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Nucleic acid sequence and protein in addition to polypeptides coding for mannitol-dehydrogenases or parts thereof and the production and use thereof in diagnosis and therapy

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Zur Erklärung der Zweibuchstaben-Codes und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

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(54) Title: NUCLEIC ACID SEQUENCE AND PROTEIN IN ADDITION TO POLYPEPTIDES CODING FOR MANNITOL-DEHYDROGENASES OR PARTS THEREOF AND THE PRODUCTION AND USE THEREOF IN DIAGNOSIS AND THERAPY

(54) Bezeichnung: NUCLEINSÄURESEQUENZ UND PROTEIN SOWIE POLYPEPTIDE KODIEREND FÜR MANNIT-DEHYDROGENASEN ODER DEREN TEILE SOWIE DEREN HERSTELLUNG UND VERWENDUNG IN DIAGNOSTIK UND THERAPIE

(57) Abstract: The invention relates to polypeptides made of mannitol-dehydrogenase of Cladosporium herbarum and Alternaria alternata nucleic acids coding therefor and the use thereof in diagnosis and therapy.

(57) Zusammenfassung: Offenbart werden Polypeptide aus der Mannit-Dehydrogenase von Cladosporium herbarum und Alternaria alternata, hierfür kodierende Nukleinsäuren und deren Verwendung in Diagnostik und Therapie.

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In certain people, allergic reactions are caused by a wide range of substances. Not only allergies to components of animals, such as, for example, cat hairs, but also allergies to plants and plant parts, such as the pollen of flowers, are known. However, allergies to microorganisms such as, for example, molds, are also known.

The present invention relates to the major allergen of the mold Cladosporium herbarum. It has been found within the scope of the present invention that, surprisingly, this major allergen is a mannitol dehydrogenase (MtDH). The present invention furthermore relates to a major allergen of Alternaria alternata.

Accordingly, a first aspect of the present invention provides an isolated polypeptide comprising the amino acid sequence of SEQ ID NO:4, 5 or 6 or comprising at least 100 consecutive amino acids from the amino acid sequence of SEQ ID NO:1 or 8, wherein the polypeptide is an allergen.

In a second aspect, the present invention provides an isolated polypeptide comprising at least 100 consecutive amino acids from the amino acid sequence of SEQ ID NO:11 wherein the polypeptide is an allergen.

In a third aspect, the present invention provides a vaccine for desensitizing patients to a mould allergy, comprising at least one allergen as defined in the first or second aspect.

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In a fourth aspect, the present invention provides the use of an allergen as defined in the first or second aspect for the preparation of a vaccine.

In a fifth aspect, the present invention provides the use of an allergen as defined in the first or second aspect for the diagnostic detection of a disease.

In a sixth aspect, the present invention provides use of a polynucleotide for detecting a mannitol dehydrogenase, wherein the polynucleotide comprises at least eight consecutive nucleotides from the nucleotide sequence of SEQ ID NO:2, 7 or 12, wherein the nucleotide sequences of SEQ ID NOS:2, 7 and 12 encode mannitol dehydrogenases.

In a seventh aspect, the present invention provides a vector for transforming a host cell, wherein the vector comprises a polynucleotide comprising at least eight consecutive nucleotides from the nucleotide sequence of SEQ ID NO:2, 7 or 12, wherein the nucleotide sequences of SEQ ID NOs:2, 7 and 12 encode mannitol dehydrogenase.

In an eighth aspect, the present invention provides a host cell transformed with a vector as defined in the seventh aspect.

In a ninth aspect, the present invention provides a method for the preparation of an allergen as defined in the first or second aspect, wherein a host cell as defined in the eighth aspect is cultured, the polypeptide expressed and purified.

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The present invention relates to a polypeptide which has at least 10 consecutive amino acids from the amino acid sequence with the sequence ID No. 1. The amino acid sequence is shown in Fig 1, as is the corresponding DNA sequence. The invention also relates to the polypeptides with the seq ID No. 4, 5 and 6.

The complete sequence of a polypeptide encoding a mannitol dehydrogenase is furthermore disclosed. Seq. ID No. 7 represents the nucleic acid sequence and seq. ID no. 8 the amino acid sequence thereof. The sequences are shown in Figure 3.

The present invention relates to a

polypeptide which has at least 10 consecutive amino acids from the amino acid sequence with the sequence ID No. 11. The amino acid sequence is shown in Fig. 4; it constitutes a major allergen which has been isolated from Alternaria alternata.

The amino acid sequence of the major allergen from Alternaria alternata, which is mannitol dehydrogenase, is shown in Fig. 5 in the one-letter code, together with the DNA sequence encoding it (sequence ID No. 12) and the flanking nucleotide sequences at the 5′ and the 3′ end.

A polypeptide according to the invention preferably has at least one epitope. An epitope is understood as meaning a region to which antibodies can bind. In principle, there are linear epitopes. In this case, the amino acids which form the epitope are arranged next to one another. However, what are known as the confirmation epitopes are more frequent. These confirmation epitopes are formed by the folding of the polypeptide. Here, amino acids which are not adjacent in the sequence can come into spatial vicinity owing to the three-dimensional folding of the polypeptide, and this surface structure is bound by an antibody.

Preferably, the epitopes are specific for one mold. In principle, antibodies against virtually all amino acid sequences can be generated with the aid of suitable techniques, for example using adjuvants. The polypeptides according to the invention, however, play an important role in diagnostics and therapy. This is why the polypeptides according to the invention preferably have specific epitopes, the epitopes being specific for one mold. Frequently, proteins or polypeptides which originate from a certain organism have similarities to a corresponding protein or polypeptide which originates from a related organism. It is therefore possible that antibodies which are

directed against an epitope of a certain mold also react with a corresponding epitope of a related mold.

The more specific an epitope, the less antibodies which are directed against it will react with an epitope of a homologous polypeptide from a related organism. Thus, the polypeptides according to the invention preferably have those epitopes which are specific for a mold. The polypeptides especially preferably have those epitopes which are specific for a mold of the genus Cladosporium. The polypeptides very especially preferably have an epitope which is specific for Cladosporium herbarum. Antibodies which are directed against such an epitope do not react with other polypeptides.

It has been found within the scope of the present invention that the polypeptide with the amino acid sequence ID No. 1 encodes a mannitol dehydrogenase. The present invention also relates to parts of this amino acid sequence with at least 11 amino acids. The polypeptides according to the invention preferably have at least 20 consecutive amino acids from the sequence with the sequence ID No. 1. More preferred are those polypeptides which have at least 50 consecutive amino acids, and very especially preferred are those polypeptides which have at least 100 consecutive amino acids from the amino acid sequence with the sequence ID No. 1.

The invention also relates to a polypeptide from the N-terminus with the sequence PGQQATKHESLLDQLS (seq. ID No. 4) and to two polypeptides from the C-terminal end with the sequence LDTGLSDFVVK (seq. ID No. 5) and MGRDGLAKEL (seq. ID No. 6).

The invention also relates to a polypeptide with the sequence ID No. 8 and to parts of this polypeptide which comprise an epitope. The parts according to the

invention of the sequence ID No. 8 have at least 11, preferably at least 20, more preferably at least 50 and very especially preferably at least 100 consecutive amino acids from the amino acid sequence with the sequence ID No. 8.

The present invention furthermore relates to a polypeptide with the sequence ID No. 11 and to parts of this polypeptide which comprise an epitope. Such epitopes are specific for Alternaria, more precisely for Alternaria alternata. The parts according to the invention of the sequence ID No. 11 have at least 11, preferably at least 20, more preferably at least 50 and especially preferably at least 100 consecutive amino acids from the amino acid sequence with the sequence ID No. 11.

These polypeptides preferably have at least one epitope. For example, it is possible, with the aid of hydrophilicity/hydrophobicity examinations, to identify those parts of the polypeptide which are especially suitable for immunological reactions. This can be done for example with the aid of suitable computer programs.

As an alternative, it is also possible to prepare fragments of the sequence with the aid of what is known as the Pepscan method and to test the short fragments for relevant epitopes by reacting them with sera from allergic patients. Moreover, it must be identified whether the epitopes are epitopes which are specific for a mold, in particular for a mold from the genus Cladosporium and/or Alternaria and in particular for Cladosporium herbarum and/or Alternaria alternata. This determination is carried out using suitable serum panels.

