NADP-DEPENDENT MANNITOL DEHYDROGENASE, A MAJOR ALLERGEN OF CLADOSPORIUM HERBARUM

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Running Title: Mannitol dehydrogenase, a major allergen of Cladosporium herbarum

Cladosporium herbarum is an important allergenic fungal species which has been reported to cause allergic diseases nearly in all climatic zones. 5-30 % of the allergic population displays IgE-antibodies against molds. Sensitization to Cladosporium has often been associated with severe asthma, and less frequently with chronic urticaria and atopic eczema. However, no dominant major allergen of this species has been found so far. We present cloning, production and characterization of NADP-dependent mannitol dehydrogenase of C. herbarum (Cla h 8) and show that this protein is a major allergen which is recognized by IgE-antibodies of about 57 % of all C. herbarum-allergic patients. This is the highest percentage of patients reacting with any C. herbarum allergen that was characterized so far. Cla h 8 was purified to homogeneity by standard chromatographic methods and both N-terminal and internal amino acid sequences of protein fragments were determined. Enzymatic analysis of the purified natural protein revealed that this allergen represents a NADP-dependent mannitol dehydrogenase (MtDH) which interconverts mannitol and D-fructose. It is a soluble, non-glycosylated cytoplasmic protein. Two-dimensional protein analysis indicated that MtDH is present as a single isoform. The cDNA encoding Cla h 8 was cloned from a lambda-ZAP library constructed from hyphae and spores. The recombinant non-fusion protein was expressed in E. coli and purified to homogeneity. Its immunological and biochemical identity with the natural protein was shown by enzyme activity tests, CD-spectroscopy, IgE immunoblots with patients’ sera, and by skin prick testing of Cladosporium-allergic patients. This protein therefore is a new major allergen of C. herbarum.

The ascomycete, Cladosporium herbarum (the name of the perfect or teleomorph form is Mycosphaerella tassiana or Davidiella tassiana) is one of the most important allergy-causing mold species worldwide. Its occurrence and association with allergic disease has been described in nearly all climatic zones of the world. In particular, sensitization to C. herbarum has been found to be high in atopic patients with severe forms of asthma (1,2). Sensitization occurs in hot and humid climates like for instance in the south of the U.S. (3), where allergic sensitization against all molds may be as high as 30 % among the allergic population. Recent evidence shows that sensitization against C. herbarum is also common in desert climates like in Saudi Arabia and Kuwait (4). C. herbarum is an indoor as well as an outdoor allergen source with seasonal peaks in late summer and autumn.

Seven C. herbarum allergens have been cloned and characterized so far (5-8). These single allergens are recognized by less than 20 % of the
C. herbarum allergic patients, thus representing minor allergens (8), but so far no major C. herbarum allergen has been reported. We are presenting here the first major allergen of C. herbarum which is a NADP-dependent mannitol dehydrogenase, a cytoplasmic non-glycosylated protein which is abundant in vegetative cells of the fungus. We produced the allergen as a recombinant non-fusion protein and tested it for enzyme activity, molecular parameters (mass and CD spectroscopy), and immunological properties, and showed that the recombinant non-fusion (rnf) protein is comparable with the natural purified allergen. Skin prick testing showed in vivo clinical relevance of the allergen.

C. herbarum is not usually an infectious agent for humans, but is a well studied plant pathogen (9,10). The physiological importance of mannitol dehydrogenase may be seen in the host-pathogen interaction between C. herbarum and the infected plant, as well as in other stress situations for the fungus. It is remarkable that mannitol dehydrogenase, like Bet v 1, the major allergen of birch pollen, is another major allergen belonging to the class of stress-inducible proteins (11,12).

Experimental Procedures

Chemicals - Unless otherwise stated all chemicals were obtained from Sigma (St. Louis, MO, USA), enzymes were obtained from Promega (Madison, WI, USA) and chromatographic media were obtained from GE Healthcare (Chalfont St. Giles, United Kingdom).

Patients and sera - C. herbarum allergic patients were selected according to a typical clinical history, a positive skin prick test to commercial C. herbarum extract and a RAST (Radio Allergo Sorbent Test) class greater than 3.

