelis-Menten-type kinetics (Fig. 4). The enzyme showed an unusually high Michaelis-Menten constant of 474 ± 80 mM for D-fructose and 18.7 ± 3.5 μ M for NADPH. A coenzyme concentration of 250 μ M NADPH was used in the assays to record the activity with varying fructose concentrations. Because of the high K_M -value for the substrate D-fructose, substrate inhibition occurred at concentrations above 750 mM and the calculated maximal enzyme activity of 148 ± 12 μ mol/(mg/min) at saturating fructose concentrations was not reached under the experimental conditions. Therefore, a fructose concentration of 400 mM was chosen for assaying activities at increasing NADPH concentrations, which gave rates of fructose reduction of about 45% of that

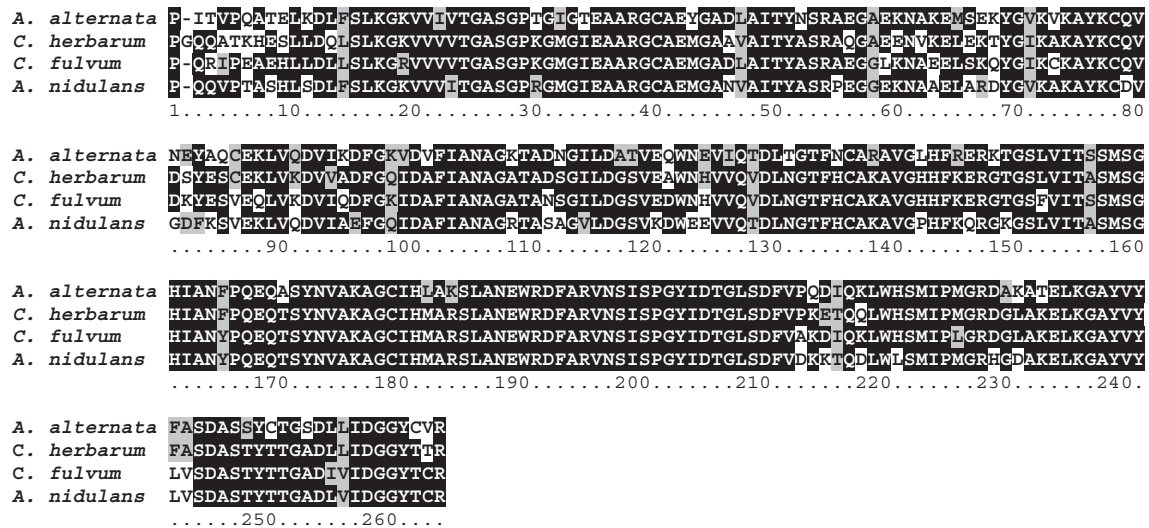


Fig. 2. Protein sequence alignment of the homologous mannitol dehydrogenases of *Alternaria alternata* (GenBank AY191815), *Cladosporium herbarum* (GenBank AY191816), *Cladosporium fulvum* (GenBank AF387300) and *Aspergillus nidulans* (GenBank AACD01000129). Identical and homologous amino acids are shown by black and grey boxes, respectively. Gaps are marked by dashes (-), and residue positions are given in the bottom line.

at the calculated saturating concentration of 0.95 M D-fructose. Also, NADPH concentrations above 500 μ M caused inhibition (data not shown).

Immunoglobulin E-immunoreactivity of the recombinant *Alternaria alternata* mannitol dehydrogenase

The IgE-immunoreactivity of the recombinant MtDH was tested with the sera of 22 *A. alternata*-allergic patients (Fig. 5a), where those patients who had a higher absorp-

tion than the negative control using the second antibody alone (lane 25) were regarded positive.

Comparable results were obtained with either the IgE-ELISA (Fig. 5c) or the IgE-immunoblot (Fig. 5b) with the purified MtDH where nine of the 22 patients (41 %) reacted positively. With *A. alternata* MtDH, some reactivity with the secondary antibody was seen, which could be reduced, when human serum was applied before incubation with the secondary antibody as apparent with the normal human serum (serum 24), serum of a birch-allergic patient (serum 23) and the sera of *A. alternata*-allergic patients not reactive to MtDH (patients 3, 6, 7, 9, 10, 12–14, 16, 18, 20–22).