Cladosporium is a fungal genus which belongs to the molds. Cladosporium species are very frequent and occur preferentially in bogs, in forests and in gardens since

they grow readily on rotten plants or on leaves. Moreover, they are found in greenhouses and in insufficiently cleaned refrigerators. Cladosporium also grows on textiles, for example linen fabrics. Cladosporium can trigger allergic reactions such as, for example, running nose, cough, sneezing, urticaria or asthma (mold allergy).

Alternaria is a fungal genus which belongs to the molds. Alternaria species occur preferentially in bogs, in forests and in gardens since they grow readily on rotten plants or on leaves. On domestic premises, they are mainly found in flour, fruit and vegetables. However, they also grow on a variety of textiles, for example linen fabrics. Alternaria can trigger allergic reactions such as, for example, running nose, cough, sneezing, urticaria or asthma (mold allergy).

Owing to the disclosure of the amino and nucleic acid sequence, it is possible, with the aid of recombinant techniques, to prepare shorter fragments of the complete sequence recombinantly in bacteria, for example in E. coli, or in higher organisms, for example insect cells, yeasts or eukaryotic cells. It is precisely short polypeptides that can also be provided readily via the chemical route with the aid of solid-phase synthesis.

The present invention furthermore relates to a vaccine which can be employed for desensitizing patients to a mold allergy. In the desensitization, patients who suffer from an allergy are brought into contact with a small amount of an antigen, whereby it is intended that neutralizing IgE antibodies are formed. The antigens with which the patient has come into contact are bound by these neutralizing antibodies. The antigen-antibody binding of antibodies of the IgE type, which trigger allergic reactions, are thereby avoided. The polypeptides according to the invention can therefore

be used for preparing a vaccine. To this end, the recombinantly produced, or else chemically produced, polypeptides can be incorporated into a suitable vaccine formulation. In addition to the polypeptides, the vaccine formulation can also comprise conventional additives and formulation auxiliaries, as well as adjuvants.

The present invention also relates to the use of a polypeptide according to the invention for a diagnostic detection of a disease. Such a disease usually takes the form of an allergy. The polypeptides are employed in a suitable diagnostic detection system. This may take the form of a radioimmunoassay (RIA), or preferably also an ELISA (enzyme-linked immunosorbent assay). The usual configuration of such a diagnostic assay is known.

Another aspect of the present invention relates to nucleotides from the nucleotide sequence with the sequence ID No. 2. The nucleotide sequence with the sequence ID No. 2 is likewise shown in Figure 1. It is part of the gene for the Cladosporium herbarum mannitol dehydrogenase according to the invention.

A further aspect of the present invention relates to polynucleotides with the nucleotide sequence of the sequence ID No. 7. Parts of this nucleotide sequence are likewise the subject-matter of the invention. With the aid of this nucleotide sequence or parts thereof, a desired polypeptide can be produced recombinantly in suitable host cells.

A further aspect of the present invention relates to polynucleotides with the nucleotide sequence, sequence ID No. 12. This is a nucleotide sequence which encodes the Alternaria alternata mannitol dehydrogenase and nucleotide sequences which are immediately adjacent to the coding region. Parts of this nucleotide sequence

are also the subject-matter of the present invention.

A polynucleotide according to the invention has at least eight consecutive nucleotides, preferably at least 12, more preferably at least 20 and most preferably at least 50 consecutive nucleotides. For some fields of application, the nucleotides must be longer, in which case the polynucleotides have at least 100 consecutive nucleotides selected from the sequence ID No. 2, ID No. 7 or sequence ID No. 12.

The polynucleotides according to the invention can be used for detecting a mannitol dehydrogenase. It is preferred to detect the presence of a gene encoding this mannitol dehydrogenase from Cladosporium herbarum and/or Alternaria alternata. These methods take the form of nucleic acid amplification methods which are known per se. A suitable example for this purpose is NASBA (nucleic acid sequence based amplification) or, more preferably, polymerase chain reaction (PCR).

Since the nucleic acid sequences encoding the Cladosporium herbarum and Alternaria alternata mannitol dehydrogenase have been disclosed, it is possible to select those nucleotide sequences for the amplification which have a very high degree of homology, or even identity. It can be expected that, when using such highly-specific primers, other mannitol dehydrogenases from related organisms are also amplified since a high degree of homology in the amino acid sequences suggests a high degree of conservation in such a gene region.

Thus, it is preferred to use such highly conserved regions for nucleic acid diagnostics in the case when the antigen is to be isolated not only from Cladosporium and/or Alternaria species, but also from other mold species.

Regions which have a low degree of homology with one

another are therefore better suited for fine diagnostics, that is to say for the distinction both between Alternaria and Cladosporium species and for the fine differentiation within Cladosporium or Alternaria species. Figure 6 shows the coding regions from Cladosporium herbarum and Alternaria alternata together. Identical nucleotide sequences are shown against a black background and identify conserved regions.

Such a method can be used for detecting the presence of the mold Cladosporium herbarum and/or Alternaria alternata. Such applications are of interest not only in medical diagnostics, but also in other fields, for example in the fields of hygiene and food testing. In this context, it must be taken into consideration that Cladosporium herbarum is capable of growth even at relatively low temperatures of up to approximately +6°C and that it can therefore constitute an undesired contamination in fields of food technology. The detection even of small amounts of Cladosporium herbarum may play an essential role in the control of foods and their quality control.

A further aspect of the present invention is the disclosure of a method for preparing a polypeptide according to the invention. First, a gene from a mold, preferably from Cladosporium herbarum or Alternaria alternata, can be amplified with the aid of the polynucleotides according to the invention and with the aid of the polymerase chain reaction. This polynucleotide can then be incorporated into a suitable vector with which a host cell is transformed. Suitable vectors multiply in the host cell, during which process the polypeptides are expressed. The host cells may take the form of conventional host cells. Suitable for this purpose are bacterial host cells such as Escherichia coli or Bacillus subtilis or yeasts such as, for example, Saccharomyces cerevisiae or Pichia pastoris.

For the purposes of the present invention, IgE immunoblots of Cladosporium herbarum crude extract were assayed with sera from 62 patients. An immunoreactive protein of molecular weight 29 kD, which was recognized by 61% of the patients' sera, was identified. The patients had been preselected in a skin test or blood test (RAST) and showed a positive response to Cladosporium herbarum extract. No other allergen in the Cladosporium herbarum extract reacted with such a high percentage of patients' sera. It is therefore assumed that this protein is the major allergen of Cladosporium herbarum.

The immunoreactive proteins disclosed within the scope of the present invention are important allergens, not only for diagnostic purposes, but also for the therapy of allergens to molds, in particular Cladosporium and Alternaria species. If appropriate, these allergens, together with other allergens, for example Alt a 1 [Unger A. et al. (1999), Clinical testing of recombinant allergens of the mold Alternaria alternata, Int. Arch. Allergy Immunol. 118, 220-221] and Enolase [Simon-Nobbe B. et al. (2000), IgE binding epitopes of enolases, a class of highly conserved fungal allergens, I. Allergy Clin. Immunol. 106, 887-895] can be employed both in diagnostics and for therapeutic purposes.

The two-dimensional separation by isoelectric focusing and SDS-PAGE showed this Cladosporium herbarum protein as a 29 kD spot and at isoelectric point at pH = 5.8.

The protein was purified to homogeneity in a conventional method (example 1). The yield amounted to 1 mg. The homogeneously purified protein was then assayed in the IgE immunoblot with a pool of six patients and was highly positive (example 2).

The protein which had been purified to homogeneity was

partially sequenced by Edmanic degradation, starting at the N terminus, and internal peptide sequences were determined after degradation with CNBr.

N-terminal and internal peptide sequences were determined after digestion with trypsin by subjecting approximately 50 µg of protein, which had been obtained by excising a spot from the two-dimensional electrophoresis, to Edman degradation.

Table 1 shows a list of all peptide sequences which were identified. The single-letter code was used. The amino acids which are shown against the black background in table 1 were found in the sequence of the Cladosporium fulvum mannitol dehydrogenase.