Preparation of C. herbarum crude extract - C. herbarum (strain 280202 from the Institut für Gärungsgewerbe, Technical University Berlin, Germany) was cultivated on YPD plates (1 % yeast extract, 2 % peptone, 2 % dextrose, 2 % agar) at 23 °C for five days. To prepare a crude extract, mycelium and spores were homogenized to a fine powder in a mortar using liquid nitrogen. Extraction was done over night in 10 mM sodium phosphate, pH 7.5, 2 mM EDTA, 3 mM NaN₃, 10 µl/ml proteases inhibitor mix (0.2 mg/ml aprotinin, 0.2 mg/ml leupeptin, 10 mg/ml bacitracin, 0.1 mg/ml antipain, 70 µg/ml pepstatin A) with constant shaking at 4°C. The mixture was centrifuged yielding the crude extract in the supernatant. Protein concentration was measured according to Bradford (13).

Purification of natural C. herbarum MtDH - The crude extract was first brought to 50 % ammonium sulfate saturation and the pH was adjusted to 6.5 with 3 M sodium acetate. After centrifugation at 15.000 g for 10 min, the supernatant was loaded onto a hydrophobic interaction chromatography column (Source PHE, GE Healthcare, 20 ml bed volume) equilibrated with buffer A (20 mM sodium phosphate, pH 6.5, 1.2 M ammonium sulfate, 2 mM EDTA, 2 mM dithiotreitol, 4 mM MgCl₂, 0.1 mM NAD⁺). The column was washed with buffer A and then eluted with 6 column volumes of a linear ammonium sulfate and pH gradient ranging from 50 to 0 % buffer B (20 mM sodium phosphate, pH 7.5, 2 mM EDTA, 4 mM dithiotreitol, 4 mM MgCl₂, 0.1 mM NAD⁺) in buffer A. Mannitol dehydrogenase eluted at about 0.84 M ammonium sulfate. Fractions containing MtDH, as visualized by SDS-PAGE were pooled, the volume was reduced by centrifugation in a Centriprep YM-10 column (Millipore, Billerica, MA, USA), desalted on a Sephadex G-25 column equilibrated with buffer B and loaded onto an anion exchange chromatography column (Source 15 Q, GE Healthcare, 8 ml bed volume) equilibrated with buffer B. After extensive washing with buffer B, MtDH was eluted with 12.5 column volumes of a linear NaCl-gradient in buffer B ranging from 0 to 300 mM NaCl. MtDH eluted at about 35 mM NaCl. After addition of 10 % glycerol the MtDH preparation was stored at -70 °C.

Enzyme kinetics - Enzyme assays were performed in 20 mM sodium phosphate buffer, pH 7.5 in 1 ml total volume at room temperature by spectrophotometrically measuring the absorption of NADPH at 340 nm. Michaelis-Menten kinetics for the reduction of D-fructose were recorded with 250 µM NADPH and varying concentrations of D-fructose. The oxidation of NADPH was measured with 735 mM D-fructose and varying concentrations of NAD⁺.

For the reverse reaction, the oxidation of D-mannitol was measured with 250 µM NAD⁺ and varying concentrations of D-mannitol whereas 200 mM D-mannitol and varying concentrations of NAD⁺ were used to monitor the reduction of NAD⁺.
1- and 2 dimensional gel-electrophoresis and immunoblot - C. herbarum extract and purified MtDH were analyzed by SDS-PAGE (14) and 2-dimensional gel-electrophoresis (15). Specific IgE-reactivity was tested by IgE-immunoblots (5).

Determination of N-terminal and internal protein sequences - The N-terminal sequence of C. herbarum MtDH was obtained by Edman degradation. The allergen was separated by 2-dimensional electrophoresis, blotted to a polyvinylidene difluoride (PVDF) membrane and stained with Coomassie brilliant blue R-250. Spots from several membranes were excised, loaded onto a standard cartridge of a Procise 491 protein sequencer (Applied Biosystems, Foster City, CA, USA) and sequenced using the pulsed liquid cycle with the manufacturer’s standard v 1.1 chemistry. Alternatively, 5 µg of the purified protein were dissolved in water, loaded onto a Procise sample preparation cartridge (Applied Biosystems) and sequenced as described above.