Except for two patients (patient 8 and patient 11), sera reactive to *A. alternata* MtDH also reacted with purified *C. herbarum* MtDH. Therefore, cross-reactivity between these two allergens was further studied in IgE-inhibition ELISA experiments with the sera of five patients reactive to *A. alternata* MtDH (Fig. 6). Three of the sera also reacted with *C. herbarum* MtDH.

When sera reactive to *A. alternata* MtDH were pre-incubated with *C. herbarum* MtDH, inhibition was observed with the serum of one patient (patient 4). Inhibition was stronger with 10 μ g/mL *C. herbarum* MtDH as compared with 1 μ g/mL. Little or no reduction of signal intensity was seen with the sera of two patients (patients 15 and 17) and, as expected, with the two sera non-reactive with *C. herbarum* MtDH (sera 8 and 11). Complete inhibition to background levels could be achieved by pre-incubating sera with *A. alternata* MtDH, indicating that the depletion was complete. Conversely, depletion of all three *C. herbarum* MtDH-reactive sera (sera 4, 15, 17) with *A. alternata* MtDH resulted in partial inhibition of

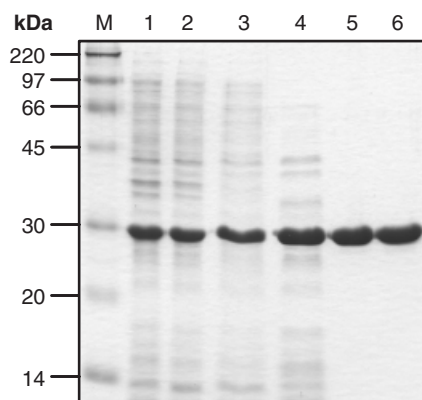


Fig. 3. Purification of recombinant *Alternaria alternata* mannitol dehydrogenase. Coomassie-stained gel showing successive steps of the purification procedure: *Escherichia coli* lysate (lane 1), soluble fraction of *E. coli* lysate (lane 2), supernatant of the 40% ammonium sulphate cut (lane 3), eluate from hydrophobic interaction chromatography at 0.5 M ammonium sulphate (lane 4), flow-through of anion exchange chromatography (lane 5) and the purified rnfMtDH after Cibacron Blue F3G-A affinity chromatography (lane 6). (M) Molecular mass marker.