Peptide sequences of the C. herbarum NADP-dependent mannitol dehydrogenase, N-terminal sequence

PGQQATKHESLLDQXSXK: a) Starting material: crude extract separated by means of 2-dimensional SDS gel
b) Analytical method: Edman sequencing

PGQQATKHESLLDQLSLKGK: a) Starting material: native purified protein
b) Analytical method: Edman sequencing

Peptide 1

HESLLDQLSLK: a) Starting material: crude extract separated by means of 2-dimensional SDS gel
b) Analytical method: MS/MS
c) Note: overlaps with the N-terminal sequence

Peptide 2

WVVVTGASGP: a) Starting material: crude extract separated by means of 2-dimensional SDS gel
b) Tryptic digest
c) Analytical method: sequencing

WVVVVTGASKR: a) Starting material: crude extract separated by means of 2-dimensional SDS gel
b) Tryptic digest
c) Analytical method: MS/MS

Peptide 3

QVDSYE: a) Starting material: crude extract separated by means of 2-dimensional SDS gel
b) Tryptic digest

c) Analytical method: sequencing

Peptide 4

LDTGLSDFVVK: a) Starting material: crude extract separated by means of 2-dimensional SDS gel
b) Tryptic digest
c) Analytical method: MS/MS

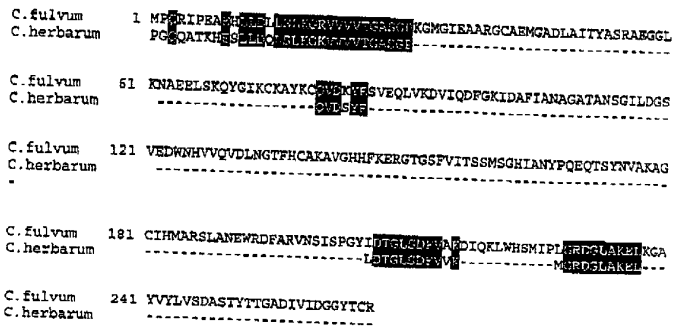
Peptide 5

MGRDGLAKEL: a) Starting material: native purified protein
b) CnBr digest
c) Analytical method: sequencing

Table 1

The peptide sequences were compared with all the protein sequences listed in the databases (Swissprot, GenBank and the like) by computer-aided homology search. All peptides showed homology with the family of the mannitol dehydrogenases. The mannitol dehydrogenase with which the peptides show the highest degree of sequence similarity is the Cladosporium fulvum mannitol dehydrogenase. A purification of Cladosporium fulvum mannitol dehydrogenase is described in Noeldner et al., Physiological and Molecular Plant Pathology (1994) pp. 281-289. The position of these peptides in the sequence can be seen in the alignment (Table 2).

Protein alignment of the C. fulvum NADP MtDH and C. herbarum peptide sequences



C.fulvum MtDH: accession number: AAK67169 (seq. ID No. 3)
Length of the coding sequence: 267 amino acids (AA)
801 base pairs (bp)
MW: 28.6 kD
pI: 6.33

Table 2

Table 2 shows the arrangement of the polypeptides with seq. ID No. 4, 5 and 6 with reference to the homology with C. fulvum.

Owing to these results, the enzyme activity of the protein which had been purified to homogeneity was assayed. The experiments reveal that the highly purified major allergen of Cladosporium herbarum is indeed a mannitol dehydrogenase which catalyzes the following metabolic reaction: Fructose + NADPH + H^+ ⇔ mannitol + $NADP^+$. Furthermore, it has been found that NADH is not active as cosubstrate and that fructose-6-phosphate is also not active as substrate. Fructose-6-phosphate has an inhibitory effect on the reaction. The method of the activity determination is described in example 3.

Then, the N-terminal peptide sequence and an internal peptide sequence of the Cladosporium herbarum mannitol dehydrogenase were used for designing PCR primers by means of back translation. The primer selection is compiled in table 3.

DNA sequence of the oligos derived from the peptides

Oligo 1:

- derived from the N-terminal sequence of the C. herbarum mannitol dehydrogenase (MtDH) (see seq. ID No. 2)
- oligo sequence: 5' CA(A/G) CA(A/G) GC(I/C) AC(I/C) AA(A/G) CA(C/T) GA 3'

Oligo 2:

- derived from peptide 4 of the C. herbarum MtDH
- oligo sequence: 5' AC(A/G) AA(A/G) TC(A/G) CT(I/C) AG(I/C) CC(A/G) GT(A/G) TC 3'

The primers are mixtures of synthetic oligonucleotides. (A/G)... means that both adenine and guanine are found in the oligonucleotides at this position. The same applies to (C/T) and (I/C), where I represents the base inosine.

Table 3

These primers which are shown in table 3 were used to carry out a PCR reaction with the DNA from a Cladosporium herbarum cDNA library constructed by the inventors (Achatz G et al., 1995, Mol. Immunol., 32; 213-27). The result was a 636 bp band. This band was sequenced by automated DNA sequencing as described by Sanger (1977, Proc. Natl. Acad. Sci. USA, 74; 5463-7) using the PCR primers as sequencing primers. Seq. ID No. 2 was identified in this process. The protein sequence (seq. ID No. 1) derived from this DNA sequence has 87% identity with the protein sequence of the

Cladosporium fulvum mannitol dehydrogenase. If the substitution by chemically related amino acids (for example I - V, isoleucine - valine and the like) is also taken into consideration, this value rises to 92%. With the plausible assumption that the Cladosporium herbarum mannitol dehydrogenase, like the Cladosporium fulvum mannitol dehydrogenase, has a total length of 267 amino acid, as much as 65% of the amino acid sequence of the major allergen (mannitol dehydrogenase) from Cladosporium herbarum were determined by firstly peptide sequencing and secondly DNA sequencing. The total sequence of this protein which is known to date, and the alignment of this sequence with the homologous Cladosporium fulvum sequence, are shown in figure 2.

Example 1

Protein purification

1. Ammonium sulfate precipitation:

Prefractioning can be achieved by an ammonium sulfate concentration of 50%, with mannitol dehydrogenase (MtDH) remaining in the supernatant.

50 mM Tris-HCl, pH 7.5 were added to the extract. Proteases were inhibited with 1 tablet of Roche Complete per 100 ml of extract and 2 mM EDTA.

The precipitation was carried out by adding solid, ground ammonium sulfate and was carried out in two steps, first 0-30%, then 30-50%. The precipitation was equilibrated for at least 45 minutes before the extract was centrifuged at 12 000 g. The supernatant was filtered and purified further via hydrophobic interaction chromatography (HIC).

2. HIC (Phenyl-Sepharose):

The supernatant from the ammonium sulfate precipitation was brought to pH 6.5 using 3 M sodium acetate. The column (8 ml Source, 15 PHE, PHARMACIA) was equilibrated with 1.2 M ammonium sulfate, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA and loaded with the sample at a flow rate of 1 ml/min. After the column had been washed with 20 ml of buffer, it was eluted with a gradient of 1.2 M ammonium sulfate to 0.6 M ammonium sulfate over 40 ml.

The mannitol dehydrogenase (MtDH) fractions were pooled and prepared for the anion exchanger. The volume is concentrated via Centricon centrifuge tubes (Millipore); buffer exchange flow 50 mM Tris-HCl, pH 7.5 with the aid of PD-10 Desalting Columns (AMERSHAM-PHARMACIA).

3. Anionic exchanger (Q-Sepharose):

The column (8 ml Source 15 Q, PHARMACIA) was equilibrated with 15 mM Tris-HCl, pH 7.5. It was eluted with a 0-300 mM NaCl gradient over 100 ml.

Example 2

Immune blot of the native purified MtDH after separation in the SDS gel

Native purified MtDH was separated by molecular weight in a reducing SDS gel (Laemmli UK, Nature, 1970; 27:680-5). Subsequently, the protein was transferred onto a PVDF membrane in a Western blot (Towbin H et al., Proc. Natl. Acad. Sci USA, 1979; 76:4350-4). After free binding sites had been saturated (30 minutes in blocking buffer: 50 mM sodium phosphate pH 7.5, 0.5% Tween 20, 0.5% BSA, 0.05% NaN_3), the membrane was incubated with patients' serum (1:10 diluted in blocking buffer). Then, the membrane was washed (with blocking buffer, 3 × 10 minutes) to remove

unspecifically bound antibodies. Specifically bound IgE-Ab were detected with the aid of an ^{125}I-labeled rabbit anti-human IgE antibody. After the membrane had been exposed to an X-ray film, the result was available.

Results:

1) The native purified MtDH reacts specifically with the IgE antibodies of C. herbarum allergy sufferers. A prominent IgE-reactive band is revealed at 29 kD.

2) The same result, viz. a prominent IgE-reactive band at 29 kD, is obtained when a C. herbarum total extract is separated in the SDS gel and subsequently incubated with patients' serum in an immune blot.

