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NADP-dependent Mannitol Dehydrogenase, a Major Allergen of *Cladosporium herbarum*[*]

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***Cladosporium herbarum* is an important allergenic fungal species that has been reported to cause allergic diseases in nearly 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 that is recognized by IgE antibodies of ~57% of all *Cladosporium* allergic patients. This is the highest percentage of patients reacting with any *Cladosporium* allergen 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 that interconverts mannitol and D-fructose. It is a soluble, non-glycosylated cytoplasmic protein. Two-dimensional protein analysis indicated that mannitol dehydrogenase is present as a single isoform. The cDNA encoding Cla h 8 was cloned from a λ-ZAP library constructed from hyphae and spores. The recombinant non-fusion protein was expressed in *Escherichia 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 sera of patients, 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, for instance in the south of the United States (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 such as 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 *C. herbarum* allergic patients, thus representing minor allergens (8), but so far no major *C. herbarum* allergen has been reported. We present here the first major allergen of *C. herbarum* that is a NADP-dependent mannitol dehydrogenase, a cytoplasmic non-glycosylated protein that 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)[2] 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; enzymes were obtained from Promega (Madison, WI), and chromatographic media were obtained from GE Healthcare.

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%

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s)AY191816.

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[2] The abbreviations used are: rnf, recombinant non-fusion; MtDH, mannitol dehydrogenase; RAST, Radio Allergo Sorbent Test.

peptone, 2% dextrose, 2% agar) at 23 °C for 5 days. To prepare a crude extract, mycelium and spores were homogenized to a fine powder in a mortar using liquid nitrogen. Extraction was done overnight in 10 mM sodium phosphate, pH 7.5, 2 mM EDTA, 3 mM NaN_3, 10 μl/ml of protease inhibitor mix (0.2 mg/ml of aprotinin, 0.2 mg/ml of leupeptin, 10 mg/ml of bacitracin, 0.1 mg/ml of antipain, 70 μg/ml of 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 Mannitol Dehydrogenase (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, 20-ml bed volume; GE Healthcare) equilibrated with buffer A (20 mM sodium phosphate, pH 6.5, 1.2 M ammonium sulfate, 2 mM EDTA, 2 mM dithiothreitol, 4 mM $MgCl_2$, 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 dithiothreitol, 4 mM $MgCl_2$, 0.1 mM NAD^+) in buffer A. Mannitol dehydrogenase eluted at ~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), desalted on a Sephadex G-25 column equilibrated with buffer B, and loaded onto an anion exchange chromatography column (Source 15 Q, 8-ml bed volume; GE Healthcare) 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 ~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 NADPH.

For the reverse reaction, the oxidation of D-mannitol was measured with 250 μM $NADP^+$ and varying concentrations of D-mannitol, whereas 200 mM D-mannitol and varying concentrations of $NADP^+$ were used to monitor the reduction of $NADP^+$.

One- and Two-dimensional Gel Electrophoresis and Immunoblot—*C. herbarum* extract and purified MtDH were analyzed by SDS-PAGE (14) and two-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 two-dimensional electrophoresis, blotted to a polyvinylidene difluoride 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), 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.

To obtain sequences of internal protein fragments, 5 μg of the purified protein were dissolved in 50 μl of 50% trifluoroacetic acid. Cleavage was performed by adding 5 μl of 5 M cyanogen bromide and incubation at 30 °C in the dark overnight. 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.

TABLE 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); I stands for inosine. BamH1, XhoI, NdeI, and EcoRI restriction sites are boldfaced.

Primer 1	5′-CA(A/G) CA(A/G) GC(I/C) qAC (I/C) AA(A/G) CA(C/T) GA-3′
Primer 2	5′-AC(A/G) AA(A/G) TC(A/G) CT(I/C) AG(I/C) CC(A/G) GT(A/G) TC-3′
Primer 3	5′-AT**G GAT CC**A TGC CTG GCC AGC AAG C-3′
Primer 4	5′-TAA **CTC GAG** TTA TCT GGT GGT GTA ACC A-3′
Primer 5	5′-GAA ATT C**CA TAT G**CC TGG CCA GCA ACG AAC-3′
Primer 6	5′-AAT **GAA TTC** TTA TCT GGT GGT GTA ACC A-3′
T3–Primer	5′-AAT TAA CCC TCA CTA AAG GG-3′
T7–Primer	5′-GTA ATA CGA CTC ACT ATA GGG C-3′

Degenerate Primer Design and PCR Amplification—Based on the protein sequence data, degenerate oligonucleotides were synthesized. Primer 1 (Table 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 of 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*-butyl alcohol/chloroform followed by precipitation with guanidinium isothiocyanate. A cDNA expression library was constructed in the Uni ZAP XR vector (Stratagene, La Jolla, CA).

