



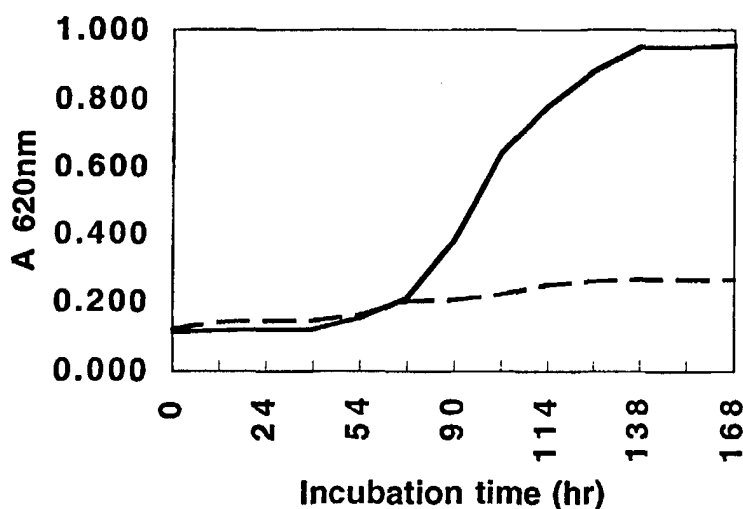
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(54) Title: ANTIFUNGAL COMPOSITION CONTAINING BETA-(1,6)-GLUCANASE, AND HOSTS INCORPORATING SAME

Paecilomyces variottii

— — B1-6/AP24 — — Ref



(57) Abstract

The present invention provides an isolated β -(1,6)-glucanase obtainable from an edible fungus which has antifungal activity and methods for isolating said protein. The invention also comprises an antifungal composition comprising a β -(1,6)-glucanase and optionally an osmotin. The invention further comprises a method for producing pathogen resistant plants incorporating chimeric DNA capable of encoding a β -(1,6)-glucanase and optionally an osmotin, and wherein the proteins are expressed.

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ANTIFUNGAL COMPOSITION CONTAINING BETA-(1,6)-GLUCANASE, AND HOSTS INCORPORATING SAME

FIELD OF THE INVENTION

5 The present invention relates to a novel glucanase protein, a combination of antifungal proteins and plants transformed with the glucanase or the combination, as well as methods of combatting fungal pathogens by causing said fungal pathogens to be contacted with said protein or proteins.

10 The invention further relates to transformed plants which show reduced susceptibility to fungal pathogens.

BACKGROUND ART

15 A protein with antifungal activity, isolated from TMV-induced tobacco leaves, which is capable of causing lysis of germinating spores and hyphal tips of *Phytophthora infestans* and which causes the hyphae to grow at a reduced rate, was disclosed in WO91/18984 A1 and in Woloshuk et al. (Plant cell 3, 619-628, 1991). This protein, an osmotin, has an apparent molecular weight of about 24 kDa and was named AP24. Comparison of its complete amino acid
20 sequence, as deduced from the nucleic acid sequence of the AP24 gene, with proteins known from databases revealed that the protein was an osmotin-like protein.

It has further been known that combination of antifungal agents such as chitinase and β -(1,3)-glucanase (EP-A 0 440 304)
25 have a synergistic effect on the ability to destroy fungal pathogens.

Recently (Lorito et al., Mol. Plant-Microb. Int. 9(3), 206-213, 1996) it has been shown that also combinations of osmotin and other antifungal agents are effective.

30 WO 95/31534 describes the β -(1,6)-glucanase (or pustulanase) from *Trichoderma harzianum* as an agent which catalyzes the cleavage of β -(1,6)-linkages in β -glucan, a major component of the cell walls of fungal cells. It has also been found to act cooperatively with cellulases and chitinases against fungal plant pathogens such
35 as *Botrytis*, *Phytophthora* and *Gibberella fujikori* (de la Cruz, J. Bacteriol. 177(7), 1864-1871, 1995).

Despite initial success in combating fungal pathogens, and the genetic engineering of plants capable of producing these

antifungal proteins with activity against fungal pathogens there remains a need to identify and isolate other proteins and/or synergistic combinations with antifungal activity.

5

SUMMARY OF THE INVENTION

The present invention provides for a new protein with β -(1,6)-glucanase activity isolated from an edible fungus. More specifically the invention describes methods to isolate such a protein.

10

The present invention further provides an antifungal composition comprising a β -(1,6)-glucanase and optionally an osmotin.

15

The present invention further provides a method for making plants pathogen resistant by transforming them with recombinant DNA comprising a sequence coding for an osmotin and a sequence coding for a β -(1,6)-glucanase.

20

More specifically the osmotin is AP24, preferably having the amino acid sequence of SEQ ID NO:2, and preferably encoded by the nucleotide sequence of SEQ ID NO:1.

25

More specifically the β -(1,6)-glucanase is of fungal origin, for instance from *Trichoderma harzianum*, or from edible fungi such as mushroom or shii-take.

30

The invention also provides a recombinant DNA sequence according to the invention further comprising a transcriptional initiation region and, optionally, a transcriptional termination region, so linked to said open reading frames as to enable the DNA to be transcribed in a living host cell when present therein, thereby producing RNAs which comprise said open reading frames. A preferred chimeric DNA sequence according to the invention is one, wherein the RNAs comprising said open reading frames are capable of being translated into protein in said host cell, when present therein, thereby producing said proteins.

35

The invention also embraces a chimeric DNA sequence comprising a DNA sequence according to the invention, which may be selected from replicons, such as bacterial cloning plasmids and vectors, such as a bacterial expression vector, a (non-integrative) plant viral vector, a Ti-plasmid vector of *Agrobacterium*, such as a binary vector, and the like, as well as a host cell comprising a

replicon or vector according to the invention, and which is capable of maintaining said replicon once present therein. Preferred according to that embodiment is a host cell which is a plant cell, said vector being a non-integrative viral vector.

5 The invention further provides a host cell stably incorporating in its genome a chimeric DNA sequence according to the invention, such as a plant cell, as well as multicellular hosts comprising such cells, or essentially consisting of such cells, such as plants. Especially preferred are plants characterised in
10 that the chimeric DNA according to the invention is expressed in at least a number of the plant's cells causing the said antifungal proteins to be produced therein.

 According to yet another embodiment of the invention a method for producing a protein with β -(1,6)-glucanase activity is
15 provided, characterised in that a host cell according to the invention is grown under conditions allowing the said protein to be produced by said host cell, optionally followed by the step of recovering the protein from the host cells.

 Another part of the invention is directed to the targeting of
20 the osmotin and/or the β -(1,6)-glucanase to the extracellular space.

 The invention also provides a method for obtaining plants with reduced susceptibility to fungi, comprising the steps of
(a) introducing into ancestor cells which are susceptible of
25 regeneration into a whole plant,

- a chimeric DNA sequence comprising open reading frames capable of encoding an osmotin and a β -(1,6)-glucanase, said open reading frames being operatively linked to one or more
transcriptional and translational regions and, optionally, a
30 transcriptional termination region, allowing the said proteins to be produced in a plant cell that is susceptible to infection by said fungus, and

- a chimeric DNA sequence capable of encoding a plant selectable marker allowing selection of transformed ancestor cells
35 when said selectable marker is present therein, and
(b) regenerating said ancestor cells into plants under conditions favouring ancestor cells which have the said selectable marker, and

(c) identifying a plant which produces the proteins, thereby reducing the susceptibility of said plant to infection.

Preferred according to the invention is a method characterised in that step (a) is performed using an *Agrobacterium tumefaciens* strain capable of T-DNA transfer to plant cells and which harbours the said chimeric DNA cloned into binary vector pMOG800; another preferred method is when step (b) is performed in the presence of an antibiotic favouring cells which have a neomycin phosphotransferase or a cyanamid hydratase gene.

Legends to the figures.

Figure 1. Purification of β 1-6 glucanase by cation exchange chromatography. Three peaks of β 1-6 glucanase activity can be seen, indicated by the numbers 1, 2, and 3 respectively. The chromatogram of one representative run is shown. Other runs showed similar, if not identical, protein patterns and distribution of β 1-6 glucanase activity. Protein is measured at A280. β 1-6 glucanase activity is measured as described and is depicted as the absorbance at 510 nanometre.

Figure 2. Purification of β 1-6 glucanase (pool no. 1, pooled fractions 4 and 5 from the cation exchange chromatography experiment; figure 1) by gel filtration chromatography. β 1-6 glucanase activity elutes from the column at fractions 24-26. Protein is measured at A280 and is depicted in milli-absorption units. β 1-6 glucanase activity is measured as described and is depicted as the absorbance at 510 nanometre.

Figure 3. SDS-PAGE analysis of the protein fractions of pool no. 1 after gel filtration chromatography (figure 2).

Figure 4. Purification of β 1-6 glucanase (pool no. 1, pooled fractions 25 and 26 from the gel filtration chromatography experiment; figure 2) by chromatophocusing chromatography on a Mono P exchanger column. β 1-6 glucanase activity elutes from the column at fractions 11-12, corresponding to a pH of about 6.7. Protein is measured at A280 and is depicted in milli-absorption units.

Figure 5. SDS-PAGE analysis of the β 1-6 glucanase activity containing fractions of pool no. 1 after chromatofocusing chromatography (figure 4).

5 Figure 6. Purification of β 1-6 glucanase (pool no. 2, pooled fractions 10, 11, and 12 from the cation exchange chromatography experiment; figure 1) by gel filtration chromatography. β 1-6 glucanase activity elutes from the column at fractions 15-17. Protein is measured at A280 and is depicted in milli-absorption
10 units. β 1-6 glucanase activity is measured as described and is depicted as the absorbance at 510 nanometre.

Figure 7. SDS-PAGE analysis of the protein fractions of pool no. 2 after gel filtration chromatography (figure 6). The arrow points
15 towards the presumed β 1-6 glucanase bands.

Figure 8. Purification of β 1-6 glucanase (pool no. 2, fraction 16 from the gel filtration chromatography experiment; figure 6) by chromatofocusing chromatography on a Mono P exchanger column. β 1-6
20 glucanase activity elutes from the column at fractions 3-5, corresponding to the flow-through of the column, meaning that the isoelectric point is above pH 6.8. Protein is measured at A280 and is depicted in milli-absorption units.

25 Figure 9. SDS-PAGE analysis of the β 1-6 glucanase activity containing fractions of pool no. 2 after chromatofocusing chromatography (figure 8). The arrow points towards the β 1-6 glucanase band.

30 Figure 10. Purification of β 1-6 glucanase (pool no. 3, pooled fractions 15, 16, and 17 from the cation exchange chromatography experiment; figure 1) by gel filtration chromatography. β 1-6 glucanase activity elutes from the column at fractions 15-18. Protein is measured at A280 and is depicted in milli-absorption
35 units. β 1-6 glucanase activity is measured as described and is depicted as the absorbance at 510 nanometre.

Figure 11. Double reciprocal plot showing β 1-6 glucanase activity as a function of pustulan concentration. Fraction number 4 from the chromatophocusing column from pool no. 2 (figure 8) was used as source of β 1-6 glucanase activity. β 1-6 glucanase activity is measured as described and is depicted as the absorbance at 510 nanometre.

Figure 12. Effect of AP24 and β 1-6 glucanase on in vitro growth of *Fusarium oxysporum*. The fungus was grown in a microtiter plate assay as described, in the presence of 5 μ g/ml AP24 plus 5 μ g/ml β 1-6 glucanase (β 1-6/AP24) or in the absence of these proteins (Ref). Fungal growth was followed in time by reading the absorbance at 620 nanometres in a microtiter plate reader.

Figure 13. Effect of AP24 and β 1-6 glucanase on in vitro growth of *Paecilomyces variottii*. Same assay as depicted in fig. 12.

Figure 14. Effect of AP24 and β 1-6 glucanase on in vitro growth of *Penicillium roqueforti*. Same assay as depicted in fig. 12.