Example 3

Immune blot of the native purified MtDH after 2-dimensional separation

Native purified MtDH was separated under denaturing conditions in an isoelectric focusing (O'Farrel PH, J. Biol. Chem., 1975; 250:4007-21) according to the net charge (isoelectric point) of the protein. Thereafter, the protein separated thus was subjected to SDS gel electrophoresis (Laemmli UK, Nature, 1970; 27:680-5), whereby a separation by molecular weight took place in addition. The protein was transferred to a PVDF membrane in a Western blot (Towbin H et al., Proc. Natl. Acad. Sci. USA, 1979; 76:4350-4). After free binding sites had been saturated (30 minutes in blocking buffer: 50 mM sodium phosphate pH 7.5, 0.5% Tween 20, 0.5% BSA, 0.05% NaN_3), the membrane was incubated with patients' serum (1:10 diluted in blocking buffer). Then, the membrane was washed (with blocking buffer, 3 × 10 minutes) to remove unspecifically bound antibodies. Specifically bound

IgE-Ab were detected with the aid of an ^{125}I-labeled rabbit anti-human IgE antibody. After the membrane had been exposed to an X-ray film, the results were available:

Results:

1) The native purified MtDH reacts specifically with the IgE antibodies of C. herbarum allergy sufferers. A prominent IgE-reactive spot was observed at a molecular weight of 29 kD and an isoelectric point of 5.8.

2) The same result, viz. a prominent IgE-reactive spot at a molecular weight of 29 kD and an isoelectric point of 5.8 is obtained when a C. herbarum total extract is separated in a two-dimensional gel and subsequently incubated with patients' serum in an immune blot. An IgE-reactive protein with a molecular weight of 29 kD and an isoelectric point of 5.6 is additionally found in the total extract. This protein could be an MtDH isoform.

Example 4

To confirm the results according to the invention, the enzyme activity was determined with the traditionally purified protein. The absorption of NADPH was measured in a photometer at 340 nm.

Reaction mix (1 ml):

50 mM Tris-HCl, pH 7.5
0.25 mM NADPH or NADH
D-fructose or fructose-6-phosphate (0.1; 0.2; 0.4; 0.6; 0.8; 1.0; 1.2 M)
H_2O to 1 ml
the reaction is started with 0.5 µl of MtDH

Results:

Reaction with fructose and NADPH
No reaction with fructose-6-phosphate and NADH

Example 5

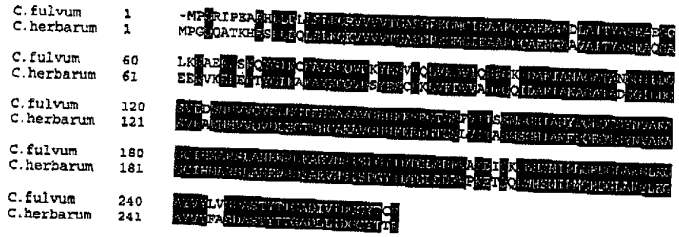
Sequence of mannitol dehydrogenase (MtDH)

The complete sequence of the Cladosporium herbarum mannitol dehydrogenase was determined as described hereinbelow.

The peptide sequences obtained by Edman degradation of the Cladosporium mannitol dehydrogenase which had been purified to homogeneity were used to synthesize primer mixtures for the PCR. The PCR resulted in a band of 636 nt which was firstly sequenced and secondly used as hybridization probe for screening our cDNA library. A complete Cladosporium herbarum mannitol dehydrogenase (MtDH) clone was isolated and sequenced. The complete sequence is shown in fig. 3; it has 84% identity with the published sequence of the C. fulvum MtDH.

Table 4 shows the sequence alignment of the two mannitol dehydrogenases of Cladosporium herbarum and Cladosporium fulvum, only the amino acids which differ being shown.

Table 4: Alignment of the amino acid sequences of the C. herbarum and C. fulvum MtDHs:



Amino acid sequences which are shown against the black background mean identical amino acid sequences, chemically similar amino acids are shown against a grey background, and amino acids which differ are shown against the normal background.

The C. fulvum sequence is represented as seq. ID No: 9, and the C. herbarum amino acid sequence as SEQ. ID No: 10.

Table 4 shows the regions of the polypeptide which may be suitable for the detection of or a vaccine for Cladosporium. They are the regions with no differences.

In the regions with pronounced differences it must be presumed that the immunological reactions differ; such regions can therefore comprise highly specific epitopes.

When determining the sequence shown in fig. 3, it was found that minor differences occurred in the nucleotide sequence in comparison with the originally isolated part-sequences. This can be attributed to differing sequences which were present in the gene library. However, these differences do not affect the present invention in any way. The invention relates to the disclosed differing sequences, since it is assumed that they are variants of the gene.

Example 6

Expression in E. coli, and reactivity with patients' serum

The open reading frame of MtDH was cloned into the following expression vectors:

a) pHis-Parallel 2 Vector (XhoI/BamHI) (Ref.: P. Sheffield, S. Garrard, and Z. Derewenda (1999). Overcoming expression and purification problems of

RhoGDI using a family of "parallel" expression vectors. Protein Expr Purif 15,34.)

b) pMW172 Vector (NdeI/EcoRI) (Ref.: M. Susani, P. Jertschin, C. Dolecek, W. R. Sperr, P. Valent, C. Ebner, D. Kraft, R. Valenta, and O. Scheiner (1995). High level expression of birch pollen profiling (Bet v 2) in Escherichia coli: purification and characterization of the recombinant allergen. Biochem Biophys Res Commun 215, 250.)

The plasmids were subsequently transformed into *Escherichia coli* strain BL21 (DE3). For the subsequent induction, 5-ml-portions of LBamp were inoculated with 50 µl of a stationary overnight culture of the two clones and the mixtures were shaken at 37°C until a OD_{600} of 0.8 had been reached (approx. 4 hours). The protein expression was induced with 0.8 mM IPTG. After incubation for 4 hours at 37°C in a shaker-incubator, the *E. coli* suspensions were spun down for 15 minutes at 4000 rpm. The bacterial pellets were subsequently resuspended in 1 ml of 1xPBS, and 6-µl-portions of the dissolved bacterial pellets were separated by SDS-PAGE and subsequently stained with Coomassie BBR250. This gave the following results: the E. coli cells which had been transformed with the expression plasmids and induced with IPTG, but not the E. coli cells without plasmid, reveal a pronounced protein band at the molecular weight expected in each case, viz. 30 kD and approximately 33 kD, respectively (apparent molecular weight).

An IgE immune blot was carried out with the polypeptides which had been separated with the aid of a gel. The serum of a Cladosporium-positive allergy sufferer was used. The bound IgE antibodies were detected with the ^{125}-I labeled rabbit anti-human IgE antibody (RAST). The two foreign proteins which were overexpressed in E. coli react strongly with the IgE of

the patient, but not the proteins of E. coli itself.

Example 7

Determination of the frequency of the response to recombinant MtDH with the aid of 30 sera of Cladosporium-positive allergy sufferers

The experiment described in example 6 was repeated, but 30 different Cladosporium-positive allergy-sufferer sera which had not been preselected were used. The control revealed a very low immune reactivity of the E. coli extract with the second antibody (RAST). This can probably be attributed to an artifact. As expected, other controls were negative.

Among 30 patients, 20 revealed an IgE-positive band at 30 kD which was more pronounced than the weak band in the control experiment. MtDH is thus recognized by approximately two thirds of the Cladosporium-positive allergy sufferers. This finding is important because this experiment demonstrates that recombinant Cladosporium herbarum MtDH can be employed as diagnostic and therapeutic for the majority of the patients.

Example 8

To clone the Alternaria alternata mannitol dehydrogenase, a cDNA bank in Lambda-ZAP (Stratagene, La Jolla, CA, USA). This cDNA cloned library was prepared with the aid of isolated mRNA from Alternaria alternata.

As described above, the cDNA library was screened with a DNA probe, with initially 24 primary clones being isolated. 5 of these clones were sequenced completely. All 5 sequences were identical in the coding region. The translation of the nucleotide sequence into an

amino acid sequence and the comparison with the amino acid sequence of the Cladosporium herbarum mannitol dehydrogenase revealed that the reading frame was complete. The sequence had 74% identity with the sequence from Cladosporium herbarum.