For the screening, 30 ng of the 636-bp-long PCR fragment were randomly labeled with [α-^{32}P]ATP using Prime a Gene® labeling system (Promega). The hybridization probe was used to screen 6×10^5 plaques of the cDNA expression library with a titer of 1.58×10^9 plaque-forming units/ml. The plating of the library and the subsequent plaque lift were done according to the 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 the *C. herbarum* MtDH was cloned into the His_6 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 primers 3 and 4 (Table 1). The recombinant non-fusion protein was cloned into pMW172 via NdeI and EcoRI using primers 5 and 6 (Table 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.

Expression and Purification of rnfMtDH and His_6-MtDH in E. coli—Luria Bertani broth with 100 mg/liter of ampicillin medium was inoculated with stationary overnight cultures of *E. coli* BL21 cells transformed with the His_6-MtDH or the rnf-MtDH constructs. Expression of the recom-

binant proteins was induced at an A_{600} of 0.8 by addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. Bacterial cells were grown for 20 h at 16 °C and harvested by centrifugation. Purification of the His_6-MtDH protein was performed according to the manufacturer's recommendations (GE Healthcare).

E. coli cells harboring rnfMtDH were lysed with lysozyme. The soluble protein fraction was brought to 50% saturation with ammonium sulfate, centrifuged, and loaded onto a hydrophobic interaction chromatography column (Source PHE; GE Healthcare). The parameters of the hydrophobic interaction chromatography and the subsequent anion 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) 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 s, and a bandwidth of 1 nm. Each circular dichroic profile represents an average of five scans. The base line obtained with buffer in the absence of protein was subtracted from the sample spectra. The data are expressed as the mean residue molar ellipticity $[\Theta]_{MRW} = \Theta/(10 \cdot C_r \cdot l)$ where Θ 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 (residues) as 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 μg/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 *Alternaria alternata* skin prick test extracts were obtained from ALK (Copenhagen, Denmark) and used in the provided dilution (100,000 SQE/ml). The weal size was recorded after 15 min. The results were regarded as positive if the mean weal diameter was at least 3 mm 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.

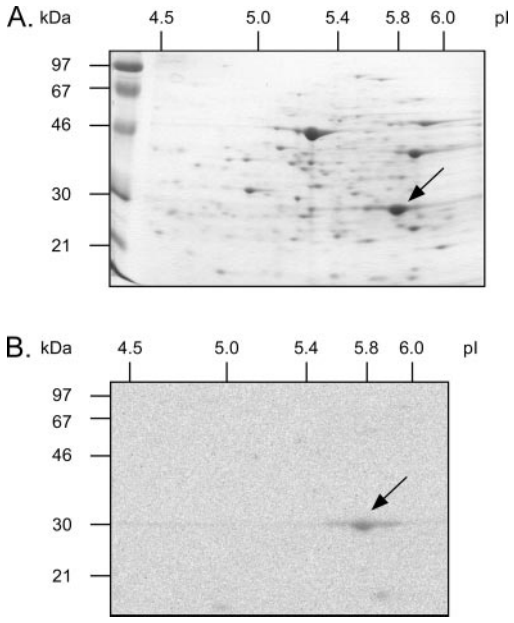

FIGURE 1. *A*, two-dimensional gel of *C. herbarum* protein extract stained with Coomassie. *B*, IgE immunoblot of *C. herbarum* protein extract. The protein spot corresponding to MtDH is marked with an *arrow*.