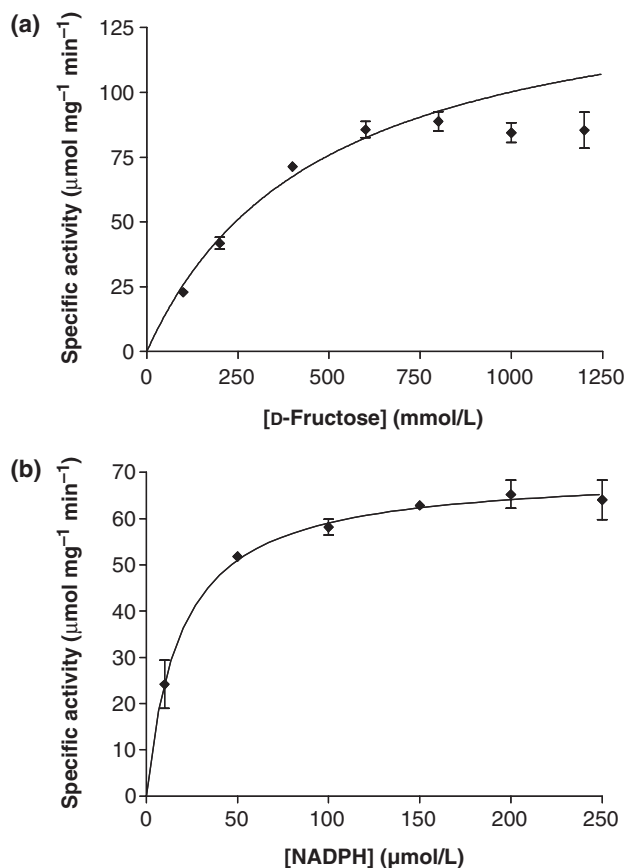


Fig. 4. Michaelis-Menten kinetics of the purified recombinant non-fusion mannitol dehydrogenase for D-fructose (a) and nicotinic adenine dinucleotide phosphate (NADPH) (b) presented as means of triplicate assays with standard errors. Curves were recorded at room temperature in 20 mM sodium phosphate buffer, pH 7.0, with 250 μ M NADPH and 400 mM D-fructose, respectively. Michaelis-Menten constants are 474 ± 80 mM D-fructose and 18.7 ± 3.5 μ M NADPH.

IgE-reactivity to *C. herbarum* MtDH, i.e. *A. alternata* MtDH-depleted sera still showed residual IgE-binding to *C. herbarum* MtDH. Again, complete inhibition was seen, when sera reactive to *C. herbarum* MtDH were pre-incubated with *C. herbarum* MtDH. For the inhibition ELISA, an additional control experiment was performed, where serum reactive to MtDH (patient 4) pre-incubated with either *A. alternata* or *C. herbarum* MtDH was applied to uncoated wells. The absence of signals in this experiment confirms that antibodies complexed with antigen are not unspecifically adsorbed to the wells. Because of the lack of serum, the ELISA experiments could only be conducted once.

The *in vivo* IgE-reactivity of Alt a 8 was tested in SPT performed with *A. alternata*-allergic patients and healthy controls. We could show that the SPTs with rnfAlt a 8 were positive in three patients. These patients also had strong weal and flare reactions with the commercial *A. alternata* extract. These results underly the clinical relevance of *A. alternata* MtDH.

The allergen was officially named Alt a 8 by the WHO-IUIS Allergen nomenclature subcommittee (<http://www.allergen.org>).

Discussion

In this study, we report the nucleotide and the deduced amino acid sequence of a new *A. alternata* allergen (Alt a 8), which was expressed as a recombinant non-fusion protein, purified and tested for its IgE-binding capacity. We show that the allergen is a MtDH that catalyses the NADPH-dependent reduction of D-fructose to D-mannitol.

Like other fungal mannitol dehydrogenases, *A. alternata* MtDH belongs to the heterogeneous group of short-chain dehydrogenases with a positionally conserved triad of Ser, Tyr and Lys residues [33]. In *A. alternata* MtDH, a conserved Ser residue was found at position 159, a Tyr residue at position 174 and a Lys residue at position 178 (Fig. 1) homologous to the catalytic triad of *A. bisporus* MtDH for which the three-dimensional structure is known [32]. Another common feature for SDR membership is the occurrence of an N-terminal nucleotide-binding fold (Rossmann-fold) with a characteristic α -helix flanked by two β -sheets and a coenzyme-binding Gly-pattern TGXXGXG where X stands for any amino acid [33]. In case of *A. alternata* MtDH, the first two glycines are separated by five amino acids, as is also the case for MtDHs of other ascomycetes (Fig. 2).

The Michaelis-Menten constant for D-fructose for this enzyme was found to be exceptionally high. Similar constants were also reported for other MtDHs, such as *C. herbarum* [22], *C. fulvum* [27], *A. bisporus* [34] and *Gibberella zeae* [35].

The absence of a putative hydrophobic signal peptide shows that *A. alternata* MtDH is a cytosolic protein. We chose *E. coli* as an expression host system, as we did not detect any N-glycosylation signals in the amino acid sequence. However, O-glycosylation was not assessed but is very unlikely for a cytosolic protein. Moreover, we could demonstrate that the homologous natural MtDH purified from the related mould *C. herbarum* is not glycosylated (our own unpublished data) and expect this to also be true for *A. alternata* MtDH.