Example 9

The open reading frame of the clone encoding the Alternaria alternata mannitol dehydrogenase was then recloned in the expression vector pHIS-parallel 2 [P. Sheffield et al. (1999), Overcoming expression and purification problems of RhoGDI using a family of "parallel" expression vectors, Protein Exp. Purif. 15, 34] using the restriction cleavage sites Bam H I (N-terminally) and Xho I (C-terminally). Upon expression in E. coli BL21 and subsequent analysis of the gene products with the aid of SDS-PAGE gel electrophoresis and Coomassie Blue staining, a pronounced protein band appeared at a molecular weight of approximately 30 kD. This corresponds approximately to the molecular weight which would be expected theoretically.

Example 10

The following procedure was chosen for purifying the recombinantly produced protein, which is provided with a poly-His fragment at the C terminus:
the E. coli cells with the expression vector, which express the foreign protein, the Alternaria alternata mannitol dehydrogenase, were first lysed in the customary manner. It was found that the recombinantly produced protein was present in insoluble form. The inclusion bodies formed by overexpression of the foreign protein were first solubilized in a buffer with 6-molar urea and subsequently purified by affinity chromatography over a nickel chelate column. The recombinantly produced mannitol dehydrogenase was applied in 6 M urea buffer. Imidazole buffer was

employed for the elution. The protein-comprising fractions were subsequently purified further by preparative SDS-PAGE gel electrophoresis and analyzed, during which process it emerged that the allergen was already purified to virtually complete homogeneity. Staining with Coomassie-BB-R only revealed on band with a molecular weight of approximately 30 kD.

Example 11

The protein prepared in accordance with example 10 was separated by gel electrophoresis and tested in an IgE immune blot with the sera of 28 patients. Al of the 28 sera originated from patients who had shown a positive response to the Alternaria alternate crude extract and who had been pretested both in a skin test and in an RAST. A pronounced band was visible in the immune blot in the case of 9 of the patients' sera tested. This corresponds to approximately 32% of the Alternaria alternate-sensitized patients.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

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The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

The claims defining the invention are as follows:

1. An isolated polypeptide comprising the amino acid sequence of SEQ ID NO:4, 5 or 6 or comprising at least 100 consecutive amino acids from the amino acid sequence of SEQ ID NO:1 or 8, wherein the polypeptide is an allergen.

2. Allergen as claimed in claim 1, comprising at least one epitope.

3. Allergen as claimed in claim 2, wherein the epitope is specific for a mould belonging to the genus Cladosporium.

4. Allergen as claimed in claim 3, wherein the mould is Cladosporium herbarum.

5. Allergen as claimed in any one of claims 1 to 4, wherein the amino acid sequence of SEQ ID NO: 1 or 8 encodes a mannitol dehydrogenase.

6. An isolated polypeptide comprising at least 100 consecutive amino acids from the amino acid sequence of SEQ ID NO:11 wherein the polypeptide is an allergen.

7. Allergen as claimed in claim 6, comprising at least one epitope.

8. Allergen as claimed in claim 6, wherein the epitope is specific for a mould belonging to the genus Alternaria.

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9. Allergen as claimed in claim 8, wherein the mould is Alternaria alternata.

10. Allergen as claimed in any one of claims 6 to 9, wherein the amino acid sequence of SEQ ID NO:11 encodes a mannitol dehydrogenase.

11. A vaccine for desensitizing patients to a mould allergy, comprising at least one allergen as claimed in any one of claims 1 to 10.

12. The use of an allergen as claimed in any one of claims 1 to 10 for the preparation of a vaccine.

13. The use of an allergen as claimed in any one of claims 1 to 10 for the diagnostic detection of a disease.

14. The use as claimed in claim 12 or 13, wherein the disease is an allergy against moulds.

15. Use of a polynucleotide for detecting a mannitol dehydrogenase, wherein the polynucleotide comprises at least eight consecutive nucleotides from the nucleotide sequence of SEQ ID NO:2, 7 or 12, wherein the nucleotide sequences of SEQ ID NOS:2, 7 and 12 encode mannitol dehydrogenases.

16. The use as claimed in claim 15, wherein at least one polynucleotide is used in a Polymerase Chain Reaction (PCR).

17. A vector for transforming a host cell, wherein the vector comprises a polynucleotide comprising at least eight consecutive nucleotides from the nucleotide sequence of SEQ ID NO:2, 7 or 12, wherein the nucleotide sequences of SEQ ID NOS:2, 7 and 12 encode mannitol dehydrogenase.

18. A host cell transformed with a vector as claimed in claim 17.

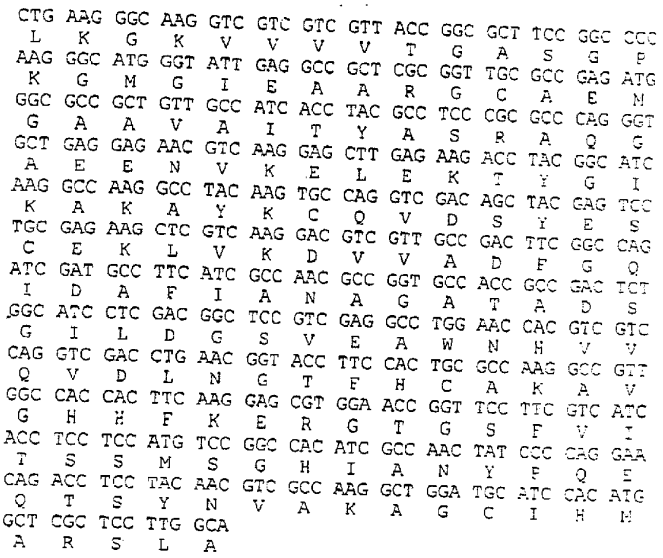
19. The host cell as claimed in claim 18, wherein the cell is Escherichia coli.

20. The host cell as claimed in claim 18, wherein the cell is Pichia pastoris.

21. A method for the preparation of an allergen as claimed in any one of claims 1 to 10, wherein a host cell as claimed in any one of claims 18 to 20 is cultured, the polypeptide expressed and purified.

22. An allergen as claimed in any one of claims 1 to 10, or a polynucleotide encoding the same, substantially as herein described with reference to the Examples.

23. Use of an allergen as claimed in any one of claims 1 to 10, or a polynucleotide encoding the same, substantially as herein described with reference to the Examples.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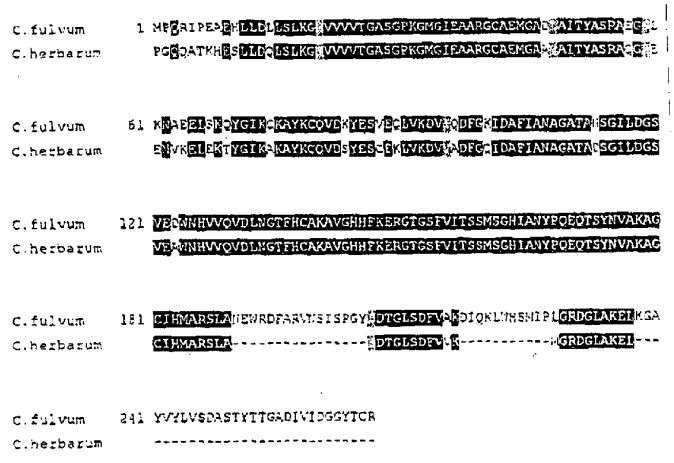
K G M G I E A A R G C A E M
GGC GCC GCT GTT GCC ATC ACC TAC GCC TCC CGC GCC CAG GGT
G A A V A I T Y A S R A Q G
GCT GAG GAG AAC GTC AAG GAG CTT GAG AAG ACC TAC GGC ATC
A E E N V K E L E K T Y G I
AAG GCC AAG GCC TAC AAG TGC CAG GTC GAC AGC TAC GAG TCC
K A K A Y K C Q V D S Y E S
TGC GAG AAG CTC GTC AAG GAC GTC GTT GCC GAC TTC GGC CAG
C E K L V K D V V A D F G Q
ATC GAT GCC TTC ATC GCC AAC GCC GGT GCC ACC GCC GAC TCT
I D A F I A N A G A T A D S
GGC ATC CTC GAC GGC TCC GTC GAG GCC TGG AAC CAC GTC GTC
G I L D G S V E A W N H V V
CAG GTC GAC CTG AAC GGT ACC TTC CAC TGC GCC AAG GCC GTT
Q V D L N G T F H C A K A V
GGC CAC CAC TTC AAG GAG CGT GGA ACC GGT TCC TTC GTC ATC
G H H F K E R G T G S F V I
ACC TCC TCC ATG TCC GGC CAC ATC GCC AAC TAT CCC CAG GAA
T S S M S G H I A N Y P Q E
CAG ACC TCC TAC AAC GTC GCC AAG GCT GGA TGC ATC CAC ATG
Q T S Y N V A K A G C I H M
GCT CGC TCC TTG GCA
A R S L A

Figure 1

Figure 2

Alignment of the C. fulvum and C. herbarum NADP-MtDHs on the basis of the available DNA and protein data



Alignment of the derived amino acid sequences of the Cladosporium herbarum and Cladosporium fulvum mannitol dehydrogenases.