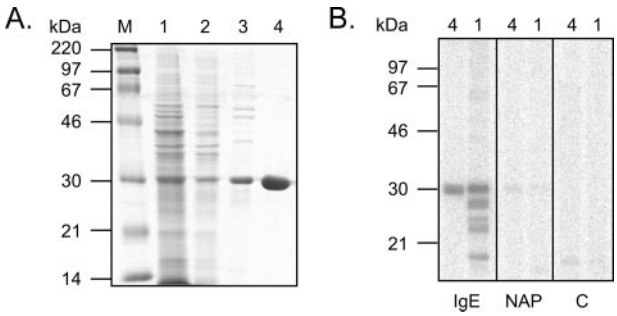

FIGURE 2. **Purification and IgE reactivity of the natural *C. herbarum* MtDH.** *A*, Coomassie-stained gel of *C. herbarum* crude extract (*lane 1*), supernatant of the 50% ammonium sulfate cut (*lane 2*), eluate of hydrophobic interaction chromatography at 0.84 M ammonium sulfate (*lane 3*), and the purified natural *C. herbarum* MtDH after anion exchange chromatography (*lane 4*). *M*, molecular mass marker. *B*, purified natural *C. herbarum* MtDH (*lane 4*) and *C. herbarum* crude extract (*lane 1*) were tested with the serum of a *C. herbarum* allergic patient (*IgE*), with the serum of a non-atopic person (*NAP*), and as a negative control with the second antibody (^{125}I-labeled rabbit anti-human IgE) (*C*).

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. 1*A*).

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. 1*B*).

Purification and Characterization of the Natural 28-kDa Allergen—The crude *C. herbarum* extract was precipitated with ammonium sulfate and purified as described under "Experimental Procedures" (Fig. 2*A*). The specific IgE reactivity of the purified natural MtDH was tested (Fig. 2*B*). 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/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 two-dimensional protein spots bound to polyvinylidene difluoride 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 polyvinylidene difluoride membrane, and sequenced with a Procise 491 protein sequencer. Finally, an N-terminal and five internal peptide sequences were obtained (Fig. 3).

Searching the GenBank™ data base 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

TABLE 2

Purification of the MtDH

A unit is defined as the amount of enzyme that will catalyze the NADP-dependent reduction of 1 μmol D-fructose/minute at a pH of 7.5 in 20 mM phosphate buffer at room temperature.

Fraction	Volume	Total protein	Total activity	Specific activity	Purification	Recovery
	ml	*mg*	*units*	*units/mg*	*-fold*	*%*
Crude extract	300	837	17230	20,6	1	100
Supernatant of the 50% ammonium sulfate cut	330	445	12073	27,1	1,3	70,0
Hydrophobic interaction chromatography	45	16,6	2448	147,5	7,2	14,2
Anion exchange chromatography	2	2,3	637	277,0	13,4	3,7

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CCGTCTACACACGCAACTTCCCGCCTCGACTCCATATCCAATCACATCAAG                          51

ATG CCT GGC CAG CAA GCA ACC AAG CAT GAG TCC CTT TTG GAC CAG CTC              99
 M   P   G   Q   Q   A   T   K   H   E   S   L   L   D   Q   L              16

TCC CTG AAG GGC AAG GTC GTC GTC GTC ACC GGC GCT TCC GGC CCC AAG             147
 S   L   K   G   K   V   V   V   V   T   G   A   S   G   P   K              32

GGC ATG GGT ATT GAG GCC GCT CGC GGT TGC GCC GAG ATG GGC GCC GCT             195
 G   M   G   I   E   A   A   R   G   C   A   E   M   G   A   A              48

GTT GCC ATC ACC TAC GCC TCC CGC GCC CAG GGT GCT GAG GAG AAC GTC             243
 V   A   I   T   Y   A   S   R   A   Q   G   A   E   E   N   V              64

AAG GAG CTT GAG AAG ACC TAC GGC ATC AAG GCC AAG GCC TAC AAG TGC             291
 K   E   L   E   K   T   Y   G   I   K   A   K   A   Y   K   C              80

CAG GTC GAC AGC TAC GAG TCC TGC GAG AAG CTC GTC AAG GAC GTC GTT             339
 Q   V   D   S   Y   E   S   C   E   K   L   V   K   D   V   V              96