In order to obtain sequences of internal protein fragments, 5 µg of the purified protein were dissolved in 50 µl 50 % trifluoroacetic acid. Cleavage was performed by adding 5 µl of 5 M cyanogen bromide and incubation at 30 °C in the dark over night. After stopping the reaction by addition of 500 µl of water, the cleaved fragments were dried, separated by 18 % SDS-PAGE, blotted, stained and sequenced as described above.

Degenerate primer design and PCR amplification - Based on the protein sequence data, degenerate oligonucleotides were synthesized. Primer 1 (Tab. 1) was deduced from the N-terminal sequence. Primer 2 was deduced from peptide 4. PCR was performed with primers 1 and 2 using C. herbarum cDNA plasmid library as template DNA.

Construction and screening of a cDNA expression library - C. herbarum mycelium was harvested and crushed with mortar and pestle under liquid nitrogen. The powder was suspended in 12 ml/g guanidinium isothiocyanate containing 1 % β-mercaptoethanol and homogenized (Ultra Turrax, IKA-Werke, Staufen, Germany). The RNA was extracted with phenol/chloroform and precipitated with isopropanol. After a digest with proteinase K and a second extraction with phenol/chloroform, RNA was precipitated with isopropanol. Finally, the RNA was precipitated with 3 M lithium chloride. A further extraction was done with n-butanol/chloroform followed by precipitation with guanidinium isothiocyanate. A cDNA expression library was constructed in the Uni ZAP XR vector (Stratagene, La Jolla, CA, USA).

For the screening 30 ng of the 636 bp long PCR fragment were randomly labeled with [α-32P]ATP using Prime a Gene® Labeling System (Promega). The hybridization probe was used to screen 6 x 10⁵ plaques of the cDNA expression library with a titer of 1.58 x 10⁹ pfu/ml. The plating of the library and the subsequent plaque lift were done according to manufacturer’s recommendations (Stratagene).

Plaques showing positive signals were in vivo excised as pBluescript SK (+/-) phagemids. To determine the size of each cDNA insert PCR was performed using the T3 and T7 promoter primers. Several MtDH clones were isolated and sequenced on both strands.

Subcloning into pHis-parallel2 and pMW172 vectors - The open reading frame of C. herbarum MtDH was cloned into the 6xHis fusion vector pHis-parallel2 (16) and the non-fusion expression vector pMW172 (17). The MtDH coding sequence was subcloned via BamHI and XhoI into the pHis-parallel2 vector after PCR amplification using primer 3 and primer 4 (Tab.1). The recombinant non-fusion protein was cloned into pMW172 via NdeI and EcoRI using primer 5 and primer 6 (Tab.1). The amplicons were digested with the respective restriction enzymes and ligated in-frame into the two vectors. The DNA sequence of the two constructs was shown to be correct by double stranded DNA-sequencing.
exchange chromatography were the same as applied for the purification of the natural *C. herbarum* MtDH (see above).

**Circular dichroism spectropolarimetry** - Circular dichroic spectra in the far UV between 190 and 260 nm wavelength were recorded at 20 °C in 10 mM sodium phosphate buffer (pH 7.4) with a J-810 spectropolarimeter (JASCO, Inc., Easton, MD, USA) in continuous scanning mode using a 1.0 mm path length, flat quartz cuvette, a sensitivity of 100 mdeg, a resolution of 1 nm, a scanning speed of 100 nm/min, a response of 1 sec and a bandwidth of 1 nm. Each circular dichroic profile represents an average of five scans. The baseline obtained with buffer in the absence of protein was subtracted from the sample spectra. The data is expressed as the mean residue molar ellipticity \([\Theta]_{MRW} = \Theta / (10 \cdot C_r \cdot l)\) where \(\Theta\) is the ellipticity in mdeg, \(l\) is the cell path length in cm and \(C_r = (n \cdot 1,000 \cdot c_g) / M_r\) is the mean residue molar concentration with the number of peptide bonds \(n\), the macromolecule concentration \(c_g\) in g/ml and the molecular mass \(M_r\) in Da.