To our knowledge, we are the first to show that a mannitol dehydrogenase is an allergenic protein recognized by patients' IgE. IgE-immunoblots and the ELISA clearly showed that the purified *A. alternata* MtDH is recognized by *A. alternata*-allergic patients with a prevalence of 41%, which classifies MtDH as the second most important allergen of *A. alternata* identified and cloned so far. The clinical significance of this finding was underlined by a positive SPT with an *A. alternata*-allergic patient showing that the rnfMtDH was able to elicit a strong immunological reaction *in vivo*.

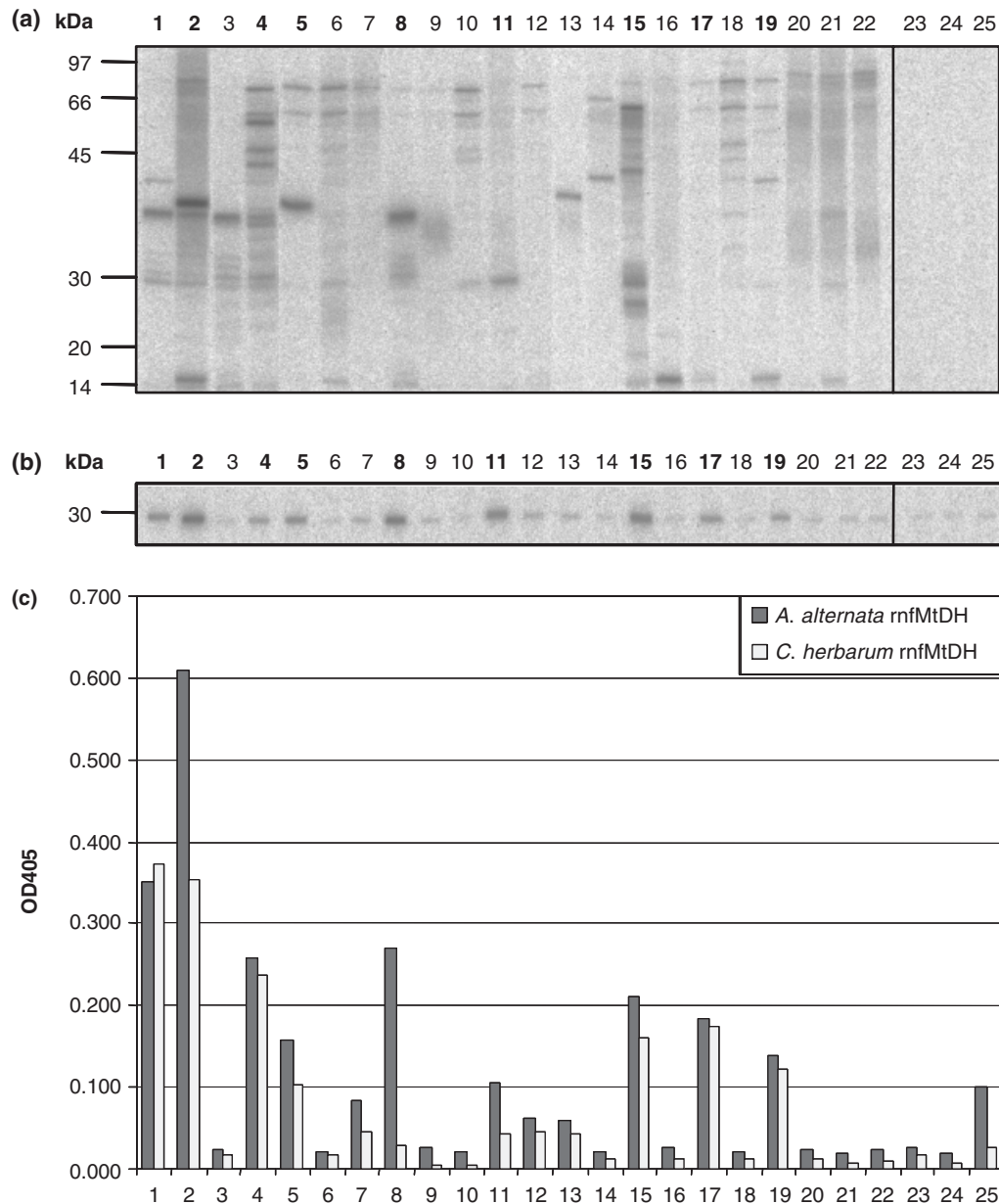


Fig. 5. IgE-immunoreactivity of 22 *Alternaria alternata*-allergic patients' sera (numbers 1–22) with *A. alternata* crude extract (a), *A. alternata* recombinant non-fusion mannitol dehydrogenase (rnfMtDH) (b, c) and *Cladosporium herbarum* rnfMtDH (c) was tested in IgE-immunoblots (a, b) and in an ELISA assay (c). Negative controls were performed with serum of a birch-allergic patient (serum 23), serum of a non-atopic person (serum 24) and the second ^{125}I -labelled rabbit anti-human IgE antibody only (25). Patients demonstrating specific IgE-reactivity in the immunoblots (a, b) are shown in bold.

Summarizing our results from the cross-inhibition ELISA, we can conclude that MtDH is cross-reactive between *A. alternata* and *C. herbarum* and that in addition to shared IgE-epitopes, there also exist mould-specific epitopes as no complete cross-inhibition could be achieved. Unfortunately, *A. alternata* MtDH reproducibly showed some reactivity with the secondary antibody. Importantly, this reactivity was not detected in the controls with normal human serum or serum from the birch-

allergic patient. An explanation for this observation could be that epitopes on *A. alternata* MtDH recognized by the anti-IgE antibody are blocked by antibodies other than IgE (e.g. IgG) when serum is applied before incubation with the secondary antibody.

Taking a closer look at the clinical histories of the patients enrolled in this study, the majority of patients were multi-allergics sensitized not only to moulds but also to pollen, foodstuffs, house dust mite and pets.