GCC GAC TTC GGC CAG ATC GAT GCC TTC ATC GCC AAC GCC GGT GCC ACC             387
 A   D   F   G   Q   I   D   A   F   I   A   N   A   G   A   T             112

GCC GAC TCT GGC ATC CTC GAC GGC TCC GTC GAG GCC TGG AAC CAC GTC             435
 A   D   S   G   I   L   D   G   S   V   E   A   W   N   H   V             128

GTC CAG GTC GAC CTG AAC GGT ACC TTC CAC TGC GCC AAG GCC GTT GGC             483
 V   Q   V   D   L   N   G   T   F   H   C   A   K   A   V   G             144

CAC CAC TTC AAG GAG CGT GGA ACC GGT TCC CTC GTC ATC ACC GCC TCC             531
 H   H   F   K   E   R   G   T   G   S   L   V   I   T   A   S             160

ATG TCC GGC CAC ATC GCC AAC TTC CCC CAG GAG CAG ACC TCC TAC AAC             579
 M   S   G   H   I   A   N   F   P   Q   E   Q   T   S   Y   N             176

GTC GCC AAG GCT GGC TGC ATC CAC ATG GCT CGC TCC CTC GCC AAC GAG             627
 V   A   K   A   G   C   I   H   M   A   R   S   L   A   N   E             192

TGG CGC GAC TTC GCC CGT GTC AAC TCC ATC TCC CCC GGT TAC ATT GAC             675
 W   R   D   F   A   R   V   N   S   I   S   P   G   Y   I   D             208

ACT GGT CTC TCC GAC TTC GTT CCC AAG GAG ACC CAG CAG CTC TGG CAC             723
 T   G   L   S   D   F   V   P   K   E   T   Q   Q   L   W   H             224

TCC ATG ATC CCC ATG GGC CGT GAC GGT CTC GCC AAG GAG CTC AAG GGC             771
 S   M   I   P   M   G   R   D   G   L   A   K   E   L   K   G             240

GCC TAC GTC TAC TTC GCC TCC GAC GCC TCC ACC TAC ACC ACC GGT GCC             819
 A   Y   V   Y   F   A   S   D   A   S   T   Y   T   T   G   A             256

GAT CTC CTC ATT GAC GGT GGT TAC ACC ACC AGA TAA                             855
 D   L   L   I   D   G   G   Y   T   T   R   *                             268

GCGACTCGCCCACAGCAAGTCGTTGAGGCGGAAGGACAAAAAAAAAAAAAAAAAAAAAAAAA              918
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FIGURE 3. **Nucleotide sequence and deduced amino acid sequence of the isolated full-length clone of *C. herbarum* MtDH.** Amino acids that have been determined by protein sequencing are *framed*. Because 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.

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 MtDH spanning 918 bp was obtained and sequenced on both strands (GenBank™ accession number AY191816) (Fig. 3). The full-length clone comprises a presequence of 51 bp preceding the start codon (ATG) and an untranslated 3′-region of 63 bp. The open reading frame covers 804 bp coding for 268 amino acids. The full-length clone of *C. herbarum* MtDH shows an identity of 83.9% and a similarity of 87.3% with the *C. fulvum* MtDH.

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-phosphate as substrate or NADH as cofactor. 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. 4, *A* and *B*, respectively. Enzyme activity was also tested in the reverse direction, with D-mannitol as substrate and $NADP^+$ as coenzyme (Fig. 4, *C* and *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: D-fructose + NADPH + H^+ ↔ D-mannitol + $NADP^+$.

The enzyme has an unusually high K_m-value for D-fructose (see also "Discussion"). Enzyme activities were determined for the natural as well