- … means that the amino acid sequence of C. herbarum still requires sequencing at this position.

Fig. 3: Complete nucleotide and protein sequence of the Cladosporium herbarum MtDH

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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
GCGACTCGCCCACAGCAAGTCGTTGAGGCGGAAGGACAAAAAAAAAAAAAAAAAAAAAAAAAA     918
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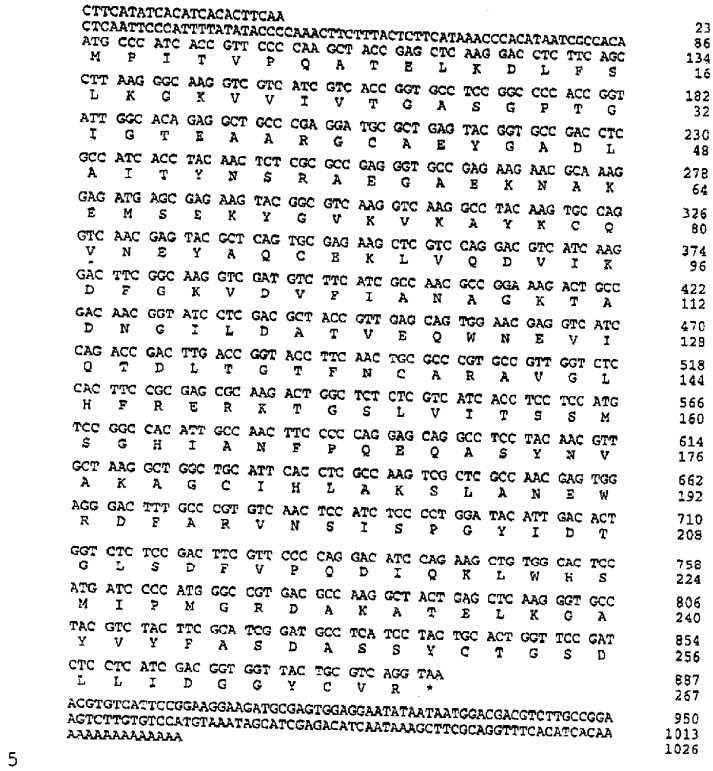
Met Pro Ile Thr Val Pro Gln Ala Thr Glu Leu Lys Asp Leu Phe Ser
 1               5                  10                  15
Leu Lys Gly Lys Val Val Ile Val Thr Gly Ala Ser Gly Pro Thr Gly
            20                  25                  30
Ile Gly Thr Glu Ala Ala Arg Gly Cys Ala Glu Tyr Gly Ala Asp Leu
        35                  40                  45
Ala Ile Thr Tyr Asn Ser Arg Ala Glu Gly Ala Glu Lys Asn Ala Lys
    50                  55                  60
Glu Met Ser Glu Lys Tyr Gly Val Lys Val Lys Ala Tyr Lys Cys Gln
65                  70                  75                  80
Val Asn Glu Tyr Ala Gln Cys Glu Lys Leu Val Gln Asp Val Ile Lys
                85                  90                  95
Asp Phe Gly Lys Val Asp Val Phe Ile Ala Asn Ala Gly Lys Thr Ala
            100                 105                 110
Asp Asn Gly Ile Leu Asp Ala Thr Val Glu Gln Trp Asn Glu Val Ile
        115                 120                 125
Gln Thr Asp Leu Thr Gly Thr Phe Asn Cys Ala Arg Ala Val Gly Leu
    130                 135                 140
His Phe Arg Glu Arg Lys Thr Gly Ser Leu Val Ile Thr Ser Ser Met
145                 150                 155                 160
Ser Gly His Ile Ala Asn Phe Pro Gln Glu Gln Ala Ser Tyr Asn Val
                165                 170                 175
Ala Lys Ala Gly Cys Ile His Leu Ala Lys Ser Leu Ala Asn Glu Trp
            180                 185                 190
Arg Asp Phe Ala Arg Val Asn Ser Ile Ser Pro Gly Tyr Ile Asp Thr
        195                 200                 205
Gly Leu Ser Asp Phe Val Pro Gln Asp Ile Gln Lys Leu Trp His Ser
    210                 215                 220
Met Ile Pro Met Gly Arg Asp Ala Lys Ala Thr Glu Leu Lys Gly Ala
225                 230                 235                 240
Tyr Val Tyr Phe Ala Ser Asp Ala Ser Ser Tyr Cys Thr Gly Ser Asp
                245                 250                 255
Leu Leu Ile Asp Gly Gly Tyr Cys Val Arg
            260                 265
```

Fig. 4: Alternaria alternata MtDH (Seq. ID No. 11)

Alternaria alternata MtDH



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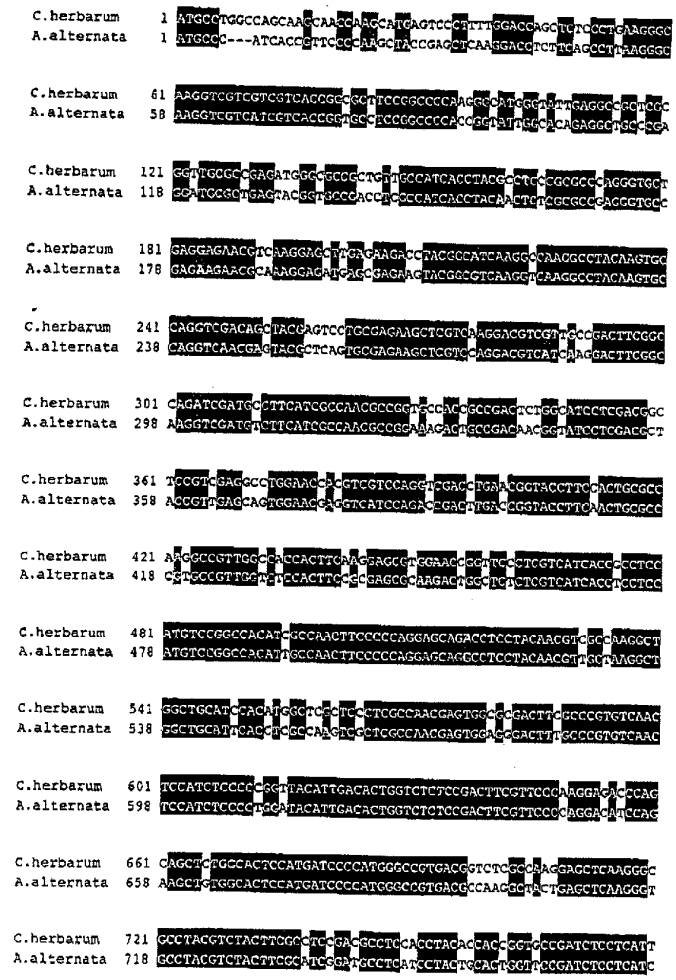
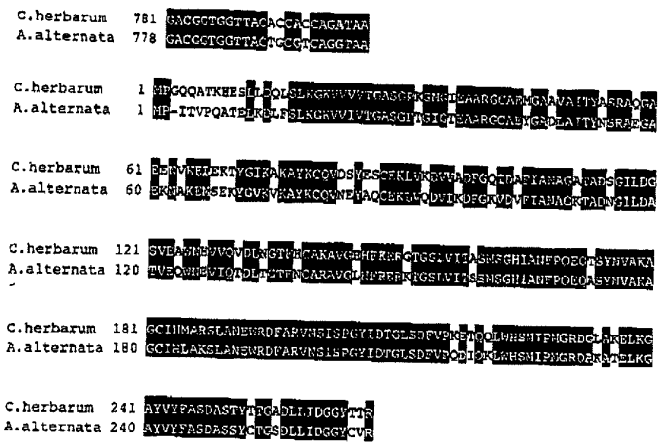
CTCAATTCCCATTTTATATACCCCAAACTTCTTTACTCTTCATAAACCCACATAATCGCCACA            86
ATG CCC ATC ACC GTT CCC CAA GCT ACC GAG CTC AAG GAC CTC TTC AGC           134
 M   P   I   T   V   P   Q   A   T   E   L   K   D   L   F   S            16
CTT AAG GGC AAG GTC GTC ATC GTC ACC GGT GCC TCC GGC CCC ACC GGT           182
 L   K   G   K   V   V   I   V   T   G   A   S   G   P   T   G            32
ATT GGC ACA GAG GCT GCC CGA GGA TGC GCT GAG TAC GGT GCC GAC CTC           230
 I   G   T   E   A   A   R   G   C   A   E   Y   G   A   D   L            48
GCC ATC ACC TAC AAC TCT CGC GCC GAG GGT GCC GAG AAG AAC GCA AAG           278
 A   I   T   Y   N   S   R   A   E   G   A   E   K   N   A   K            64
GAG ATG AGC GAG AAG TAC GGC GTC AAG GTC AAG GCC TAC AAG TGC CAG           326
 E   M   S   E   K   Y   G   V   K   V   K   A   Y   K   C   Q            80
GTC AAC GAG TAC GCT CAG TGC GAG AAG CTC GTC CAG GAC GTC ATC AAG           374
 V   N   E   Y   A   Q   C   E   K   L   V   Q   D   V   I   K            96
GAC TTC GGC AAG GTC GAT GTC TTC ATC GCC AAC GCC GGA AAG ACT GCC           422
 D   F   G   K   V   D   V   F   I   A   N   A   G   K   T   A           112
GAC AAC GGT ATC CTC GAC GCT ACC GTT GAG CAG TGG AAC GAG GTC ATC           470
 D   N   G   I   L   D   A   T   V   E   Q   W   N   E   V   I           128
CAG ACC GAC TTG ACC GGT ACC TTC AAC TGC GCC CGT GCC GTT GGT CTC           518
 Q   T   D   L   T   G   T   F   N   C   A   R   A   V   G   L           144
CAC TTC CGC GAG CGC AAG ACT GGC TCT CTC GTC ATC ACC TCC TCC ATG           566
 H   F   R   E   R   K   T   G   S   L   V   I   T   S   S   M           160
TCC GGC CAC ATT GCC AAC TTC CCC CAG GAG CAG GCC TCC TAC AAC GTT           614
 S   G   H   I   A   N   F   P   Q   E   Q   A   S   Y   N   V           176
GCT AAG GCT GGC TGC ATT CAC CTC GCC AAG TCG CTC GCC AAC GAG TGG           662
 A   K   A   G   C   I   H   L   A   K   S   L   A   N   E   W           192
AGG GAC TTT GCC CGT GTC AAC TCC ATC TCC CCT GGA TAC ATT GAC ACT           710
 R   D   F   A   R   V   N   S   I   S   P   G   Y   I   D   T           208

GGT CTC TCC GAC TTC GTT CCC CAG GAC ATC CAG AAG CTG TGG CAC TCC           758
 G   L   S   D   F   V   P   Q   D   I   Q   K   L   W   H   S           224
ATG ATC CCC ATG GGC CGT GAC GCC AAG GCT ACT GAG CTC AAG GGT GCC           806
 M   I   P   M   G   R   D   A   K   A   T   E   L   K   G   A           240
TAC GTC TAC TTC GCA TCG GAT GCC TCA TCC TAC TGC ACT GGT TCC GAT           854
 Y   V   Y   F   A   S   D   A   S   S   Y   C   T   G   S   D           256
CTC CTC ATC GAC GGT GGT TAC TGC GTC AGG TAA                               887
 L   L   I   D   G   G   Y   C   V   R   *                               267
ACGTGTCATTCCGGAAGGAAGATGCGAGTGGAGGAATATAATAATGGACGACGTCTTGCCGGA           950
AGTCTTGTGTCCATGTAAATAGCATCGAGACATCAATAAAGCTTCGCAGGTTTCACATCACAA          1013
AAAAAAAAAAAAA                                                            1026
```