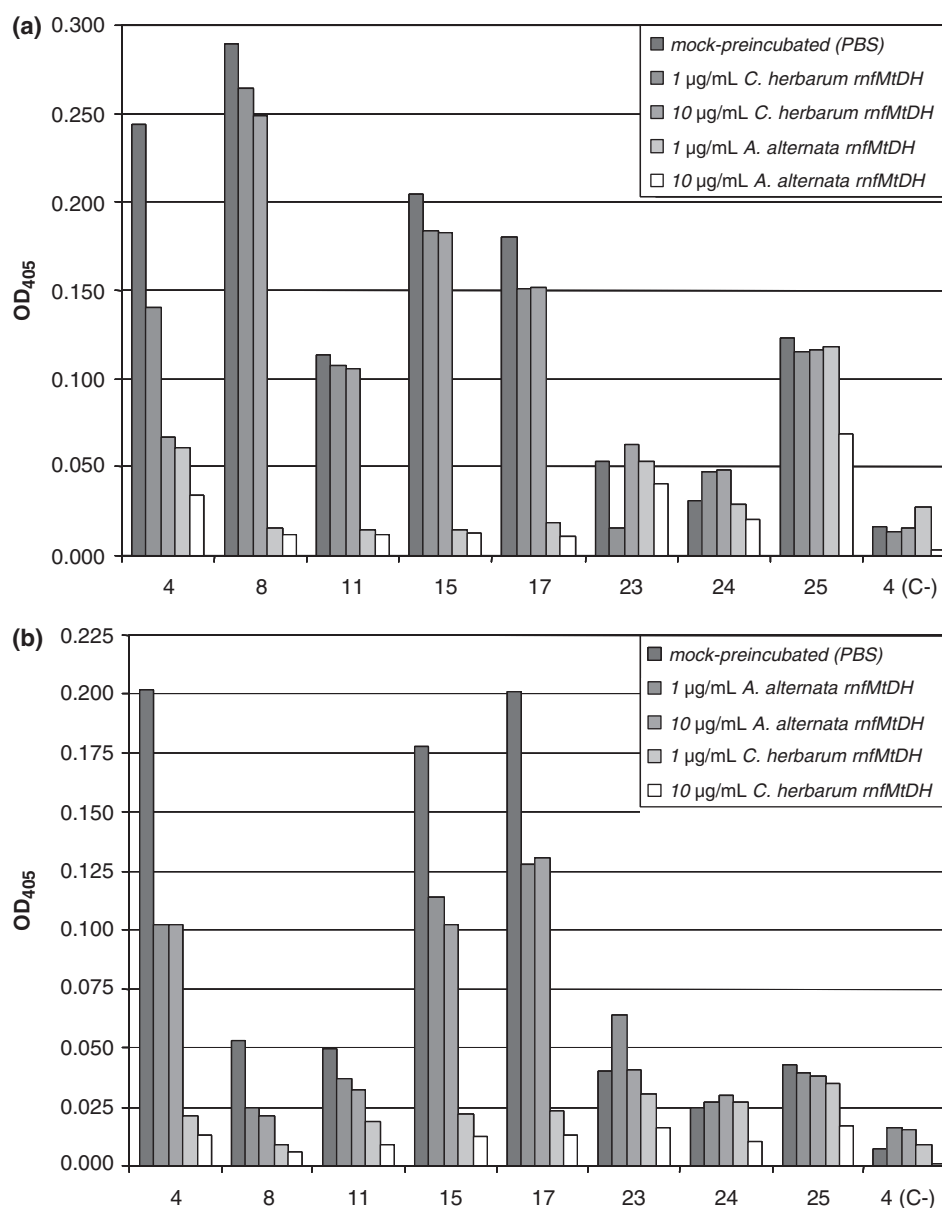


Fig. 6. In the IgE-inhibition ELISA, either *A. alternata* recombinant non-fusion mannitol dehydrogenase (rnf MtDH) (a) or *Cladosporium herbarum* rnfMtDH (b) was coated to the microtitre plate. Sera 4, 8, 11, 15 and 17 were depleted with different amounts of *A. alternata* or *C. herbarum* rnfMtDH (see insert). Negative controls were performed with serum of a birch-allergic patient (23), serum of a non-atopic person (24) and the second ^{125} I-labelled rabbit anti-human IgE antibody only (25). In an additional control experiment, a serum reactive to MtDH (serum 4) was applied to uncoated wells [4 (C-)].

Interestingly, more than 50% of the patients presented themselves with asthma. Other common symptoms involved bronchitis and rhinoconjunctivitis. Symptoms did not correlate with specific or total IgE levels.

At present, diagnosis of *A. alternata* allergic patients is still hampered by the use of ill-defined fungal extracts in SPT because of strain-to-strain variabilities and difficulties with growth and extraction procedures [36]. On the other hand, the advantages of a molecule-based approach are obvious. Unger et al. [13] showed that seven of seven *A. alternata*-allergic patients could be positively tested