**Skin prick test of *C. herbarum* allergic patients with rnfMtDH** - Skin prick tests were performed in duplicate in inverse order on the volar surface of the forearm. The patient was tested with the purified rnfMtDH \((c = 100 \mu g/ml)\), which had been tested for purity by mass spectrometry, CD-spectroscopy and SDS-PAGE. Its immunological reactivity was shown by IgE-immunoblot. The preparation was free of endotoxins and non-cytotoxic. The *C. herbarum* and *A. alternata* skin prick test-extracts were obtained from ALK (Copenhagen, Denmark) and used in the provided dilution \((100,000 \text{ SQE/ml})\). The weal size was recorded after 15 minutes. The results were regarded as positive if the mean weal diameter was at least 3mm in the absence of a reaction with the saline control (18). The study design was approved by the ethical committee of the University of Zurich. A full oral and written explanation of the procedure was given to the participants, and their written consent was obtained before testing.

**RESULTS**

Detection of a new 28 kDa *C. herbarum* allergen by two dimensional gel electrophoresis - *C. herbarum* extract was dialyzed against water and subjected to isoelectric focusing using the capillary IEF system of Biometra (Göttingen, Germany). After SDS-PAGE the gel was stained with Coomassie-Brilliant Blue showing the complex protein pattern of *C. herbarum* with a major protein spot at 28 kDa (Fig. 1A).

To determine the molecular mass and the isoelectric point of the new allergen an IgE-immunoblot of the *C. herbarum* extract was performed. The blot was incubated with the serum of a *C. herbarum* allergic patient, who was known to react with a 28 kDa protein. The immunoblot revealed a single immunoreactive protein spot at a molecular mass of 28 kDa and an isoelectric point of 5.8. No isoforms of the protein were detectable (Fig. 1B).

Purification and characterization of the natural 28 kDa allergen - The crude *C. herbarum* extract was precipitated with ammonium sulfate and purified as described in the Experimental Procedures (Fig. 2A). The specific IgE-reactivity of the purified natural MtDH was tested (Fig. 2B). The specific activity of NADP-dependent MtDH was measured in each fraction as shown in Table 2. Finally, the recovery was 7.6 mg of purified MtDH per 1l of *C. herbarum* crude extract.

Protein sequencing and cloning of the 28 kDa allergen - The N-terminal sequence of the new allergen was determined by Edman degradation starting from 2D protein spots bound to PVDF membrane and stained with Coomassie Brilliant Blue.

Internal protein sequences were obtained by cleaving the purified natural protein with cyanogen bromide. Peptides were separated by SDS-PAGE, blotted to PVDF membrane and sequenced with a Procise 491 protein sequencer. Finally, an N-terminal and 5 internal peptide sequences were obtained (Fig. 3).

Searching GenBank database for proteins with homology to the peptide sequences revealed homology to the NADP-dependent mannitol dehydrogenase of *Cladosporium fulvum* (GenBank Accession Number AAK67169). Based on the protein sequence information (Fig. 3) degenerate primers were designed and PCR was performed using an *in vivo* excised *C. herbarum* λ-ZAP cDNA library as template. A 636 bp long PCR product was obtained, radioactively labeled and used for screening the λ-ZAP cDNA expression library. A full-length clone of *C. herbarum* NADP-dependent mannitol dehydrogenase (MtDH) spanning 918 bp was obtained and sequenced on both strands (GenBank Accession Number AAK67169).
The correct three dimensional folding of the rnfMtDH was investigated by far-UV circular dichroism spectropolarimetry. The circular dichroism spectra of the purified nMtDH and the rnfMtDH were recorded and are almost superimposable indicating a native-like folding of the recombinant protein (Fig. 5).