DETAILED DESCRIPTION OF THE INVENTION

The β -(1,6)-glucanase protein according to the present invention may be obtained by isolating it from any suitable edible fungus source material containing it. Edible fungi in this respect means fungi which are commonly eaten. A particularly suitable source comprises fruit bodies and or mycelium of mushrooms (*Agaricus spp.*), oyster mushrooms (*Pleurotus ostreatus*), shii-take (*Lentinus edodes*) or of other commonly eaten fungi such as *Volvariella spp.*, *Tricholoma matsutake*, *Tuber uncinatum*, *Tuber melanosporum*, *Tuber spp.*, *Stephensia spp.*, *Cantharellus spp.*, *Pleurotus spp.*, *Coprinus spp.*, *Lepiota spp.*, *Marasmius spp.*, *Polysporus spp.*, *Verpa spp.*, *Tricholoma spp.*, *Terfezia spp.*, *Morchella spp.*, *Cyttaria spp.* and fungi routinely used in the production of cheese such as *Penicillium roqueforti* and *P. camemberti*.

Alternatively, proteins according to the invention may be obtained by cloning DNA comprising an open reading frame capable of encoding said protein, or the precursor thereof, linking said open reading frame to a transcriptional, and optionally a translational initiation and transcriptional termination region, inserting said DNA into a suitable host cell and allowing said host cell to produce said protein. Subsequently, the protein may be recovered from said host cells, preferably after secretion of the protein into the culture medium by said host cells. Alternatively, said host cells may be used directly in a process of combating fungal pathogens according to the invention as a pesticidal acceptable composition.

Host cells suitable for use in a process of obtaining a protein according to the invention may be selected from prokaryotic microbial hosts, such as bacteria e.g. *Agrobacterium*, *Bacillus*, *Cyanobacteria*, *E.coli*, *Pseudomonas*, and the like, as well as eukaryotic hosts including yeasts, e.g. *Saccharomyces cerevisiae*, fungi, e.g. *Trichoderma* and plant cells, including protoplasts.

The word protein means a sequence of amino acids connected through peptide bonds. Polypeptides or peptides are also considered to be proteins. Muteins of the protein of the invention are proteins that are obtained from the proteins depicted in the sequence listing by replacing, adding and/or deleting one or more amino acids, while still retaining their glucanase activity. Such muteins can readily be made by protein engineering *in vivo*, e.g. by changing the open reading frame capable of encoding the protein such that the amino acid sequence is thereby affected. As long as the changes in the amino acid sequences do not altogether abolish the glucanase activity such muteins are embraced in the present invention.

The present invention provides a chimeric DNA sequence which comprises an open reading frame capable of encoding the protein according to the invention or a combination of osmotin and β -(1,6)-glucanase. The expression chimeric DNA sequence shall mean to comprise any DNA sequence which comprises DNA sequences not naturally found in nature. For instance, chimeric DNA shall mean to comprise DNA comprising the said open reading frame in a non-natural location of the host genome, notwithstanding the fact that

said host genome normally contains a copy of the said open reading frame in its natural chromosomal location. Similarly, the said open reading frame may be incorporated in the host genome wherein it is not naturally found, or in a replicon or vector where it is not naturally found, such as a bacterial plasmid or a viral vector. Chimeric DNA shall not be limited to DNA molecules which are replicable in a host, but shall also mean to comprise DNA capable of being ligated into a replicon, for instance by virtue of specific adaptor sequences, physically linked to the open reading frame according to the invention. The open reading frame may or may not be linked to its natural upstream and downstream regulatory elements.

The open reading frame may be derived from a genomic library. In this latter it may contain one or more introns separating the exons making up the open reading frame that encodes a protein according to the invention. The open reading frame may also be encoded by one uninterrupted exon, or by a cDNA to the mRNA encoding a protein according to the invention. Open reading frames according to the invention also comprise those in which one or more introns have been artificially removed or added. Each of these variants is embraced by the present invention.

The open reading frame coding for the osmotin preferably comprises a nucleotide sequence that codes for a protein as is depicted in SEQ ID NO:2. Most preferably the nucleotide sequence comprises the nucleotide sequence of the open reading frame depicted in SEQ ID NO:1.

The open reading frame coding for the β -(1,6)-glucanase can either be composed of the sequence coding for the protein which can be derived from *Trichoderma harzianum*, or it can be derived from an edible fungus or a plant.

In order to be capable of being expressed in a host cell a chimeric DNA according to the invention will usually be provided with regulatory elements enabling it to be recognised by the biochemical machinery of the host and allowing for the open reading frame to be transcribed and/or translated in the host. It will usually comprise a transcriptional initiation region which may be suitably derived from any gene capable of being expressed in the host cell of choice, as well as a translational initiation region

for ribosome recognition and attachment. In eukaryotic cells, an expression cassette usually comprises in addition a transcriptional termination region located downstream of said open reading frame, allowing transcription to terminate and polyadenylation of the primary transcript to occur. In addition, the codon usage may be adapted to accepted codon usage of the host of choice. The principles governing the expression of a chimeric DNA construct in a chosen host cell are commonly understood by those of ordinary skill in the art and the construction of expressible chimeric DNA constructs is now routine for any sort of host cell, be it prokaryotic or eukaryotic.

In order for the open reading frame to be maintained in a host cell it will usually be provided in the form of a replicon comprising said open reading frame according to the invention linked to DNA which is recognised and replicated by the chosen host cell. Accordingly the selection of the replicon is determined largely by the host cell of choice. Such principles as govern the selection of suitable replicons for a particular chosen host are well within the realm of the ordinary skilled person in the art.

A special type of replicon is one capable of transferring itself, or a part thereof, to another host cell, such as a plant cell, thereby co-transferring the open reading frame according to the invention to said plant cell. Replicons with such capability are herein referred to as vectors. An example of such vector is a Ti-plasmid vector which, when present in a suitable host, such as *Agrobacterium tumefaciens*, is capable of transferring part of itself, the so-called T-region, to a plant cell. Different types of Ti-plasmid vectors (*vide*: EP 0 116 718 B1) are now routinely being used to transfer chimeric DNA sequences into plant cells, or protoplasts, from which new plants may be generated which stably incorporate said chimeric DNA in their genomes. A particularly preferred form of Ti-plasmid vectors are the so-called binary vectors as claimed in (EP 0 120 516 B1 and US 4,940,838). Other suitable vectors, which may be used to introduce DNA according to the invention into a plant host, may be selected from the viral vectors, e.g. non-integrative plant viral vectors, such as derivable from the double stranded plant viruses (e.g. CaMV) and single stranded viruses, gemini viruses and the like. The use of

such vectors may be advantageous, particularly when it is difficult to stably transform the plant host. Such may be the case with woody species, especially trees and vines.

5 The expression "host cells incorporating a chimeric DNA sequence according to the invention in their genome" shall mean to comprise cells, as well as multicellular organisms comprising such cells, or essentially consisting of such cells, which stably incorporate said chimeric DNA into their genome thereby maintaining the chimeric DNA, and preferably transmitting a copy of such
10 chimeric DNA to progeny cells, be it through mitosis or meiosis. According to a preferred embodiment of the invention plants are provided, which essentially consist of cells which incorporate one or more copies of said chimeric DNA into their genome, and which are capable of transmitting a copy or copies to their progeny,
15 preferably in a Mendelian fashion. By virtue of the transcription and translation of the chimeric DNA according to the invention in some or all of the plant's cells, those cells as produce the antifungal proteins will show enhanced resistance to fungal infections. Although the principles as govern transcription of DNA
20 in plant cells are not always understood, the creation of chimeric DNA capable of being expressed in substantially a constitutive fashion, that is, in substantially most cell types of the plant and substantially without serious temporal and/or developmental restrictions, is now routine. Transcription initiation regions
25 routinely in use for that purpose are promoters obtainable from the cauliflower mosaic virus, notably the 35S RNA and 19S RNA transcript promoters and the so-called T-DNA promoters of *Agrobacterium tumefaciens*, in particular to be mentioned are the nopaline synthase promoter, octopine synthase promoter (as
30 disclosed in EP 0 122 791 B1) and the mannopine synthase promoter. In addition plant promoters may be used, which may be substantially constitutive, such as the rice actin gene promoter, or e.g. organ-specific, such as the root-specific promoter. Alternatively, pathogen-inducible promoters may be used such as the PRP1 promoter
35 (also named *gst1* promoter) obtainable from potato (Martini N. et al. (1993), Mol. Gen. Genet. 263, 179-186). The choice of the promoter is not essential, although it must be said that constitutive high-level promoters are slightly preferred. It is

further known that duplication of certain elements, so-called enhancers, may considerably enhance the expression level of the DNA under its regime (vide for instance: Kay R. et al. (1987), Science 236, 1299-1302: the duplication of the sequence between -343 and -90 of the CaMV 35S promoter increases the activity of that promoter). In addition to the 35S promoter, singly or doubly enhanced, examples of high-level promoters are the light-inducible ribulose biphosphate carboxylase small subunit (*rbcSSU*) promoter and the chlorophyll a/b binding protein (*Cab*) promoter. Also envisaged by the present invention are hybrid promoters, which comprise elements of different promoter regions physically linked. A well known example thereof is the so-called CaMV enhanced mannopine synthase promoter (US Patent 5,106,739), which comprises elements of the mannopine synthase promoter linked to the CaMV enhancer.

As regards the necessity of a transcriptional terminator region, it is generally believed that such a region enhances the reliability as well as the efficiency of transcription in plant cells. Use thereof is therefore strongly preferred in the context of the present invention.

Another aspect of gene expression in transgenic plants concerns the targeting of antifungal proteins to the extracellular space (apoplast). Naturally intracellularly occurring proteins, among which proteins according to the present invention, may be caused to be targeted to the apoplast by removal of the C-terminal propeptide, e.g. by modifying the open reading frame at its 3' end such that protein is caused to be C-terminally truncated. A certain number of amino acids of the C-terminal part of the protein was found to be responsible for targeting of the protein to the vacuole (e.g. vide WO91/18984 A1). By introducing a translational stopcodon in the open reading frame the truncated protein is caused to be targeted to the apoplast.

The invention can be practiced in all plants, even in plant species that are presently not amenable for transformation, as the amenability of such species is just a matter of time and because transformation as such is of no relevance for the principles underlying the invention. Hence, plants for the purpose of this

description shall include angiosperms as well as gymnosperms, monocotyledonous as well as dicotyledonous plants, be they for feed, food or industrial processing purposes; included are plants used for any agricultural or horticultural purpose including forestry and flower culture, as well as home gardening or indoor gardening, or other decorative purposes.

Transformation of plant species is now routine for an impressive number of plant species, including both the *Dicotyledoneae* as well as the *Monocotyledoneae*. In principle any transformation method may be used to introduce chimeric DNA according to the invention into a suitable ancestor cell. Methods may suitably be selected from the calcium/polyethylene glycol method for protoplasts (Krens, F.A. et al., 1982, *Nature* 296, 72-74; Negrutiu I. et al, June 1987, *Plant Mol. Biol.* 8, 363-373), electroporation of protoplasts (Shillito R.D. et al., 1985 *Bio/Technol.* 3, 1099-1102), microinjection into plant material (Crossway A. et al., 1986, *Mol. Gen. Genet.* 202, 179-185), (DNA or RNA-coated) particle bombardment of various plant material (Klein T.M. et al., 1987, *Nature* 327, 70), infection with (non-integrative) viruses, in planta *Agrobacterium tumefaciens* mediated gene transfer by infiltration of adult plants or transformation of mature pollen or microspores (EP 0 301 316) and the like. A preferred method according to the invention comprises *Agrobacterium*-mediated DNA transfer. Especially preferred is the use of the so-called binary vector technology as disclosed in EP A 120 516 and U.S. Patent 4,940,838).

Although considered somewhat more recalcitrant towards genetic transformation, monocotyledonous plants are amenable to transformation and fertile transgenic plants can be regenerated from transformed cells or embryos, or other plant material. Presently, preferred methods for transformation of monocots are microprojectile bombardment of embryos, explants or suspension cells, and direct DNA uptake or (tissue) electroporation (Shimamoto, et al, 1989, *Nature* 338, 274-276). Transgenic maize plants have been obtained by introducing the *Streptomyces hygroscopicus* bar-gene, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension

culture by microprojectile bombardment (Gordon-Kamm, 1990, Plant Cell, 2, 603-618). The introduction of genetic material into aleurone protoplasts of other monocot crops such as wheat and barley has been reported (Lee, 1989, Plant Mol. Biol. 13, 21-30).