with only two recombinant allergens of this mould, namely the major allergen Alt a 1 [14] and enolase (Alt a 6) [16]. Considering the rather small number of patients enrolled in this clinical study and sensitization rates of 80% for Alt a 1 and 22% for Alt a six among *A. alternata*, allergic patients, it is obvious that more allergens have to be included to further improve the diagnostic accuracy in a molecule-based approach. In a study of Asturias et al. [21], 42 *A. alternata*-allergic patients were tested with natural and recombinant Alt a 1, rAlt a 2 and rAlt a 6 (enolase) showing that 41 of 42 patients tested positively

reacted with rAlt a 1 and six out of 42 patients had specific IgE against rAlt a 6, whereas rAlt a 2 was not detected by any *A. alternata*-allergic patient. As 2–20% [13, 21] of the *A. alternata*-allergic patients do not react with Alt a 1 and only 15–28% react with *A. alternata* enolase, a further allergen is required for a component-resolved diagnostic and therapeutic approach. One promising candidate is mannitol dehydrogenase, the second most important *A. alternata* allergen described so far besides Alt a 2, whose incidence of reactivity (61%) [20] could not be confirmed by others [21].

Acknowledgements

This work was supported by project S8812-MED to B. Simon-Nobbe. The project was part of the National Research Network S88-MED of the Austrian Science Fund (FWF).

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