Fig. 5: DNA sequence (seq. ID No. 12) of the Alternaria alternata mannitol dehydrogenase



5 Fig. 6: Alignment of the C. herbarum and A. alternata DNA sequences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A.alternata 778 GACGGTGGTTAC
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A.alternata 1 MP-ITVPQATE
C.herbarum 61
A.alternata 60
C.herbarum 121
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A.alternata 180
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A.alternata 240 AYVYFASDASSY

SEQUENZPROTOKOLL

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             20                  25                  30

Ala Ile Thr Tyr Ala Ser Arg Ala Gln Gly Ala Glu Glu Asn Val Lys
         35                  40                  45

Glu Leu Glu Lys Thr Tyr Gly Ile Lys Ala Lys Ala Tyr Lys Cys Gln
     50                  55                  60

Val Asp Ser Tyr Glu Ser Cys Glu Lys Leu Val Lys Asp Val Val Ala
 65                  70                  75                  80

Asp Phe Gly Gln Ile Asp Ala Phe Ile Ala Asn Ala Gly Ala Thr Ala
                 85                  90                  95

Asp Ser Gly Ile Leu Asp Gly Ser Val Glu Ala Trp Asn His Val Val
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            100                 105                 110

Gln Val Asp Leu Asn Gly Thr Phe His Cys Ala Lys Ala Val Gly His
        115                 120                 125

His Phe Lys Glu Arg Gly Thr Gly Ser Phe Val Ile Thr Ser Ser Met
    130                 135                 140

Ser Gly His Ile Ala Asn Tyr Pro Gln Glu Gln Thr Ser Tyr Asn Val
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Ala Lys Ala Gly Cys Ile His Met Ala Arg Ser Leu Ala
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cagggtgctg aggagaacgt caaggagctt gagaagacct acggcatcaa ggccaaggcc 180
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ctcgacggct ccgtccaggc ctggaaccac gtcgtccagg tcgacctgaa cggtaccttc 360
cactgcgcca aggccgttgg ccaccacttc aaggagcgtg gaaccggttc cttcgtcatc 420
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Leu Lys Gly Arg Val Val Val Val Thr Gly Ala Ser Gly Pro Lys Gly
            20                  25                  30

Met Gly Ile Glu Ala Ala Arg Gly Cys Ala Glu Met Gly Ala Asp Leu
        35                  40                  45

Ala Ile Thr Tyr Ala Ser Arg Ala Glu Gly Gly Leu Lys Asn Ala Glu
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     50                  55                  60

Glu Leu Ser Lys Gln Tyr Gly Ile Lys Cys Lys Ala Tyr Lys Cys Gln
 65                  70                  75                  80

Val Asp Lys Tyr Glu Ser Val Glu Gln Leu Val Lys Asp Val Ile Gln
                85                  90                  95

Asp Phe Gly Lys Ile Asp Ala Phe Ile Ala Asn Ala Gly Ala Thr Ala
            100                 105                 110

Asn Ser Gly Ile Leu Asp Gly Ser Val Glu Asp Trp Asn His Val Val
        115                 120                 125

Gln Val Asp Leu Asn Gly Thr Phe His Cys Ala Lys Ala Val Gly His
    130                 135                 140

His Phe Lys Glu Arg Gly Thr Gly Ser Phe Val Ile Thr Ser Ser Met
145                 150                 155                 160

Ser Gly His Ile Ala Asn Tyr Pro Gln Glu Gln Thr Ser Tyr Asn Val
                165                 170                 175

Ala Lys Ala Gly Cys Ile His Met Ala Arg Ser Leu Ala Asn Glu Trp
            180                 185                 190

Arg Asp Phe Ala Arg Val Asn Ser Ile Ser Pro Gly Tyr Ile Asp Thr
        195                 200                 205

Gly Leu Ser Asp Phe Val Ala Lys Asp Ile Gln Lys Leu Trp His Ser
    210                 215                 220

Met Ile Pro Leu Gly Arg Asp Gly Leu Ala Lys Glu Leu Lys Gly Ala
225                 230                 235                 240

Tyr Val Tyr Leu Val Ser Asp Ala Ser Thr Tyr Thr Thr Gly Ala Asp
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Ile Val Ile Asp Gly Gly Tyr Thr Cys Arg
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<210> 4
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<213> Cladosporium herbarum

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ggccaccact tcaaggagcg tggaaccggt tccctcgtca tcaccgcctc catgtccggc 540
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Gly Met Gly Ile Glu Ala Ala Arg Gly Cys Ala Glu Met Gly Ala Ala
35 40 45