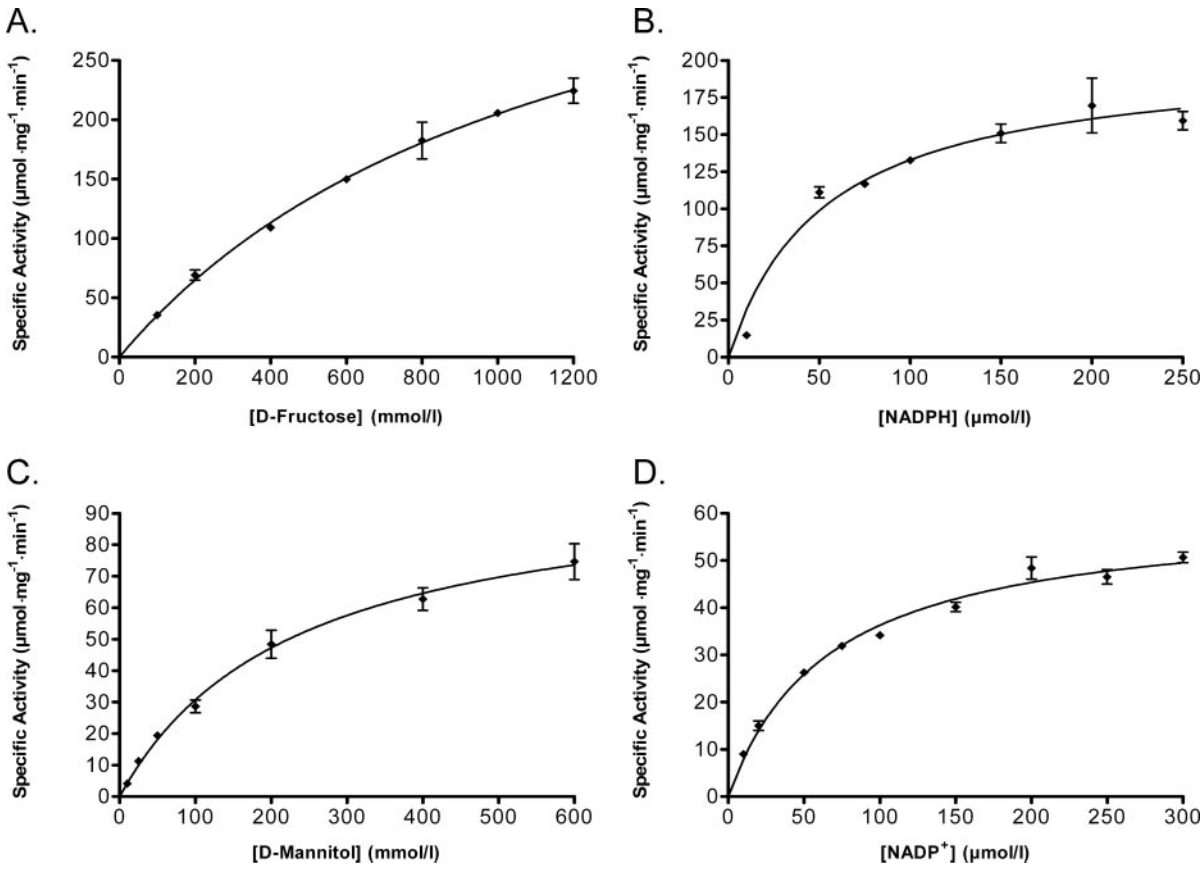

FIGURE 4. **Michaelis-Menten kinetics of the natural *C. herbarum* MtDH for D-fructose (*A*), NADPH (*B*), D-mannitol (*C*), and $NADP^+$ (*D*).** Michaelis-Menten constants are 1.17 ± 0.20 M D-fructose, 0.23 ± 0.05 M D-mannitol, 53 ± 10 µM NADPH, and 67 ± 7 µM $NADP^+$. Curves were recorded in 20 mM 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.