Biochemical characterization of the C. herbarum MtDH - To prove the enzymatic activity of the putative C. herbarum mannitol dehydrogenase, MtDH activity was measured by spectrophotometry showing that this allergen is specific for D-fructose and NADPH (Fig. 4). No reaction was observed with Fructose-6-Phosphat as substrate or NADH as co-factor. In the assay a D-Fructose concentration of 735 mM and a NADPH coenzyme concentration of 250 µM were used, the pH in the assay was 7.5. Typical Michaelis-Menten-type kinetics for D-fructose and NADPH are plotted in Fig. 4A and 4B, respectively. Enzyme activity was also tested in the reverse direction, with D-mannitol as substrate and NADP⁺ as coenzyme (Fig. 4C, D). The calculated Michaelis-Menten constants for D-fructose, D-mannitol, NADPH and NADP⁺ were 1.17 ± 0.20 M, 0.23 ± 0.05 M, 53 ± 10 µM and 67 ± 7 µM, respectively. The reaction equation is as follows:

\[
\text{D-fructose} + \text{NADPH} + \text{H}^+ \leftrightarrow \text{D-mannitol} + \text{NADP}^+ 
\]

The enzyme has an unusually high \(K_m\)-value for D-fructose (see also Discussion). Enzyme activities were determined for the natural as well as for the recombinant non-fusion protein and no significant differences were observed.

Computer based analysis of the protein sequence using the ExPASy proteomics server revealed a calculated molecular mass of 28.332 kDa and an isoelectric point of 5.9. No signal peptide was found in the N-terminal region. Binding of the coenzyme is provided by a glycine-rich TGXXXGXXG motif, where X can be any amino acid as predicted by the Pattern and profile search in the InterPro database. The predicted catalytically active triad consists of a serine (S) at position 160, a tyrosine (Y) at position 175 and a lysine (K) at position 179. This triad was determined based on the sequence homology between the MtDHs from C. herbarum and Agaricus bipolaris from which the crystallographic structure has been solved (19).

The immunological reactivity of MtDH was also tested in vivo in a skin prick test. The skin prick test revealed that a C. herbarum allergic patient, who was skin prick test positive with a commercial C. herbarum extract, also showed a strong weal and flare reaction with the purified rnfMtDH (Fig. 7) demonstrating its ability to induce an immediate type skin reaction.

DISCUSSION

Summarizing the immunological and allergologic data, we have shown that NADP-dependent MtDH of C. herbarum is indeed a major allergen since about 57 % of the C. herbarum allergic patients recognized by a positive IgE-immunoblot using C. herbarum total protein extract, display specific IgE-reactivity with the pure recombinant MtDH. Thus MtDH is the first major allergen from C. herbarum because all other allergens characterized so far (5,7,20-23) are recognized by less than 22 %. In 2D immunobLOTS no isoforms have been identified, thus simplifying the use of rnfMtDH in diagnosis and therapy. In vivo testing of rnfMtDH by skin prick test clearly showed the immunological reactivity of the protein, since we...
could demonstrate that the new major *C. herbarum* allergen does not only bind IgE *in vitro*, but also induces mediator release from effector cells leading to a weal and flare reaction *in vivo*.

As already mentioned, MtDH of fungi including the enzyme described here is stress-inducible and we have to consider the function of this enzyme in environmental stress defence of the organism. Under stress conditions mannitol is an abundant low molecular mass substance of molds (24,25). Physicochemical as well as physiological *in vivo* data show that mannitol can serve as an osmolyte protecting fungal cells as well as plant cells in conditions of high salinity (26). For instance, transgenic tobacco plants could be rendered salt-tolerant by expressing a prokaryotic mannitol dehydrogenase (27). However, mannitol is also a scavenger for hydroxyl radicals and other reactive oxygen species and is produced in plant pathogenic fungi after infection, as a defence against the superoxide production of the plant. It was shown that mannitol production by fungal plant pathogens is an important virulence factor. The remarkably low affinity (K_m about 1.2 M) of NADP-dependent MtDH for its substrate, D-fructose, as found in the present paper was also seen by others (28). However, when the reaction was measured in then reverse direction, the K_m for mannitol was found to be about five times lower (0.23 M). One explanation for this remarkable difference could be that fructose in aqueous solution is predominantly present in the hemiacetal ring form, while the open chain form is the true substrate of the enzyme. Therefore, the equilibrium between the two forms influences the apparent K_m for fructose (19).