Wheat plants have been regenerated from embryogenic suspension culture by selecting embryogenic callus for the establishment of the embryogenic suspension cultures (Vasil, 1990 Bio/Technol. 8, 429-434). The combination with transformation systems for these crops enables the application of the present invention to monocots.

Monocotyledonous plants, including commercially important crops such as rice and corn are also amenable to DNA transfer by *Agrobacterium* strains (vide WO 94/00977; EP 0 159 418 B1; Gould J, Michael D, Hasegawa O, Ulian EC, Peterson G, Smith RH, (1991) Plant. Physiol. 95, 426-434).

To obtain transgenic plants capable of constitutively expressing more than one chimeric gene, a number of alternatives are available including the following:

A. The use of DNA, e.g. a T-DNA on a binary plasmid, with a number of modified genes physically coupled to a second selectable marker gene. The advantage of this method is that the chimeric genes are physically coupled and therefore migrate as a single Mendelian locus.

B. Cross-pollination of transgenic plants each already capable of expressing one or more chimeric genes, preferably coupled to a selectable marker gene, with pollen from a transgenic plant which contains one or more chimeric genes coupled to another selectable marker. Afterwards the seed, which is obtained by this crossing, maybe selected on the basis of the presence of the two selectable markers, or on the basis of the presence of the chimeric genes themselves. The plants obtained from the selected seeds can afterwards be used for further crossing. In principle the chimeric genes are not on a single locus and the genes may therefore segregate as independent loci.

C. The use of a number of a plurality chimeric DNA molecules, e.g. plasmids, each having one or more chimeric genes and a selectable marker. If the frequency of co-transformation is high, then selection on the basis of only one marker is sufficient. In other

cases, the selection on the basis of more than one marker is preferred.

- D. Consecutive transformation of transgenic plants already containing a first, second, (etc), chimeric gene with new chimeric DNA, optionally comprising a selectable marker gene. As in method 5 B, the chimeric genes are in principle not on a single locus and the chimeric genes may therefore segregate as independent loci.
- E. Combinations of the above mentioned strategies.

The actual strategy may depend on several considerations as maybe easily determined such as the purpose of the parental lines (direct growing, use in a breeding programme, use to produce hybrids) but is not critical with respect to the described invention.

It is known that practically all plants can be regenerated from cultured cells or tissues. The means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Shoots may be induced directly, or indirectly from callus via organogenesis or embryogenesis and subsequently rooted. Next to the selectable marker, the culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype and on the history of the culture. If these three variables are controlled regeneration is usually reproducible and repeatable.

After stable incorporation of the transformed gene sequences into the transgenic plants, the traits conferred by them can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

To select or screen for transformed cells, it is preferred to include a marker gene linked to the plant expressible gene according to the invention to be transferred to a plant cell. The choice of a suitable marker gene in plant transformation is well within the scope of the average skilled worker; some examples of

5 routinely used marker genes are the neomycin phosphotransferase genes conferring resistance to kanamycin (EP-B 131 623), the glutathion-S-transferase gene from rat liver conferring resistance to glutathione derived herbicides (EP-A 256 223), glutamine
10 synthetase conferring upon overexpression resistance to glutamine synthetase inhibitors such as phosphinothricin (WO 87/05327), the acetyl transferase gene from *Streptomyces viridochromogenes* conferring resistance to the selective agent phosphinothricin (EP-A 275 957), the gene encoding a 5-enolshikimate-3- phosphate synthase
15 (EPSPS) conferring tolerance to N-phosphonomethylglycine, the *bar* gene conferring resistance against Bialaphos (e.g. WO 91/02071), the cyanamid hydratase gene and the like. The actual choice of the marker is not crucial as long as it is functional (i.e. selective) in combination with the plant cells of choice.

15 The marker gene and the gene of interest do not have to be linked, since co-transformation of unlinked genes (U.S. Patent 4,399,216) is also an efficient process in plant transformation.

Preferred plant material for transformation, especially for dicotyledonous crops are leaf-discs which can be readily
20 transformed and have good regenerative capability (Horsch R.B. et al., (1985) Science 227, 1229-1231).

Another embodiment of the present invention is an antifungal composition comprising a β -(1,6)-glucanase and an osmotin. It has been found that both components act synergistically to yield an
25 antifungal effect *in vitro*. Such antifungal effects are very useful in food applications. Especially low-fat spreads, cheese and other dairy products, tea-based beverages, fruit- and tomato-based products are vulnerable food products in this respect. Combinations of antifungal compounds which act synergistically are preferable to
30 combat fungi in food applications because less preservative substance is needed. Furthermore, it is possible to use components from a natural source, which allows reduction of non-natural food additives which is preferable both from a nutritional and from a occupational health viewpoint.

35 It will be understood that from a regulatory viewpoint it would be preferable to use the β -(1,6)-glucanase which, as put forward in this invention, can be derived from edible fungi, such as mushrooms and shii-take.

Beside the active ingredients the composition according to the invention may contain auxiliary ingredients which are usual for fungi combatting compositions and which may include solid diluents, solvents, stabilizers and pH-regulators. The composition may be in the form of a powder, a paste or a liquid, depending on the envisaged way of application.

The composition is particularly suitable as a preservative for combatting fungi in food, but it can be used as well for preventing or combatting undesired fungal growth on other products, such as cosmetic products, where the fungal growth inhibiting composition is not only useful to keep the surface of the cosmetic product (e.g. soap) free of fungi, but also for an advantageous effect on the skin which is treated with such product, e.g. a shampoo being applied to a scalp with a fungal affliction.

Both the antifungal composition according to the invention as the plant transformed with the gene coding for the protein of the invention may contain additional antifungal components.

Examples of proteins that may be used in combination with the proteins according to the invention include, but are not limited to, β -(1,3)-glucanases and chitinases which are obtainable from barley (Swegle M. *et al.*, 1989, *Plant Mol. Biol.* 12, 403-412; Balance G.M. *et al.*, 1976, *Can. J. Plant Sci.* 56, 459-466 ; Hoj P.B. *et al.*, 1988, *FEBS Lett.* 230, 67-71; Hoj P.B. *et al.*, 1989, *Plant Mol. Biol.* 13, 31-42 1989), bean (Boller T. *et al.*, 1983, *Planta* 157, 22-31; Broglie K.E. *et al.* 1986, *Proc. Natl. Acad. Sci. USA* 83, 6820-6824; Vögeli U. *et al.*, 1988 *Planta* 174, 364-372); Mauch F. & Staehelin L.A., 1989, *Plant Cell* 1, 447-457); cucumber (Motraux J.P. & Boller T. (1986), *Physiol. Mol. Plant Pathol.* 28, 161-169); leek (Spanu P. *et al.*, 1989, *Planta* 177, 447-455); maize (Nasser W. *et al.*, 1988, *Plant Mol. Biol.* 11, 529-538), oat (Fink W. *et al.*, 1988, *Plant Physiol.* 88, 270-275), pea (Mauch F. *et al.* 1984, *Plant Physiol.* 76, 607-611; Mauch F. *et al.*, 1988, *Plant Physiol.* 87, 325-333), poplar (Parsons, T.J. *et al.* 1989, *Proc. Natl. Acad. Sci. USA* 86, 7895-7899), potato (Gaynor J.J. 1988, *Nucl. Acids Res.* 16, 5210; Kombrink E. *et al.* 1988, *Proc. Natl. Acad. Sci. USA* 85, 782-786; Laflamme D. and Roxby R., 1989, *Plant Mol. Biol.* 13, 249-250), tobacco (e.g. Legrand M. *et al.* 1987,

Proc. Natl. Acad. Sci. USA 84, 6750-6754; Shinshi H. *et al.* 1987, Proc. Natl. Acad. Sci. USA 84, 89-93), tomato (Joosten M.H.A. & De Wit P.J.G.M. 1989, Plant Physiol. 89, 945-951), wheat (Molano J. *et al.*, 1979, J. Biol. Chem. 254, 4901-4907), and the like.

5 In this context it should be emphasised that plants already containing chimeric DNA capable of encoding antifungal proteins may form a suitable genetic background for introducing chimeric DNA according to the invention, for instance in order to enhance resistance levels, or broaden the resistance. The cloning of other
10 genes corresponding to proteins that can suitably be used in combination with DNA, and the obtention of transgenic plants, capable of relatively over-expressing same, as well as the assessment of their effect on pathogen resistance *in planta*, is now within the scope of the ordinary skilled person in the art.

15 The obtention of transgenic plants capable of expressing, or relatively over-expressing, proteins according to the invention is a preferred method for counteracting the damages caused by pathogens such as fungi, as will be clear from the above description.

20 Plants, or parts thereof, which relatively over-express the protein combination according to the invention, including plant varieties, with improved resistance against diseases, especially diseases caused by fungi may be grown in the field, in the greenhouse, or at home or elsewhere. Plants or edible parts thereof
25 may be used for animal feed or human consumption, or may be processed for food, feed or other purposes in any form of agriculture or industry. Agriculture shall mean to include horticulture, arboriculture, flower culture, and the like. Industries which may benefit from plant material according to the
30 invention include but are not limited to the pharmaceutical industry, the paper and pulp manufacturing industry, sugar manufacturing industry, feed and food industry, enzyme manufacturers and the like.

35 The advantages of the plants, or parts thereof, according to the invention are the decreased need for fungicide treatment, thus lowering costs of material, labour, and environmental pollution, or prolonging shelf-life of products (e.g. fruit, seed, and the like) of such plants. Plants for the purpose of this invention shall mean

multicellular organisms capable of photosynthesis, and subject to some form of fungal disease. They shall at least include angiosperms as well as gymnosperms, monocotyledonous as well as dicotyledonous plants.

5 The phrase "plants which relatively over-express a protein" shall mean plants which contain cells expressing a transgene-encoded protein which is either not naturally present in said plant, or if it is present by virtue of an endogenous gene encoding an identical protein, not in the same quantity, or not in the same
10 cells, compartments of cells, tissues or organs of the plant. It is known for instance that proteins which normally accumulate intracellularly may be targeted to the apoplastic space. The following state of the art may be taken into consideration, especially as illustrating the general level of skill in the art to
15 which this invention pertains.
EP-A 392 225 A2; EP-A 440 304 A1; EP-A 460 753 A2; WO90/07001 A1;
US Patent 4,940,840.

Evaluation of transgenic plants

20 Subsequently transformed plants are evaluated for the presence of the desired properties and/or the extent to which the desired properties are expressed. A first evaluation may include the level of expression of the newly introduced genes, the level of fungal resistance of the transformed plants, stable heritability of
25 the desired properties, field trials and the like.

 Secondly, if desirable, the transformed plants can be crossbred with other varieties, for instance varieties of higher commercial value or varieties in which other desired characteristics have already been introduced, or used for the
30 creation of hybrid seeds, or be subject to another round of transformation and the like.

EXPERIMENTAL PART

35 Standard methods for the isolation, manipulation and amplification of DNA, as well as suitable vectors for replication of recombinant DNA, suitable bacterium strains, selection markers, media and the like are described for instance in Maniatis et al., molecular

cloning: A Laboratory Manual 2nd. edition (1989) Cold Spring Harbor Laboratory Press; DNA Cloning: Volumes I and II (D.N. Glover ed. 1985); and in: From Genes To Clones (E.-L. Winnacker ed. 1987).

5

Enzyme assay

β (1,6) glucanase activity was determined by measuring the amount of reducing sugars released from pustulan at 37°C. Pustulan was dissolved in hot buffer (50mM Kac, pH 5.0) and, after cooling, remained in solution without precipitation. The standard assay
10 (0.5ml) contained the enzyme preparation, 1.3 mg of pustulan and 50mM Kac, pH 5.0.

β (1,3) glucanase activity was assayed with laminarin as the reaction substrate (1.25 mg of laminarin in 0.5ml 50mM NaAc, pH 5.5).

15

The reducing sugars were determined according to Nelson, N., J. Biol. Chem. 153, 375-380, 1944.

EXAMPLE 1

20

Purification of β (1,6) glucanase from edible mushrooms via pustulan precipitation.