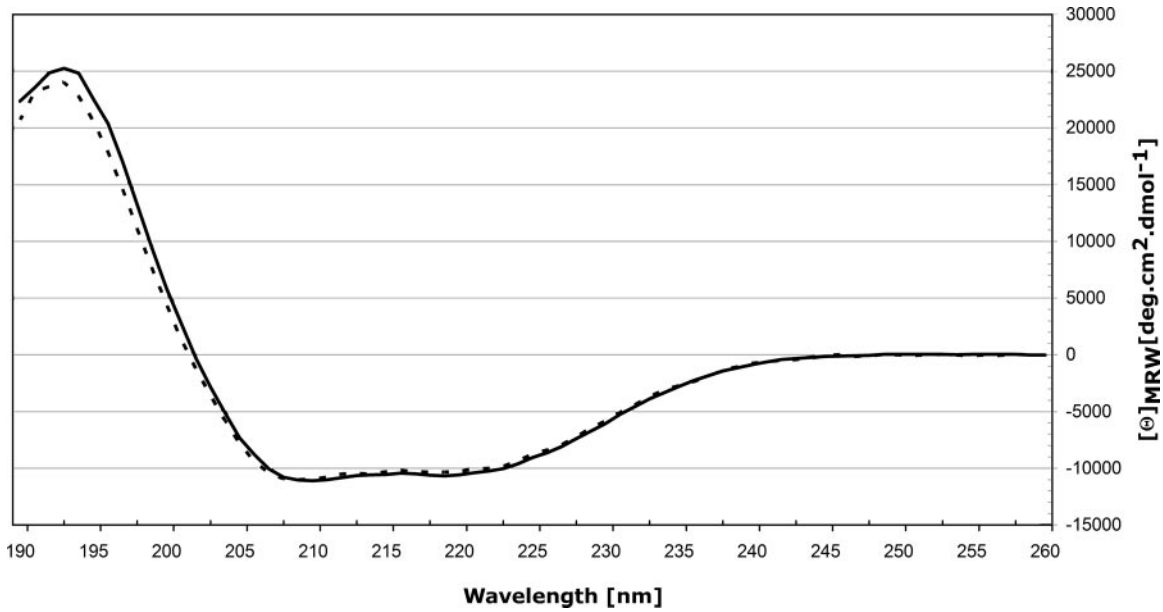

FIGURE 5. **Circular dichroism spectra of the natural and the recombinant non-fusion MtDH recorded at wavelengths between 190 and 260 nm in 10 mM sodium phosphate buffer (pH 7. 4) at 20 °C.** The CD spectrum of the nMtDH is shown with *dashed line*; the *continuous line* corresponds to rnfMtDH.

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 (www.expasy.org/) 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 T*G*XXX*G*X*G* motif, where *X* can be any amino acid as predicted by a pattern and profile search in the InterPro data base.

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.

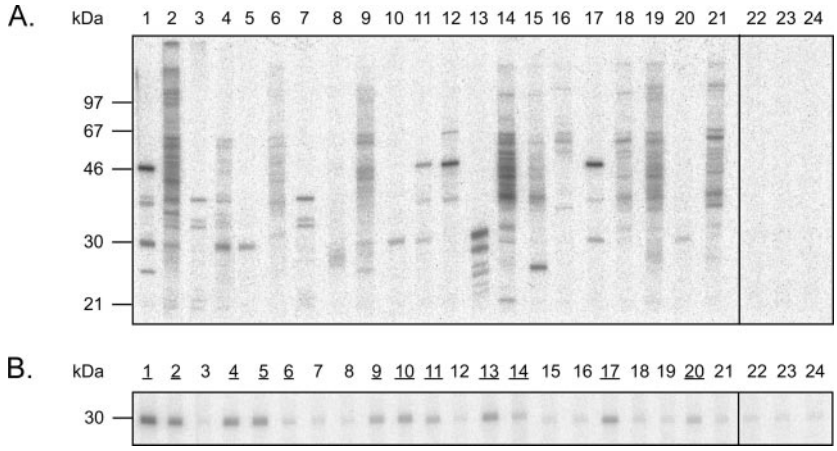

FIGURE 6. **IgE immunoblots of *C. herbarum* crude extract (*A*) and purified rnfMtDH (*B*).** 21 *C. herbarum* allergic patients (*lanes 1–21*) were investigated. Serving as controls were the serum of an atopic patient (*lane 22*), the serum of a non-atopic person (*lane 23*), and secondary ^{125}I-labeled rabbit anti-human IgE antibody alone (*lane 24*). Numbers for patients with a specific IgE reactivity with the MtDH are *underlined*.