The thermodynamic equilibrium for the reaction equation (D-fructose + NADPH +H^+ ↔ D-mannitol + NADP^+) is such that under stress conditions leading to increased NADPH synthesis the production of mannitol is favoured. Fungi accumulate mannitol to up to 100 mM in the cytoplasm, they secrete it during the defence reaction (29) but they do not secrete MtDH which is a cytoplasmic enzyme (our own unpublished observations).

The MtDH allergen described here belongs to the class of short chain mannitol dehydrogenases which attack carbon atom 2 and use NADP(H) and not NAD(H). A totally different MtDH is activated in the plant after fungal infection, leading to degradation of mannitol via a different reaction. This is a NAD-dependent mannitol-1-dehydrogenase, a medium chain mannitol dehydrogenase which catalyzes a reaction that under the given physiological circumstances leads to mannose production (29). Summarizing, we can state that mannitol and mannitol dehydrogenases, are important stress molecules of fungi, which occur in relatively large amounts and this might be the one of the reasons why *C. herbarum* MtDH is the major allergen of this fungal species.

Finally, we want to discuss the patients whose sera were analyzed in this study with respect to case history to detect any correlation between sensitization to *C. herbarum* and particular allergic diseases. In countries with very high incidence of atopic disease and asthma (for instance Portland, USA), it was shown that patients with severe asthma are in up to 30 % of the cases sensitized to molds (3), in particular to *Cladosporium herbarum* and *Alternaria alternata*. In the total allergic population, sensitization to molds may vary from 5 % to about 30 % (30,31). Therefore, sensitization to *C. herbarum* and *A. alternata* is an important risk factor for severe asthma in these countries (2). Quite unexpectedly, sensitization to the two molds is the major risk factor for developing asthma also in countries with a desert climate, like Saudi Arabia and Kuwait (32,33). Having a look on the case histories of the patients tested no correlation between RAST-classes, pattern of IgE-reactivity and the patients’ symptoms could be found. The symptoms observed were asthma, bronchitis, rhinoconjunctivitis and atopic dermatitis. In order to give some epidemiologic data on the prevalence of *C. herbarum* allergy in the area of Salzburg we are now discussing the 5310 patients who attended the Salzburg outpatient allergy clinic and were tested for type I allergy in the years 2003 and 2004. Of these patients, 115 (2.1 %) tested positive in the *C. herbarum* RAST. 42 of them (36,5 %; 24 female, 18 male) displayed a RAST class ≥ 3. The only consistent pattern which we observed among those patients was that practically all of them were multiallergics not only sensitized to *C. herbarum* but also to pollen, foodstuffs and house dust mite. The symptoms also were atopic dermatitis, rhinoconjunctivitis and asthma with no particular correlation between RAST class, symptoms and the severity of the disease. There is no recognizable pattern in these 42 patients pointing to a particular mode of sensitization.
Since MtDH is the first major allergen of *C. herbarum*, it is together with a set of minor allergens (e.g. enolase) the most promising candidate for a component resolved diagnosis and therapy of *C. herbarum* allergy.

REFERENCES

ACKNOWLEDGEMENT

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We are grateful to P. Sheffield for providing us the pHIS-Parallel2 vector and to an anonymous reviewer for useful suggestions concerning enzyme activity measurements.

FOOTNOTES

1The abbreviations used are: CD, circular dichroism; MtDH, mannitol dehydrogenase; n, natural; PCR, polymerase chain reaction; pl, isoelectric point; PVDF, Polyvinyliden fluoride; RAST, Radio Allergo Sorbent Test; r, recombinant; rnf, recombinant non-fusion.

The DNA sequence coding for the \textit{C. herbarum} NADP-dependent mannitol dehydrogenase was deposited in GenBank database (GenBank Accession Number AY191816).

FIGURE LEGENDS

\textbf{Fig. 1.} \textit{A}, 2D-gel of \textit{C. herbarum} protein extract stained with Coomassie and \textit{B}, IgE-immunoblot of \textit{C. herbarum} protein extract. The protein spot corresponding to MtDH is marked with an arrow.