Val Ala Ile Thr Tyr Ala Ser Arg Ala Gln Gly Ala Glu Glu Asn Val
50 55 60

Lys Glu Leu Glu Lys Thr Tyr Gly Ile Lys Ala Lys Ala Tyr Lys Cys
65 70 75 80

Gln Val Asp Ser Tyr Glu Ser Cys Glu Lys Leu Val Lys Asp Val Val
85 90 95

Ala Asp Phe Gly Gln Ile Asp Ala Phe Ile Ala Asn Ala Gly Ala Thr
100 105 110

Ala Asp Ser Gly Ile Leu Asp Gly Ser Val Glu Ala Trp Asn His Val
115 120 125

Val Gln Val Asp Leu Asn Gly Thr Phe His Cys Ala Lys Ala Val Gly
130 135 140

His His Phe Lys Glu Arg Gly Thr Gly Ser Leu Val Ile Thr Ala Ser
145 150 155 160

Met Ser Gly His Ile Ala Asn Phe Pro Gln Glu Gln Thr Ser Tyr Asn
165 170 175

Val Ala Lys Ala Gly Cys Ile His Met Ala Arg Ser Leu Ala Asn Glu
180 185 190

Trp Arg Asp Phe Ala Arg Val Asn Ser Ile Ser Pro Gly Tyr Ile Asp
195 200 205

Thr Gly Leu Ser Asp Phe Val Pro Lys Glu Thr Gln Gln Leu Trp His
210 215 220

5

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Ser Met Ile Pro Met Gly Arg Asp Gly Leu Ala Lys Glu Leu Lys Gly
225                 230                 235                 240

Ala Tyr Val Tyr Phe Ala Ser Asp Ala Ser Thr Tyr Thr Thr Gly Ala
                245                 250                 255

Asp Leu Leu Ile Asp Gly Gly Tyr Thr Thr Arg
            260                 265
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<210> 9
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Leu Lys Gly Arg Val Val Val Val Thr Gly Ala Ser Gly Pro Lys Gly
             20                  25                  30

Met Gly Ile Glu Ala Ala Arg Gly Cys Ala Glu Met Gly Ala Asp Leu
         35                  40                  45

Ala Ile Thr Tyr Ala Ser Arg Ala Glu Gly Gly Leu Lys Asn Ala Glu
     50                  55                  60

Glu Leu Ser Lys Gln Tyr Gly Ile Lys Cys Lys Ala Tyr Lys Cys Gln
 65                  70                  75                  80

Val Asp Lys Tyr Glu Ser Val Glu Gln Leu Val Lys Asp Val Ile Gln
                 85                  90                  95

Asp Phe Gly Lys Ile Asp Ala Phe Ile Ala Asn Ala Gly Ala Thr Ala
            100                 105                 110

Asn Ser Gly Ile Leu Asp Gly Ser Val Glu Asp Trp Asn His Val Val
        115                 120                 125

Gln Val Asp Leu Asn Gly Thr Phe His Cys Ala Lys Ala Val Gly His
    130                 135                 140

His Phe Lys Glu Arg Gly Thr Gly Ser Phe Val Ile Thr Ser Ser Met
145                 150                 155                 160

Ser Gly His Ile Ala Asn Tyr Pro Gln Glu Gln Thr Ser Tyr Asn Val
                165                 170                 175
```

```
Ala Lys Ala Gly Cys Ile His Met Ala Arg Ser Leu Ala Asn Glu Trp
            180                 185                 190

Arg Asp Phe Ala Arg Val Asn Ser Ile Ser Pro Gly Tyr Ile Asp Thr
        195                 200                 205

Gly Leu Ser Asp Phe Val Ala Lys Asp Ile Gln Lys Leu Trp His Ser
    210                 215                 220

Met Ile Pro Leu Gly Arg Asp Gly Leu Ala Lys Glu Leu Lys Gly Ala
225                 230                 235                 240

Tyr Val Tyr Leu Val Ser Asp Ala Ser Thr Tyr Thr Thr Gly Ala Asp
                245                 250                 255

Ile Val Ile Asp Gly Gly Tyr Thr Cys Arg
            260                 265
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<210> 10
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Ser Leu Lys Gly Lys Val Val Val Val Thr Gly Ala Ser Gly Pro Lys
             20                  25                  30

Gly Met Gly Ile Glu Ala Ala Arg Gly Cys Ala Glu Met Gly Ala Ala
         35                  40                  45

Val Ala Ile Thr Tyr Ala Ser Arg Ala Gln Gly Ala Glu Glu Asn Val
     50                  55                  60

Lys Glu Leu Glu Lys Thr Tyr Gly Ile Lys Ala Lys Ala Tyr Lys Cys
 65                  70                  75                  80

Gln Val Asp Ser Tyr Glu Ser Cys Glu Lys Leu Val Lys Asp Val Val
                 85                  90                  95

Ala Asp Phe Gly Gln Ile Asp Ala Phe Ile Ala Asn Ala Gly Ala Thr
            100                 105                 110

Ala Asp Ser Gly Ile Leu Asp Gly Ser Val Glu Ala Trp Asn His Val
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```
        115                 120                 125

Val Gln Val Asp Leu Asn Gly Thr Phe His Cys Ala Lys Ala Val Gly
    130                 135                 140

His His Phe Lys Glu Arg Gly Thr Gly Ser Leu Val Ile Thr Ala Ser
145                 150                 155                 160

Met Ser Gly His Ile Ala Asn Phe Pro Gln Glu Gln Thr Ser Tyr Asn
                165                 170                 175

Val Ala Lys Ala Gly Cys Ile His Met Ala Arg Ser Leu Ala Asn Glu
            180                 185                 190

Trp Arg Asp Phe Ala Arg Val Asn Ser Ile Ser Pro Gly Tyr Ile Asp
        195                 200                 205

Thr Gly Leu Ser Asp Phe Val Pro Lys Glu Thr Gln Gln Leu Trp His
    210                 215                 220

Ser Met Ile Pro Met Gly Arg Asp Gly Leu Ala Lys Glu Leu Lys Gly
225                 230                 235                 240

Ala Tyr Val Tyr Phe Ala Ser Asp Ala Ser Thr Tyr Thr Thr Gly Ala
                245                 250                 255

Asp Leu Leu Ile Asp Gly Gly Tyr Thr Thr Arg
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             20                  25                  30

Ile Gly Thr Glu Ala Ala Arg Gly Cys Ala Glu Tyr Gly Ala Asp Leu
         35                  40                  45

Ala Ile Thr Tyr Asn Ser Arg Ala Glu Gly Ala Glu Lys Asn Ala Lys
     50                  55                  60
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Glu Met Ser Glu Lys Tyr Gly Val Lys Val Lys Ala Tyr Lys Cys Gln
 65                  70                  75                  80

Val Asn Glu Tyr Ala Gln Cys Glu Lys Leu Val Gln Asp Val Ile Lys
                85                  90                  95

Asp Phe Gly Lys Val Asp Val Phe Ile Ala Asn Ala Gly Lys Thr Ala
            100                 105                 110

Asp Asn Gly Ile Leu Asp Ala Thr Val Glu Gln Trp Asn Glu Val Ile
        115                 120                 125

Gln Thr Asp Leu Thr Gly Thr Phe Asn Cys Ala Arg Ala Val Gly Leu
    130                 135                 140

His Phe Arg Glu Arg Lys Thr Gly Ser Leu Val Ile Thr Ser Ser Met
145                 150                 155                 160

Ser Gly His Ile Ala Asn Phe Pro Gln Glu Gln Ala Ser Tyr Asn Val
                165                 170                 175

Ala Lys Ala Gly Cys Ile His Leu Ala Lys Ser Leu Ala Asn Glu Trp
            180                 185                 190

Arg Asp Phe Ala Arg Val Asn Ser Ile Ser Pro Gly Tyr Ile Asp Thr
        195                 200                 205

Gly Leu Ser Asp Phe Val Pro Gln Asp Ile Gln Lys Leu Trp His Ser
    210                 215                 220

Met Ile Pro Met Gly Arg Asp Ala Lys Ala Thr Glu Leu Lys Gly Ala
225                 230                 235                 240

Tyr Val Tyr Phe Ala Ser Asp Ala Ser Ser Tyr Cys Thr Gly Ser Asp
                245                 250                 255

Leu Leu Ile Asp Gly Gly Tyr Cys Val Arg
            260                 265
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cctcctacaa cgttgctaag gctggctgca ttcacctcgc caagtcgctc gccaacgagt 660
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10