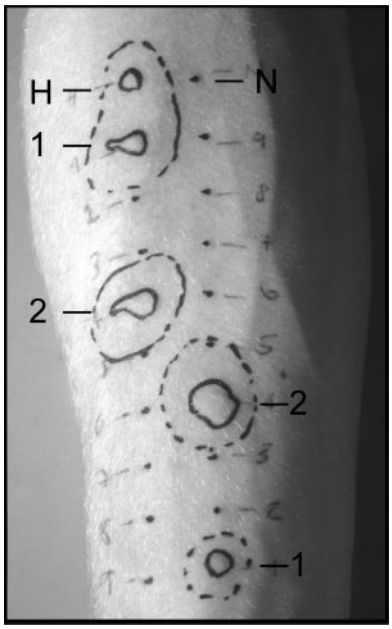

FIGURE 7. **Skin prick test of a *C. herbarum* allergic patient with commercial *C. herbarum* extract and rnfMtDH.** 0.9% saline (*N*), 0.01% histamine dihydrochloride (*H*), *C. herbarum* extract (*1*), and *C. herbarum* MtDH (*2*).

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 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).

Immunological Reactivity of C. herbarum MtDH—Determination of the prevalence of IgE reactivity using rnfMtDH revealed that 12 of 21 (57%) *C. herbarum* allergic patients specifically reacted with the protein (Fig. 6). The same result was obtained testing the His_6-MtDH from *C. herbarum* for its IgE reactivity. The allergen therefore represents a major allergen and was given the abbreviation "Cla h 8" by the World Health Organization-International Union of Immunological Societies allergen nomenclature subcommittee (http://www.allergen.org/).

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.

Genomic Sequence—Genomic DNA from *C. herbarum* was used as template DNA for PCR amplification with the same primers that were used for cloning the MtDH coding sequence. The sequence of the genomic clone was determined on both strands. The comparison of the genomic sequence with the cDNA sequence revealed the absence of introns.

DISCUSSION

Summarizing the immunological and allergologic data, we have shown that NADP-dependent MtDH of *C. herbarum* is indeed a major allergen, because ~57% of the *C. herbarum* allergic patients defined 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 <22% of patients. In two-dimensional 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, because we could demonstrate that the new major *C. herbarum* allergen not only binds 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 defense 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 defense 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 ~1.2 M) of NADP-dependent MtDH for its substrate, D-fructose, as found in the present report was also seen by others (28). However, when the reaction was measured in reverse direction, the K_m for mannitol was found to be ~5 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, whereas 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 favored. Fungi accumulate mannitol to up to 100 mM in the cytoplasm and secrete it during the defense reaction (29), but they do not secrete MtDH, which is a cytoplasmic enzyme.[3]

The MtDH allergen described here belongs to the class of short chain mannitol dehydrogenases that 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 that 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 that occur in relatively large amounts and this might be the one of the reasons *C. herbarum* MtDH is the major allergen of this fungal species.

Finally, we 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 incidences of atopic disease and asthma (for instance, Portland, OR in the United States), it was shown that patients with severe asthma are in up to 30% of the cases sensitized to molds (3), in particular to *C. herbarum* and *A. alternata*. In the total allergic population, sensitization to molds may vary from 5 to ~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 also the major risk factor for developing asthma in countries with a desert climate, such as Saudi Arabia and Kuwait (32, 33). The case histories of the patients tested showed no correlation between RAST classes, pattern of IgE reactivity, and the patients' symptoms. The symptoms observed were asthma, bronchitis, rhinoconjunctivitis, and atopic dermatitis. Epidemiologic data on the prevalence of *C. herbarum* allergy in the area of Salzburg were acquired through 5310 patients who attended the Salzburg outpatient allergy clinic and were tested for type I allergy in 2003 and 2004. Of these patients, 115 (2.1%) tested positive in the *C. herbarum* RAST. 42 of them (24 female, 18 male) displayed a RAST class ≥3. The only consistent pattern that we observed was that practically all the patients were multiallergics not only sensitized to *C. herbarum* but also to pollen, foodstuffs, and house dust mite. The symptoms were also 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 (indoor/outdoor, farm environment). Summarizing, the correlation between sensitization to molds and severe asthma that was found in other countries (2, 3, 32, 33) was not found among the relatively small sample of Austrian allergic patients, which might have to do with a multiplicity of factors that could differ between countries (lifestyle, genetics, climate, etc.). Because 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 of and therapy for *C. herbarum* allergy.

[3] M. Breitenbach, B. Simon-Nobbe, U. Denk, and P. B. Schneider, unpublished observations.

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