\textbf{Fig. 2.} Purification and IgE-reactivity of the natural \textit{C. herbarum} MtDH. \textit{A}, Coomassie stained gel of \textit{C. herbarum} crude extract (\textit{lane 1}), supernatant of the 50\% ammonium sulfate cut (\textit{lane 2}), eluate of hydrophobic interaction chromatography at 0.84 M ammonium sulfate (\textit{lane 3}) and the purified natural \textit{C. herbarum} MtDH after anion exchange chromatography (\textit{lane 4}). \textit{(M)} Molecular weight marker. \textit{B}, Purified natural \textit{C. herbarum} MtDH (\textit{lane 4}) and \textit{C. herbarum} crude extract (\textit{lane 1}) were tested with the serum of a \textit{C. herbarum} allergic patient (IgE), with the serum of a non-atopic person (NAP) and as a negative control with the second antibody (\textit{\textsuperscript{125}I}-labeled rabbit anti-human IgE) \textit{(C)}. 
Fig. 3. Nucleotide sequence and deduced amino acid sequence of the isolated full-length clone of C. herbarum MtDH. Amino acids which have been determined by protein-sequencing are framed. Since the protein sequence of peptide 1 overlaps the N-terminal sequence, peptide 1 is shown in dark gray. Amino acids forming the catalytic triad are shown in bold and underlined, whereas amino acids making up the coenzyme binding site are displayed in italics and underlined. The stop codon is shown as an asterisk (*). Numbers on the right denote nucleotide and amino acid positions.

Fig. 4. A, Michaelis-Menten kinetics of the natural C. herbarum MtDH for D-fructose, B, NADPH, C, D-Mannitol, and D, NADP⁺. Michaelis-Menten constants are 1.17 ± 0.20 M D-fructose, 0.23 ± 0.05 M D-mannitol, 53 ± 10 µM NADPH and 67 ± 7 µM NADP⁺. Curves were recorded in 20 mM phosphate buffer (pH 7.5) at room temperature with 250 µM NADPH (A), 735 mM D-fructose (B), 250 µM NADP⁺ (C) and 200 mM D-mannitol (D). Values are presented as means of triplicate assays with standard errors.

Fig. 5. Circular dichroism spectra of the natural and the recombinant non-fusion MtDH recorded at wavelengths between 190 nm and 260 nm in 10 mM sodium phosphate buffer (pH 7.4) at 20 °C. The CD spectrum of the nMtDH is shown with dashed line and the continuous line corresponds to rnfMtDH.

Fig. 6. A, IgE-immunoblots of C. herbarum crude extract and B, purified rnfMtDH. In the immunoblots 21 C. herbarum allergic patients (lanes 1-21) were investigated. As controls served the serum of an atopic patient (lane 22), the serum of a non-atopic person (lane 23) and the secondary 125I labeled rabbit anti-human IgE antibody alone (lane 24). Patients with a specific IgE-reactivity with the MtDH are underlined.

Fig. 7. Skin prick test of a C. herbarum allergic patient with commercial C. herbarum extract and rnfMtDH. 0.9 % saline (N), 0.01 % histamine dihydrochloride (H), C. herbarum extract (I), C. herbarum MtDH (2).

TABLE LEGENDS

Tab. 1. Oligonucleotides used as primers in PCR and sequencing. (A/G) means that either adenine or guanine was incorporated at the given position. The same is true for (C/T) and (I/C), whereby I stands for inosine. BamH1, XhoI, NdeI and EcoRI restriction sites are framed.

Tab. 2. Purification of the MtDH. A unit is defined as the amount of enzyme, which will catalyze the NADP-dependent reduction of 1 µmol D-Fructose per minute at a pH of 7.5 in 20 mM phosphate buffer at room temperature.
Figure 2

A. 

B. 

kDa

M 1 2 3 4

IgE NAP C
### Table 1

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NADP-dependent mannitol dehydrogenase, a major allergen of cladosporium herbarum
Birgit Simon-Nobbe, Ursula Denk, Peter Bernhard Schneider, Christian Radauer, Markus Teige, Reto Crameri, Thomas Hawranek, Roland Lang, Klaus Richter, Peter Schmid-Grendelmeier, Stephan Nobbe, Arnulf Hartl and Michael Breitenbach

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