It was investigated whether edible mushrooms contained β -(1,6)-glucanase activity. We were able to demonstrate β -(1,6)-glucanase
25 activity in mushrooms. As a positive control we used a *Trichoderma* extract that is sold commercially as Novozym 234. This extract is known to contain substantial amounts of β (1,6)glucanase enzymatic activity.

30

Table 1 β -(1,6)-glucanase activities in different extracts

Sample	specific activity (nkat/mg)
<i>Agaricus bisporus</i>	1.8
TMV infected tobacco	0
Sunflower	0
Novozym 234	17

Mushrooms do contain β -(1,6)-glucanase activity, its specific activity being about one order of magnitude lower compared to the *Trichoderma* derived enzyme. In protein fractions of sunflower and
 5 TMV- infected tobacco no activity could be detected.

Five different mushrooms were tested for β -(1,6)-glucanase activity and two mushrooms with the highest specific activity were used for purification of the enzyme.

10

Table 2

Mushroom extract	Specific activity (nkat/mg protein)
<i>Agaricus bisporus</i>	0.35
Cave mushrooms	0.9
Chestnut mushrooms	0.5
Oyster mushrooms	1.4
Shii Take	2.6

The newly determined specific activity for the *Agaricus bisporus* extract is substantial lower than that depicted in table 1. The
 15 extract used for the data of Table 2 is a different extract, and we do not know the reason for this discrepancy. The extracts of Oyster mushrooms and Shii Take were used for further purification. Purification is as follows: An extract of fungi is made by chopping and grinding them in a buffer containing 0.5 M NaAc pH=5.2 + 0.1%
 20 β -mercaptoethanol, then freezing in liquid nitrogen and mincing the fungi in a blender. Then the crude extract was filtered through 5 layers of cheese cloth. Particles were removed from this solution by centrifugation for 1 hour at 9500 rpm in a Sorvall S-30 rotor. The crude protein extract (homogenate) was then further purified by
 25 ammonium sulfate precipitation, the β -(1,6)-glucanase activity was precipitated at 80% saturation. The pellet was dialysed into 50 mM NaAc (pH=5.2) buffer. For pustulan adsorption and digestion, this extract was mixed with finely dispersed pustulan particles, incubated for 20 minutes at room temperature, and the mixture
 30 centrifuged for 10 minutes at maximum speed in a microfuge

centrifuge. Then the pellets were washed twice in 70 mM Potassium phosphate buffer pH 6.0 containing 1 M NaCl. The enzyme was released by overnight incubation of the particles in a buffer containing 50 mM Kac (pH=5.5) in the presence of protease inhibitors and azide to prevent microbial growth. Further purification of the extract was performed by chromatofocusing on a Mono-P column (Pharmacia), in a buffer containing 25 mM imidazole-HCl with a pH gradient of 7.4 to 4.0. Elution of the Oystermushroom enzyme occurred around pH 5.6. The Shiitake enzyme eluted at pH 4.5. Data obtained with the mentioned purification scheme are depicted in table 3.

Table 3

Oyster mushrooms	Total activity (nkat)	Specific activity (nkat/mg)
Homogenate	233.5	1.43
AS fraction	134.9	2.22
Pustulan fraction	0.68	>50
Mono-P peak	5.4	>180
Shiitake		
Homogenate	60.1	2.6
AS fraction	21.1	0.85
Pustulan fraction	0.26	>30
Mono-P peak	0.46	>230

The crude fungi homogenates contain, besides the β -(1,6)-glucanase activity (see above), also β -(1,3)-glucanase activity and chitinase activity. Specific activities for β -(1,3)-glucanase are 9.1 and 30.2 nkat/mg for Oyster mushrooms and Shiitake respectively. The activities for chitinase are 13.8 and 53.5 OD(550nm) units/mg respectively. The purified proteins (Mono-P fractions) contained no detectable activity for β -(1,3)-glucanase, however, a small amount of chitinase activity was still present.

Both Mono-P peak fractions were subjected to SDS-PAGE. No protein bands were detectable in the Shiitake β -(1,6)-glucanase peak fraction. The Oyster mushroom β -(1,6)glucanase peak fraction

contained two closely spaced protein bands of approximately 50-51kDa after silver staining of the gel. β -(1,6)-glucanase from *Trichoderma* is approximately 43kDa.

5

Example 2**Purification of β (1,6) glucanase from edible mushrooms**

Oyster mushrooms (*Pleurotus ostreatus*) were homogenized at 4°C in 50mM KAc pH 5.0, 0.1% β -mercaptoethanol (mushrooms : buffer = 1.5 : 1 (w/v)), using a Waring blender. The homogenate was
10 centrifuged for 30 minutes at 9,000 g at 4°C in a Sorvall SS34 rotor. Subsequently, solid ammonium sulphate was added gradually to a concentration of 2M. Aggregated proteins were removed by centrifugation for 30 minutes at 9,000 g at 4°C in a Sorvall SS34
15 rotor.

Hydrophobic interaction chromatography

The supernatant was filtered over a paper filter and applied to a phenyl-sepharose 6FastFlow High sub column (1.6x15cm) pre-equilibrated with 2M ammonium sulphate in 50mM potassium-acetate
20 buffer, pH 5.0 at a flow rate of 10 ml/min. The column was washed with at least 10 column volumes of buffer A after which bound protein was eluted with a negative linear gradient ending at 50mM potassium acetate buffer pH 5.0. This was followed by a positive
25 gradient starting with 50mM potassium acetate buffer and ended with the same acetate buffer containing 50% ethylene glycol (flow rate 5 ml/min.). Fractions of 10 ml were collected and assayed for β -(1,6)-glucanase activity. Fractions containing β -(1,6)-glucanase activity were pooled and concentrated in an Amicon ultra-filtration
30 device with a molecular weight cut-off point of 10 kDa.

Cation exchange chromatography

The concentrated protein solution was diluted 10 times with 25mM sodium acetate buffer pH 4.0 and subjected to cation exchange
35 chromatography on a small Resource S column (Pharmacia). Unbound protein was washed off with 2 column volumes of 25mM sodium acetate buffer pH 4.0. Retained proteins were eluted with a linear gradient ending at 250 mM NaCl in 25mM sodium acetate pH 4.0 after 40 column

volumes. Fractions of 2 ml were collected and and assayed for β -(1,6)-glucanase activity. Three protein peaks containing β -(1,6)-glucanase activity were found (see fig. 1). Fractions containing these protein peaks were pooled seperately, resulting in three
5 pools (pool no. 1; 2; 3) containing β -(1,6)-glucanase activity. The pooled protein fractions were brought to pH 5.0 by adding 1/10 volume of 1M potassium acetate pH 5.0. Each pool was concentrated in an Amicon ultra-filtration device with a molecular cut-off point of 10 kDa to about 0.5-1.0 ml.

Gelfiltration chromatography and subsequent chromatofocusing

The pools were subjected to gelfiltration chromatography (Superdex 75, 10/30, Pharmacia), with 200mM NaCl in 50mM potassium
15 acetate, pH 5.0 as the running buffer. The sample volume was 200 μ l; flow rate 0.5 ml/min, fractions of 0.5 ml were collected.

Pool # 1.

The antifungal activity elutes from the gel-filtration column (fig. 2) at about 12kDa, based on standard marker proteins. Comparison of the active fractions with the protein pattern on SDS-PAGE (fig. 3) reveals a 40-45 kDa protein as the most likely
20 candidate for β -(1,6)-glucanase. This result, i.e. the low apparent molecular weight after gel filtration, could be due to an affinity for the column material. It has been reported that fungal cell wall hydrolases can display affinity for Sephacryl supports (Cruz de la, J. et al., J. Bacteriology 177, No 7, 1864-1871, 1995; Cruz de la, J. et al., Eur. J. Biochem. 206, 856-867, 1992; Tangarore, H. et al., Appl. Environ. Microbiol. 55, 177-184, 1989) and thus are
25 retarded to a certain extend on a gel filtration column. β -(1,6)-glucanase containing fractions were pooled, concentrated and subjected to Mono P chromatophocussing chromatography. Starting pH was pH 7.0. Proteins were eluted with a negative pH gradient ending at pH 4.0. The β -(1,6)-glucanase activity eluted from the column at
30 pH 6.7 (fig. 4). SDS-PAGE showed one band with an apparent molecular weight of 40-45kDa (fig. 5).

Pool # 2.

The antifungal activity elutes from the gel-filtration column (fig. 6) at about 88kDa, based on standard marker proteins.

Comparison of the active fractions with the protein pattern on SDS-PAGE (fig. 7) did not result in the determination of a single protein band as the most likely candidate for β -(1,6)-glucanase.

The fraction containing the least protein bands (fraction 16) was subsequently subjected to Mono P chromatofocussing chromatography starting at pH 7.0. Proteins were eluted with a negative pH gradient ending at pH 4.0. The β -(1,6)-glucanase activity was found

in the flow-through of the column, indicating an isoelectric point of 6.8 or higher (fig. 8). SDS-PAGE of the β -(1,6)-glucanase containing fractions (fig. 9) showed two bands. Comparison of this gel (fig. 9) with the SDS-PAGE patterns after gel-filtration chromatography (fig. 7) and the respective β -(1,6)-glucanase

activities, showed that β -(1,6)-glucanase corresponds to a protein band with an apparent molecular weight of about 50kDa. This band is indicated with an arrow in fig. 7 and fig. 9. These fractions did not contain β (1,3) glucanase activity.

Pool # 3.

The antifungal activity elutes from the gel-filtration column at the same position as for pool no.2, 88kDa (fig. 10).

Kinetic properties of β -(1,6)-glucanase activities.

The enzyme activity was measured at different substrate concentrations. A K_m of 0.13mg pustulan per ml was calculated (fig. 11), which is about one order of magnitude lower than the K_m described for β -(1,6)-glucanase from *Trichoderma harzianum* (Cruz de la, J. et al., J. Bacteriology 177, No 7, 1864-1871, 1995; and patent no WO95/31534).

Example 3

In vitro antifungal assay on fungi.

The antifungal activity of proteins is measured in a microtiter plate assay. Each well of a 96-well microtiter dish contained 75 μ l of potato dextrose agar containing 450 spores. The spore solution is added to PDA (0.75% agar, pH 4.9 at 39°C) just

prior to filling of the microtiterplate wells. On top of this, 75µl filter sterilized (0.22 µm filter) protein solution is added. The tests are carried out in microtiter plates at 20°C. At several time points after the initiation of incubation the fungus is monitored in a microtiter plate reader at 620nm.

Synergy in antifungal properties of AP24 and β 1-6 glucanase

Spores of *Fusarium oxysporum*, *Penicillium roqueforti*, or *Paecilomyces variotii* are used in the antifungal assays. AP24 or β -(1-6)-glucanase from *Trichoderma harzianum* are added separately at a final concentration of 5µg/ml or are added in combination (5µg/ml of each). AP24 was purified according to WO91/18984, β -(1-6)-glucanase was purified from a *Pichia pastoris* expression system according to Lora, J.M. et al., Mol. Gen. Genet., 247, 639-645, 1995; Bom, I.J. et al., Biochim. Biophys. Acta, General subjects, submitted 1998. AP 24 alone or β -(1-6)-glucanase alone did not result in inhibition of fungal growth. AP24 in combination with β -(1-6)-glucanase (5µg/ml of each) resulted in strong inhibition of fungal growth (see figures 12, 13, 14).

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- 5 (i) APPLICANT:
- (A) NAME: MOGEN International nv
(B) STREET: Einsteinweg 97
(C) CITY: Leiden
10 (E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 2333 CB
(G) TELEPHONE: (31)-71-5258282
(H) TELEFAX: (31)-71-5221471
- 15 (ii) TITLE OF INVENTION: Antifungal composition, and hosts
incorporating same.
- (iii) NUMBER OF SEQUENCES: 2
- 20 (iv) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
25 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)
- (2) INFORMATION FOR SEQ ID NO: 1:
- (i) SEQUENCE CHARACTERISTICS:
- 30 (A) LENGTH: 741 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
- 35 (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
- 40 (vi) ORIGINAL SOURCE:
- (A) ORGANISM: *Nicotiana tabacum*
- (vii) IMMEDIATE SOURCE:
- 45 (B) CLONE: pMOG404
- (ix) FEATURE:
- (A) NAME/KEY: CDS
50 (B) LOCATION: 1..739

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

5	1	ATG GGC AAC TTG AGA TCT TCT TTT GTT TTC TTC CTC CTT GCC TTG GTG	48
		Met Gly Asn Leu Arg Ser Ser Phe Val Phe Phe Leu Leu Ala Leu Val	
		5 10 15	
10		ACT TAT ACT TAT GCT GCC ACT ATC GAG GTC CGA AAC AAC TGT CCG TAC	96
		Thr Tyr Thr Tyr Ala Ala Thr Ile Glu Val Arg Asn Asn Cys Pro Tyr	
		20 25 30	
		ACC GTT TGG GCG GCG TCG ACA CCC ATA GGC GGT GGC CGG CGT CTC GAT	144
		Thr Val Trp Ala Ala Ser Thr Pro Ile Gly Gly Gly Arg Arg Leu Asp	
		35 40 45	
15		CGA GGC CAA ACT TGG GTG ATC AAT GCG CCA CGA GGT ACT AAA ATG GCA	192
		Arg Gly Gln Thr Trp Val Ile Asn Ala Pro Arg Gly Thr Lys Met Ala	
		50 55 60	
20		CGT GTA TGG GGC CGT ACT AAT TGT AAC TTC AAT GCT GCT GGT AGG GGT	240
		Arg Val Trp Gly Arg Thr Asn Cys Asn Phe Asn Ala Ala Gly Arg Gly	
		65 70 75 80	
25		ACG TGC CAA ACC GGT GAC TGT GGT GGA GTC CTA CAG TGC ACC GGG TGG	288
		Thr Cys Gln Thr Gly Asp Cys Gly Gly Val Leu Gln Cys Thr Gly Trp	
		85 90 95	
30		GGT AAA CCA CCA AAC ACC TTG GCT GAA TAC GCT TTG GAC CAA TTC AGT	336
		Gly Lys Pro Pro Asn Thr Leu Ala Glu Tyr Ala Leu Asp Gln Phe Ser	
		100 105 110	
		GGT TTA GAT TTC TGG GAC ATT TCT TTA GTT GAT GGA TTC AAC ATT CCG	384
		Gly Leu Asp Phe Trp Asp Ile Ser Leu Val Asp Gly Phe Asn Ile Pro	
		115 120 125	
35		ATG ACT TTC GCC CCG ACT AAC CCT AGT GGA GGG AAA TGC CAT GCA ATT	432
		Met Thr Phe Ala Pro Thr Asn Pro Ser Gly Gly Lys Cys His Ala Ile	
		130 135 140	
40		CAT TGT ACG GCT AAT ATA AAC GGC GAA TGT CCC CGC GAA CTT AGG GTT	480
		His Cys Thr Ala Asn Ile Asn Gly Glu Cys Pro Arg Glu Leu Arg Val	
		145 150 155 160	
45		CCC GGA GGA TGT AAT AAC CCT TGT ACT ACA TTC GGA GGA CAA CAA TAT	528
		Pro Gly Gly Cys Asn Asn Pro Cys Thr Thr Phe Gly Gly Gln Gln Tyr	
		165 170 175	
50		TGT TGC ACA CAA GGA CCT TGT GGT CCT ACA TTT TTC TCA AAA TTT TTC	576
		Cys Cys Thr Gln Gly Pro Cys Gly Pro Thr Phe Phe Ser Lys Phe Phe	
		180 185 190	
		AAA CAA AGA TGC CCT GAT GCC TAT AGC TAC CCA CAA GAT GAT CCT ACT	624
		Lys Gln Arg Cys Pro Asp Ala Tyr Ser Tyr Pro Gln Asp Asp Pro Thr	
		195 200 205	
55		AGC ACT TTT ACT TGC CCT GGT GGT AGT ACA AAT TAT AGG GTT ATC TTT	672
		Ser Thr Phe Thr Cys Pro Gly Gly Ser Thr Asn Tyr Arg Val Ile Phe	
		210 215 220	

TGT CCT AAT GGT CAA GCT CAC CCA AAT TTT CCC TTG GAA ATG CCT GGA 720
 Cys Pro Asn Gly Gln Ala His Pro Asn Phe Pro Leu Glu Met Pro Gly
 225 230 235 240

5 AGT GAT GAA GTG GCT AAG T AG 741
 Ser Asp Glu Val Ala Lys
 245

10 (2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 246 amino acids

(B) TYPE: amino acid

15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

20 Met Gly Asn Leu Arg Ser Ser Phe Val Phe Phe Leu Leu Ala Leu Val
 1 5 10 15

25 Thr Tyr Thr Tyr Ala Ala Thr Ile Glu Val Arg Asn Asn Cys Pro Tyr
 20 25 30

Thr Val Trp Ala Ala Ser Thr Pro Ile Gly Gly Gly Arg Arg Leu Asp
 35 40 45

30 Arg Gly Gln Thr Trp Val Ile Asn Ala Pro Arg Gly Thr Lys Met Ala
 50 55 60

Arg Val Trp Gly Arg Thr Asn Cys Asn Phe Asn Ala Ala Gly Arg Gly
 65 70 75 80

35 Thr Cys Gln Thr Gly Asp Cys Gly Gly Val Leu Gln Cys Thr Gly Trp
 85 90 95

Gly Lys Pro Pro Asn Thr Leu Ala Glu Tyr Ala Leu Asp Gln Phe Ser
 40 100 105 110

Gly Leu Asp Phe Trp Asp Ile Ser Leu Val Asp Gly Phe Asn Ile Pro
 115 120 125

45 Met Thr Phe Ala Pro Thr Asn Pro Ser Gly Gly Lys Cys His Ala Ile
 130 135 140

His Cys Thr Ala Asn Ile Asn Gly Glu Cys Pro Arg Glu Leu Arg Val
 145 150 155 160

50 Pro Gly Gly Cys Asn Asn Pro Cys Thr Thr Phe Gly Gly Gln Gln Tyr
 165 170 175

Cys Cys Thr Gln Gly Pro Cys Gly Pro Thr Phe Phe Ser Lys Phe Phe
 55 180 185 190

Lys Gln Arg Cys Pro Asp Ala Tyr Ser Tyr Pro Gln Asp Asp Pro Thr
 195 200 205

Ser Thr Phe Thr Cys Pro Gly Gly Ser Thr Asn Tyr Arg Val Ile Phe
210 215 220

5 Cys Pro Asn Gly Gln Ala His Pro Asn Phe Pro Leu Glu Met Pro Gly
225 230 235 240

Ser Asp Glu Val Ala Lys
245

CLAIMS

1. An isolated protein which has β -(1,6)-glucanase activity, which is obtainable from an edible fungus.
- 5
2. An isolated protein according to claim 1 and which can be purified by performing the following steps:
- a. making an extract of material from an edible fungus;
- b. precipitate the extract with ammonium sulphate
- 10 c. purify the precipitate by adsorption to pustulan particles,
- d. chromatofocus the purified material on a Mono P column, and, if necessary,
- e. gel filtration.
- 15
3. An isolated protein according to claim 1 and which can be purified by performing the following steps:
- a. making an extract of material from an edible fungus;
- b. separating fractions on hydrophobic interaction chromatography;
- c. separating fractions on cation exchange chromatography;
- 20 d. gel filtration; and optionally
- e. chromatofocusing; thereby
- after step b) to e) assaying the fractions obtained on β -(1,6)-glucanase activity and continuing with the fractions found to have said activity.
- 25
4. The protein according to any of claims 1 to 3, characterized in that it has a molecular weight of about 50-51 kD.
5. The protein according to any of claims 1 to 4, characterized
- 30 in that the edible fungus is selected from the group consisting of *Lentinus edodes*, *Volvariella* spp., *Tricholoma matsutake*, *Agaricus* spp., *Tuber uncinatum*, *Tuber melanosporum*, *Tuber* spp., *Stephensia* spp., *Cantharellus* spp., *Pleurotus* spp., *Coprinus* spp., *Lepiota* spp., *Marasmius* spp., *Polysporus* spp., *Verpa* spp., *Tricholoma* spp.,
- 35 *Terfezia* spp., *Morchella* spp., *Cyttaria* spp and fungi routinely used in the production of cheese such as *Penicillium roqueforti* and *P. camemberti*

6. Antifungal composition comprising a β -(1,6)-glucanase.
7. Antifungal composition of claim 6, characterized in that the β -(1,6)-glucanase is the protein according to any of claim 1-5.
- 5 8. Antifungal composition according to claim 6 or 7, further comprising an osmotin.
9. Antifungal composition according to claim 8, characterized in
10 that the osmotin is AP24.
10. Method for the production of pathogen resistant plants by transforming them with a chimeric DNA comprising a sequence coding for a β -(1,6)-glucanase.
- 15 11. Method according to claim 10, characterized in that the plants are also transformed with a chimeric DNA comprising a sequence coding for an osmotin.
12. Method according to claim 11, characterized in that the
20 osmotin comprises the amino acid sequence of SEQ ID NO:2.
13. Method according to claim 12, characterized in that the
25 sequence coding for the osmotin comprises the nucleotide sequence of SEQ ID NO:1.
14. Method according to any of claims 10 to 13, characterized in that the β -(1,6)-glucanase is of fungal origin.
- 30 15. Method according to claim 14, characterized in that the fungus is *Trichoderma harzianum*.
16. Method according to claim 14, characterized in that the
35 fungus is an edible mushroom.

17. Method according to claim 16, characterized in that the fungus is selected from the group consisting of *Lentinus edodes*, *Volvariella* spp., *Tricholoma matsutake*, *Agaricus* spp., *Tuber uncinatum*, *Tuber melanosporum*, *Tuber* spp., *Stephensia* spp.,
5 *Cantharellus* spp., *Pleurotus* spp., *Coprinus* spp., *Lepiota* spp., *Marasmius* spp., *Polysporus* spp., *Verpa* spp., *Tricholoma* spp., *Terfezia* spp., *Morchella* spp., *Cyttaria* spp and fungi routinely used in the production of cheese such as *Penicillium roqueforti* and *P. camemberti*.
- 10 18. Method according to any of claims 10-17 characterized in that the osmotin or the β -(1,6)-glucanase is targeted to the extracellular space.
- 15 19. Method according to any of claims 10-18, characterized in that the plants are also transformed with a sequence coding for a protein selected from the group consisting of chitinases, β -(1,3)-glucanases, chitobiases, N-acetyl- β -glucosaminidases, basic PR-1, antifungal proteins (AFP's), phospholipase B, proteases, defensins,
20 cecropins, thionins, mellitins, magainins, attacins, dipterins, cacrutins, xenopsins, and hybrids thereof.

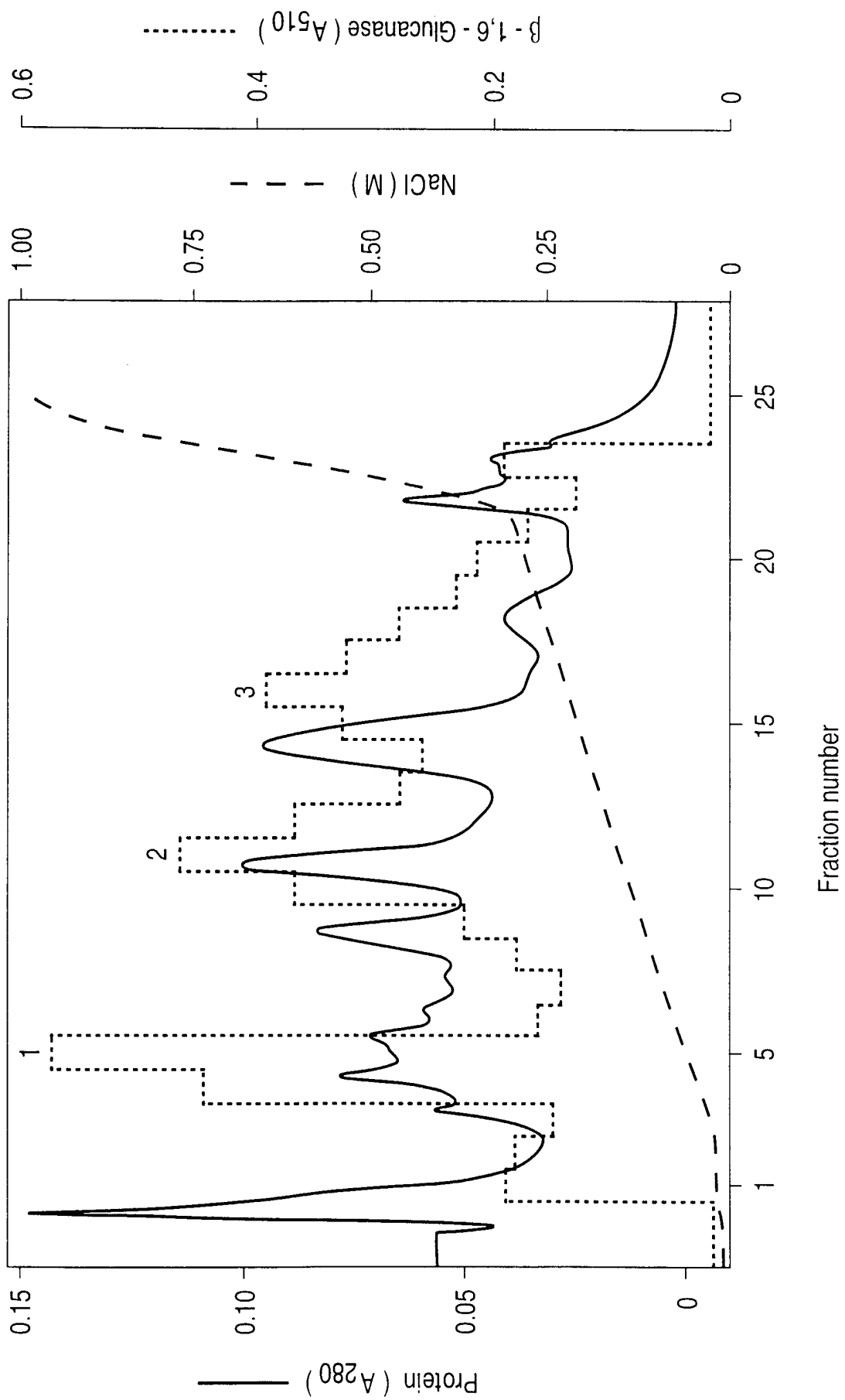


Fig. 1

Gelfiltration on SD75 of pool 1

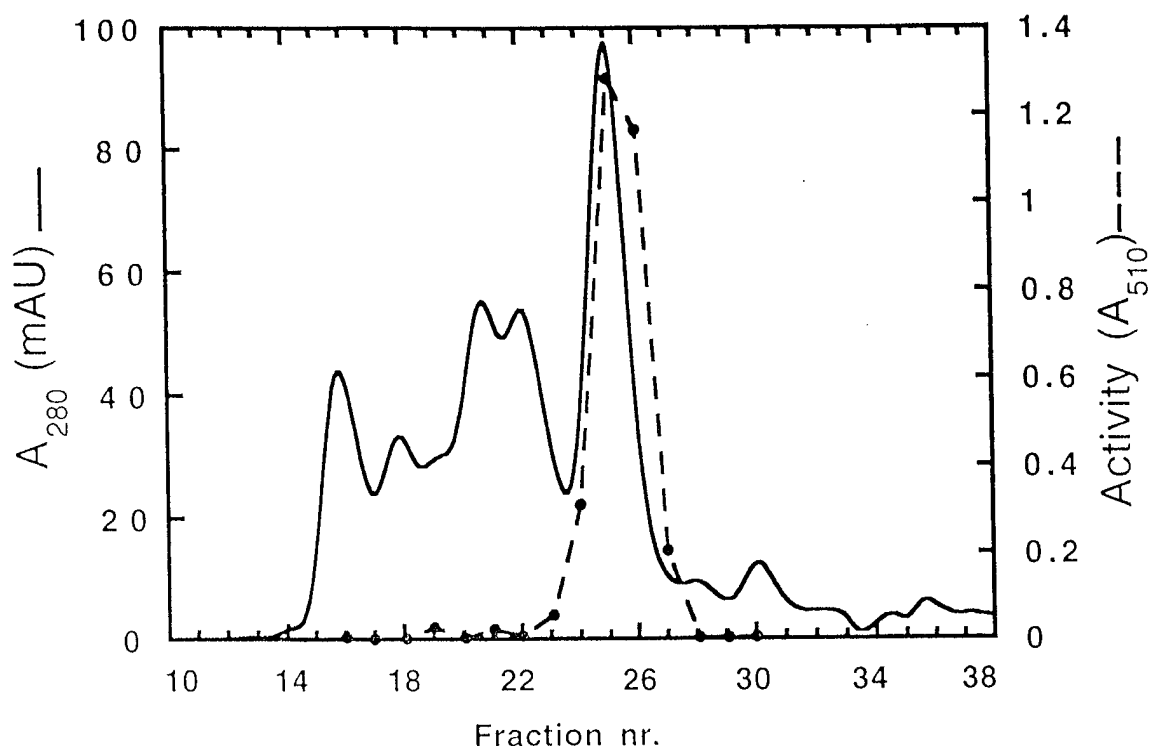


Fig. 2

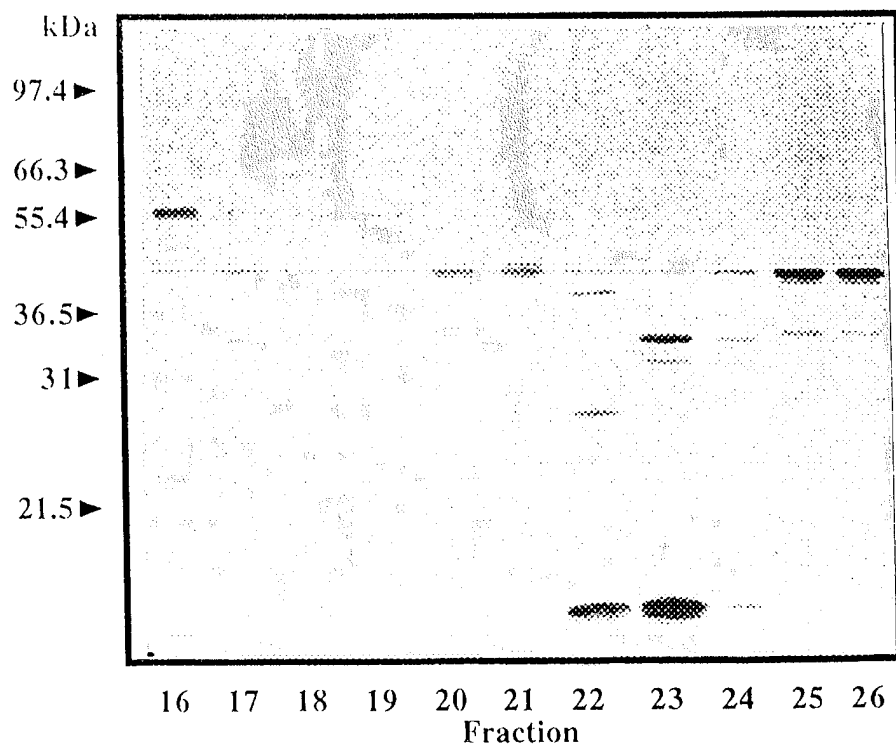


Fig. 3

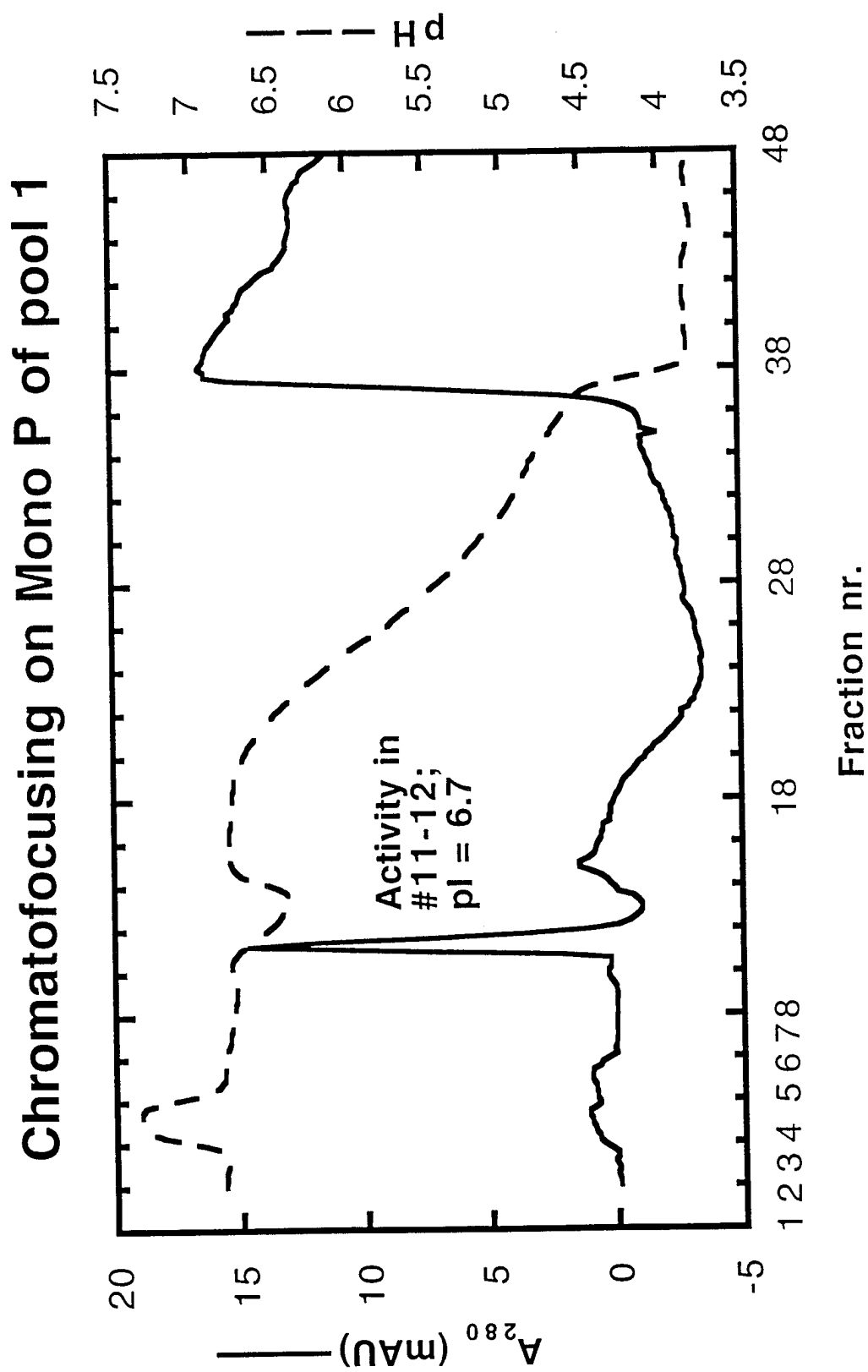
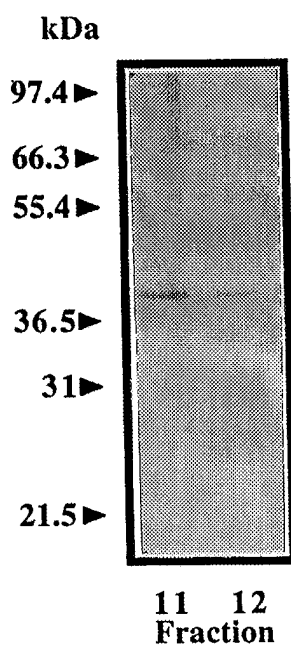


Fig. 4

**Fig. 5**

Gelfiltration on SD75 of pool 2

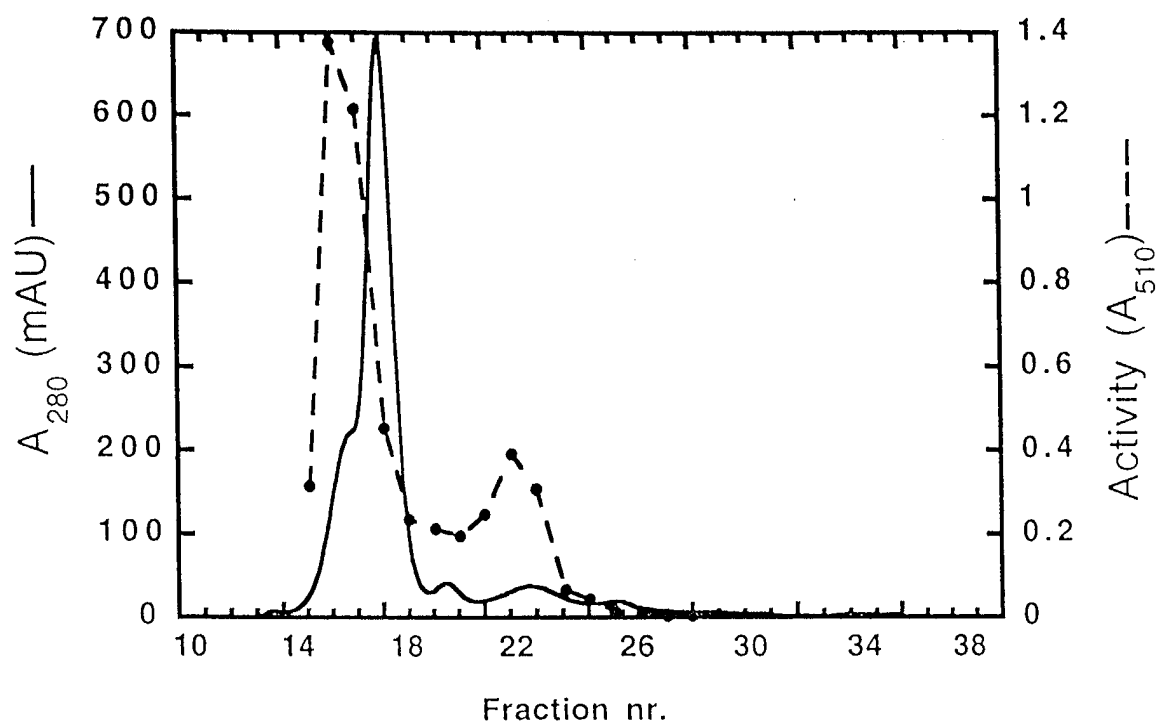


Fig. 6

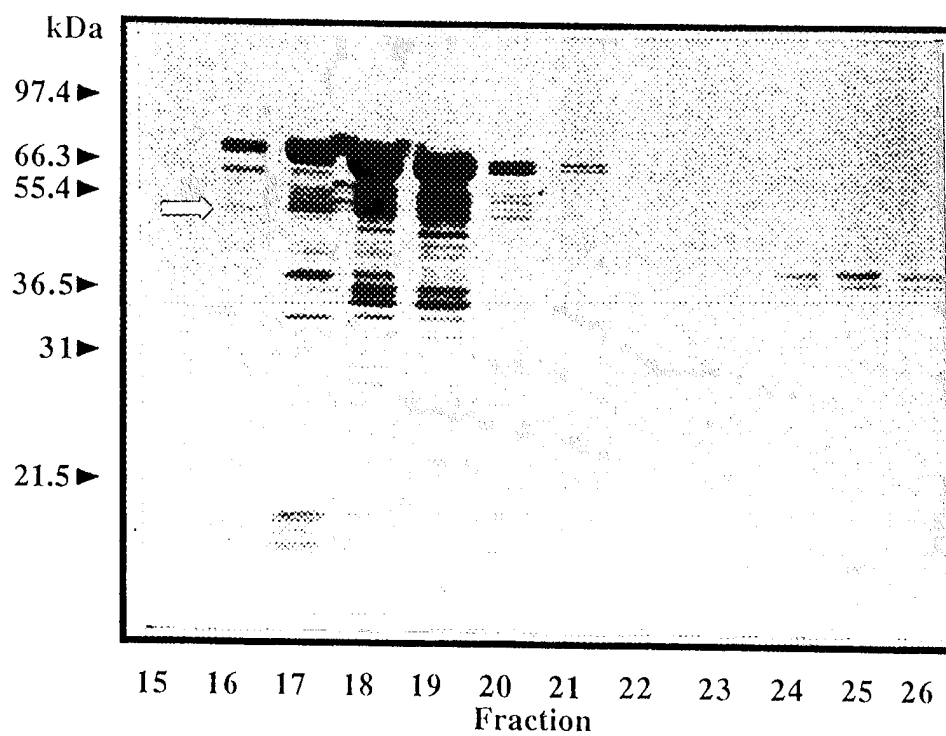


Fig. 7

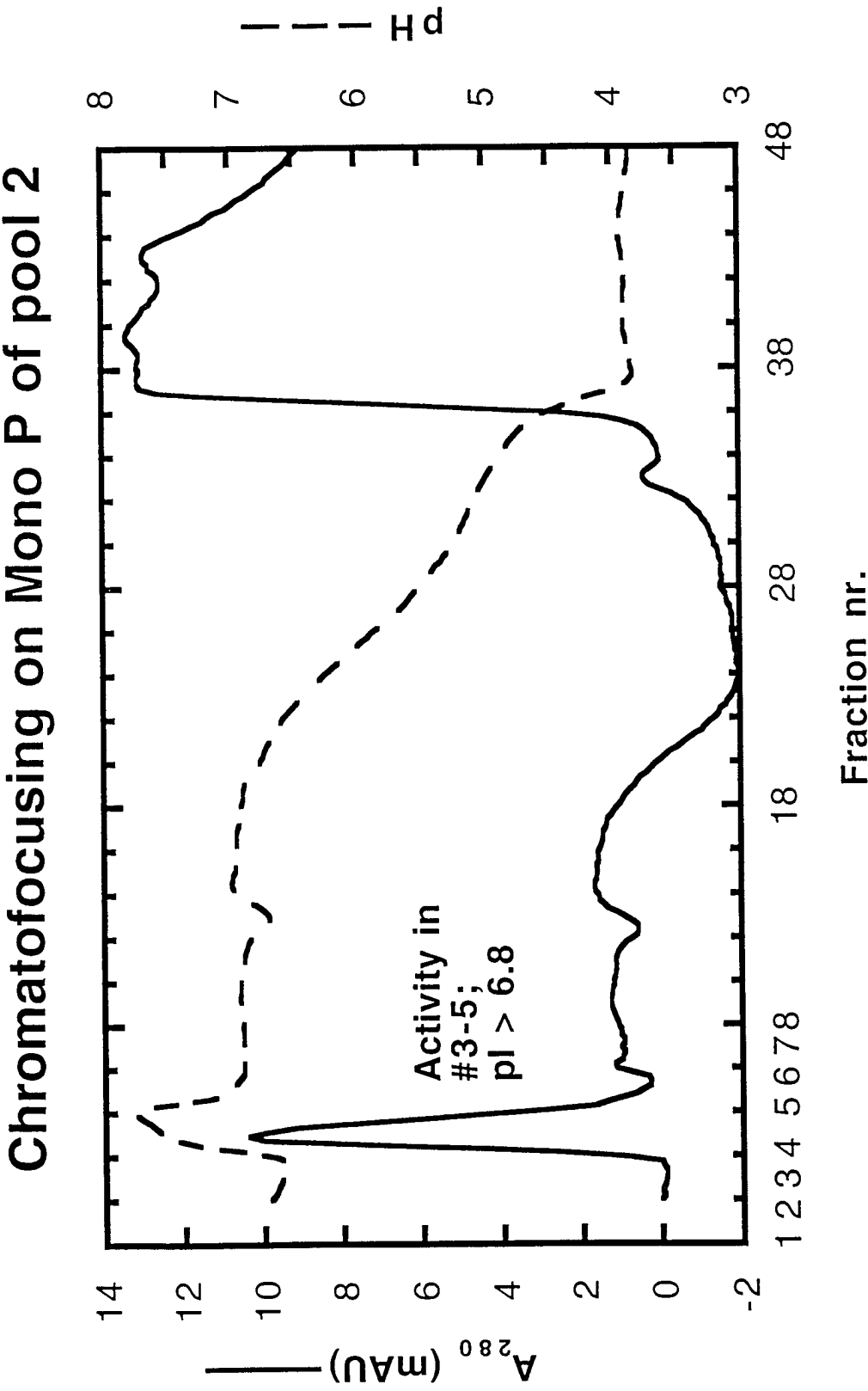


Fig. 8

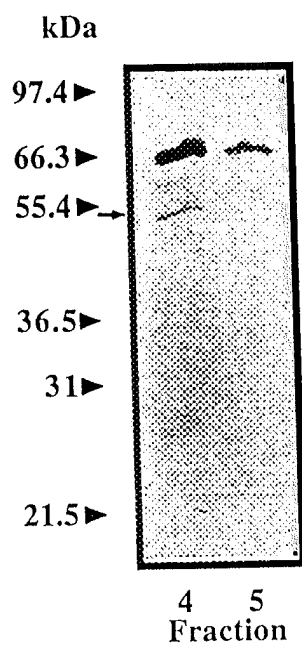


Fig. 9

Gelfiltration on SD75 of pool 3

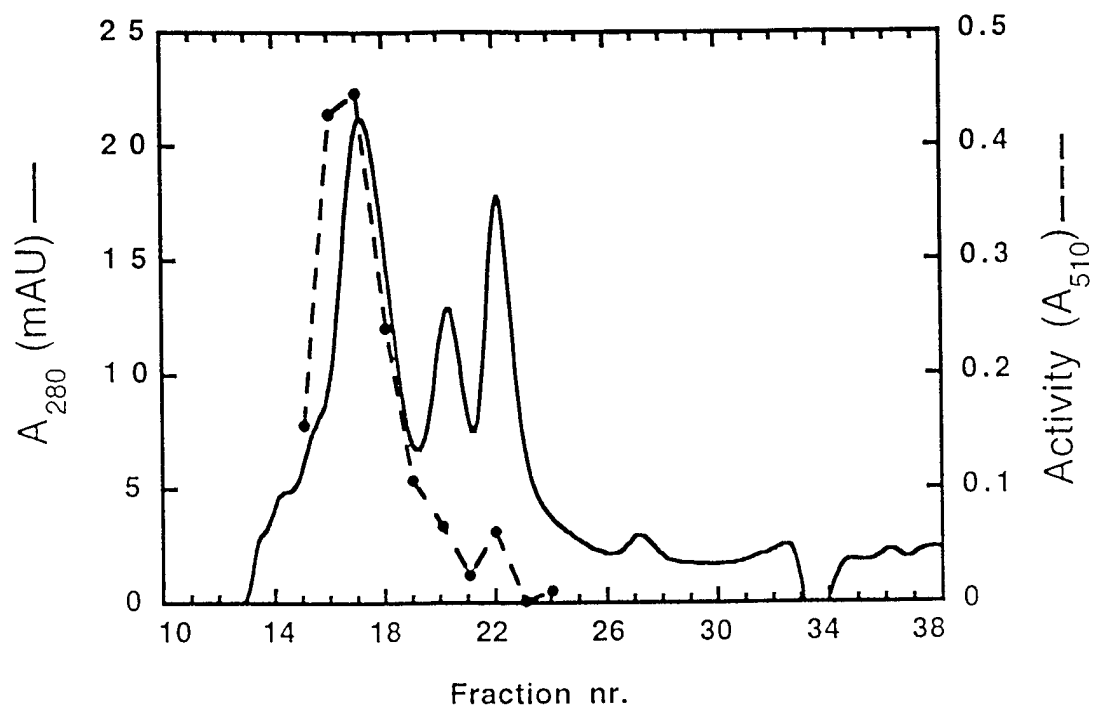


Fig. 10

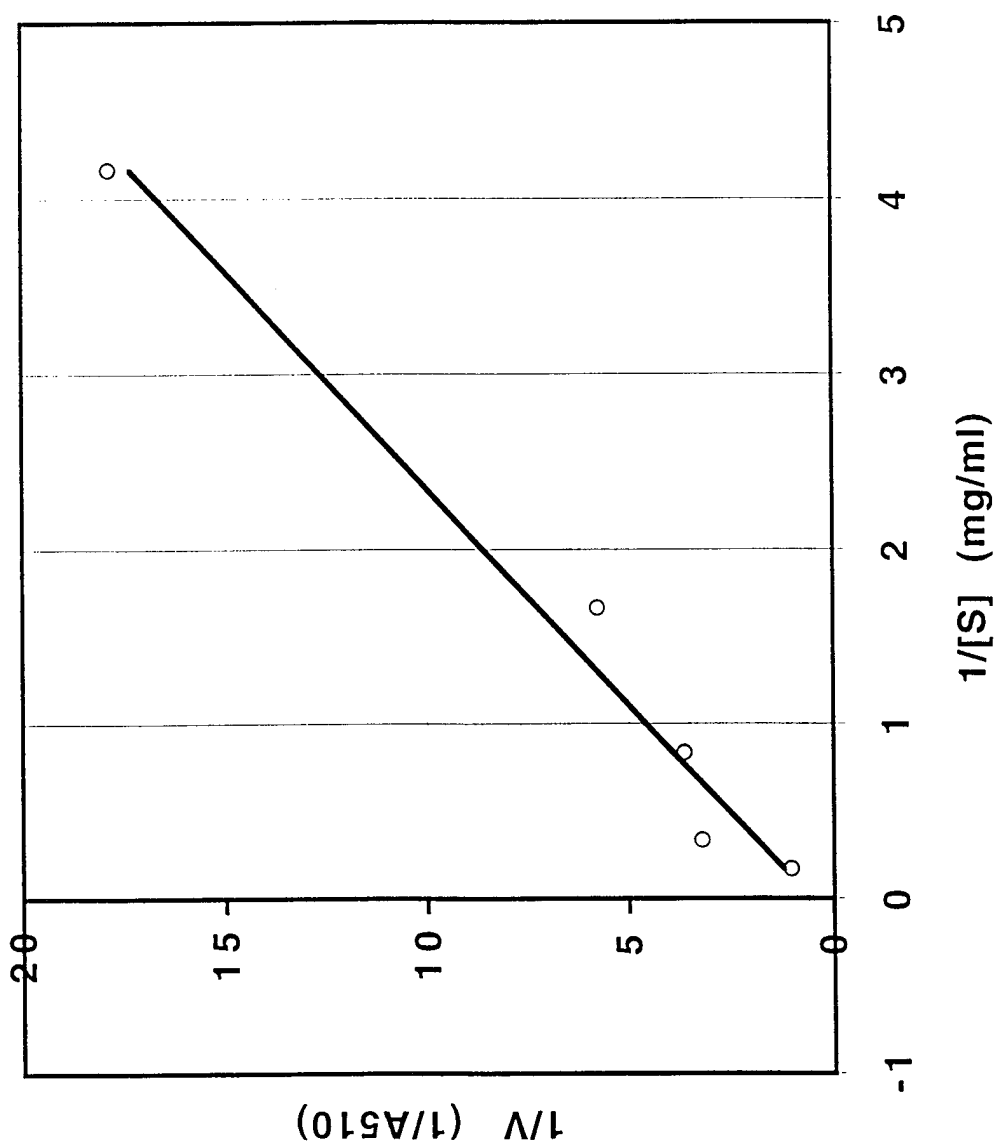
Lineweaver-Burk plot β 1,6-Glucanase pool 2

Fig. 11

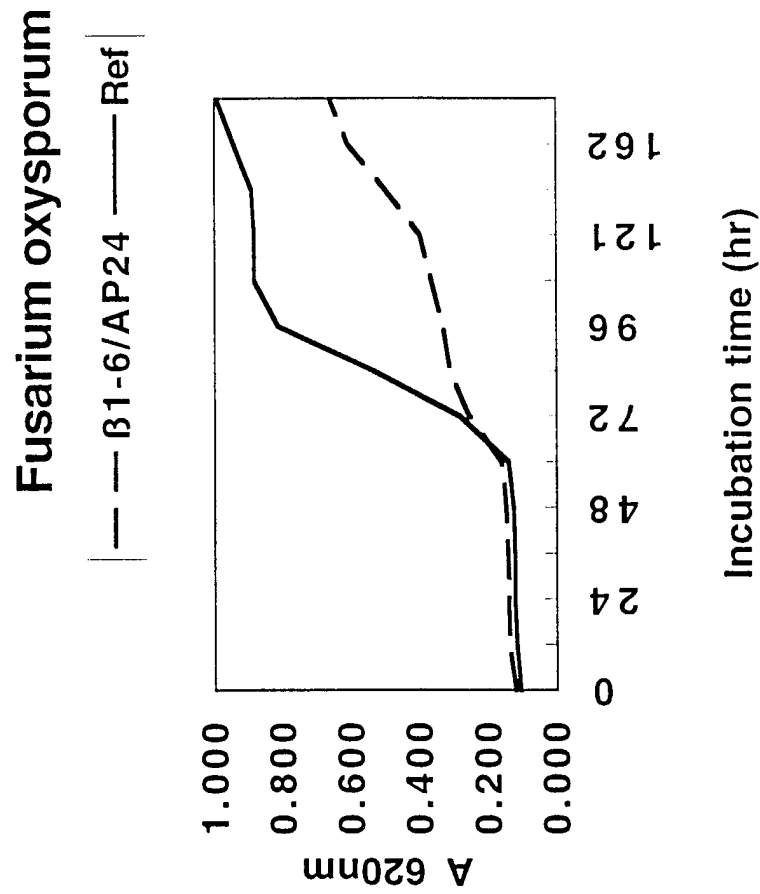


Fig. 12

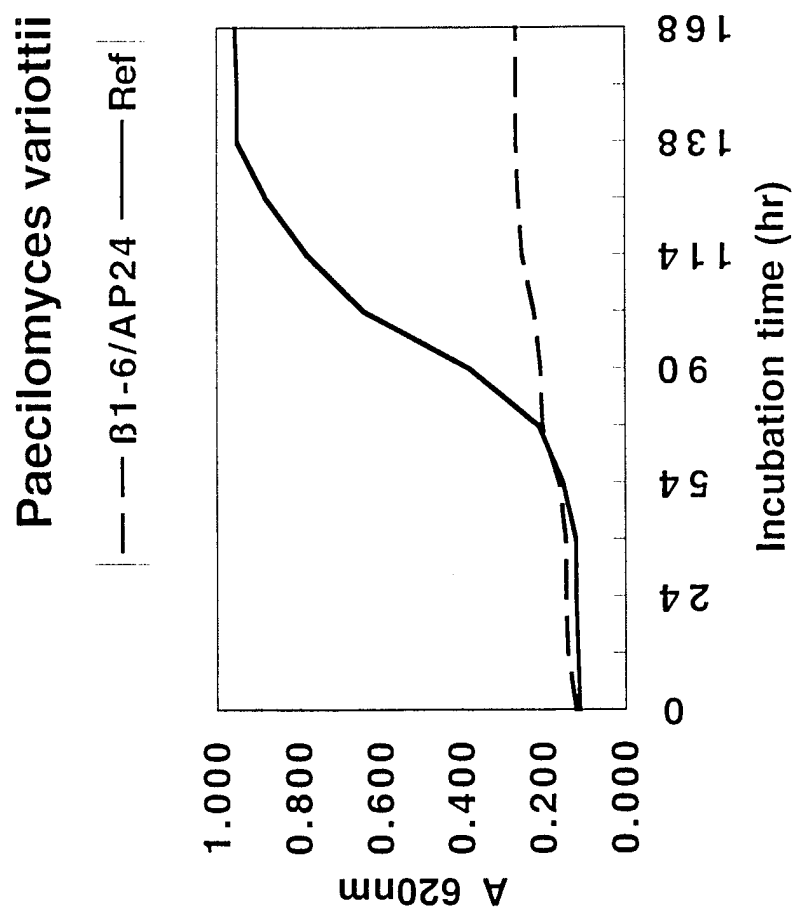


Fig. 13

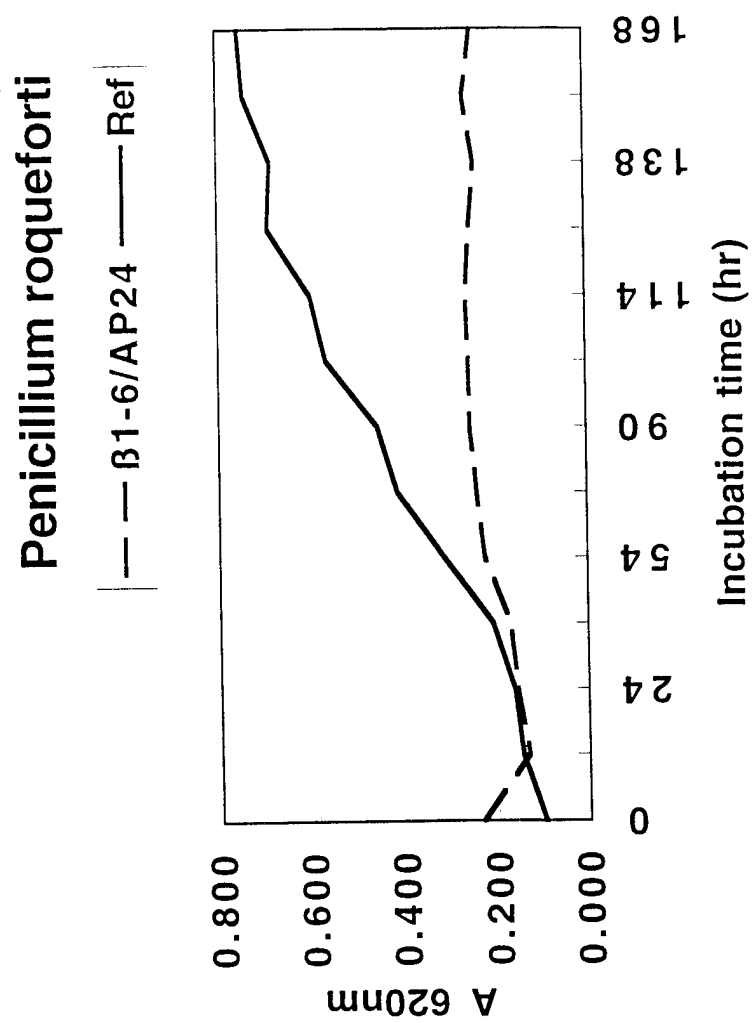


Fig. 14

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/02580

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/82 C12N15/56 C12N9/24 A01N63/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 31534 A (NOVO NORDISK A/S) 23 November 1995 cited in the application	1,4-7
Y	abstract; page 1,2; page 3, line 22; page 6,7; page 8, line 1-11; page 13, line 17; page 14, line 4; examples 3,12; page 34; claim 4,21,27	10,14,15
X	--- MULENGA, D.K., ET AL. : "isolation and characterization of a unique endo-beta-1,6-glucanase from yeast Saccharomycopsis fibuligera NCYC 451" MICROBIOS, vol. 80, no. 324, 1994, pages 143-154, XP002076682 see the whole document --- -/--	1,4

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

10 September 1998

Date of mailing of the international search report

25/09/1998

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/02580

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 02319 A (SALK INST FOR BIOLOGICAL STUDI) 26 January 1995 page 3-8; page 9, line 1-10; examples; claim 9 ---	10, 14, 15
A	WO 94 24271 A (CORNELL RES FOUNDATION INC) 27 October 1994 pages 1,3,4; examples 2 and 3 ---	1-19
A	US 4 748 021 A (CHET ILAN ET AL) 31 May 1988 see the whole document ---	1-19
A	WO 94 08010 A (MONSANTO CO) 14 April 1994 page 2; page 2, line 18-20; page 10-11; page 14-16 ---	1-19
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P,X	PANDEY M ET AL: "Expression and characterization of Pichia etchellsii beta -glucosidase in Escherichia coli" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, vol. 190, no. 1, 29 April 1997, page 45-51 XP004064383 see the whole document -----	1,4

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Information on patent family members

International Application No

PCT/EP 98/02580

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