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(54) Title: LYTIC ENZYMES USEFUL FOR TREATING FUNGAL INFECTIONS

(57) Abstract: The present invention features a new method for isolating and purifying lytic enzymes useful for treating fungal infections from *Trichoderms*. The present invention further features methods of treating fungal infections in mammals including humans by administering one or more lytic enzymes and compositions comprising the same.

LYTIC ENZYMES USEFUL FOR TREATING FUNGAL INFECTIONS

FIELD OF THE INVENTION

The present invention is in the field of medicine, carbohydrate chemistry and biochemistry. Specifically, the present invention features a new method for isolating and purifying lytic enzymes from *Trichoderma spp.* The present invention further features methods of treating fungal infections in animals and humans by administering lytic enzymes and compositions comprising the same.

BACKGROUND OF THE INVENTION

Carbohydrates play many important roles in the functioning of living organisms. In addition to their metabolic roles, carbohydrates are structural components of the cell walls and membranes of plants, animals and microbes. Fungal cell walls commonly contain polymers of D-glucose called glucans and polymers of nitrogen containing N-acetyl-D-glucosamine called chitin. Bartnicki-Garcia, *Ann Review Microbiol.* 22:87-108 (1968). Glucans may be chemically linked in various ways, most commonly through anomeric (or) C1 carbons to the C2, C3, C4 or C6 carbons of a neighboring glucose moiety. This variation in linkage may be expressed as a " 1-3 glucan" or a " -6 glucan." Chitins, however, are only linked in a 1-4 fashion. The glucans and chitins of fungal cell wall are primarily involved as structural components, maintaining rigidity and conferring protection. They may also be degraded and used as nutritional sources after exhaustion of external nutrients.

While the majority of the fungi carry out essential activities in nature, some are pathogenic to plants, animals and humans. Fungi cause diseases in animals and humans through several mechanisms. First, some fungi elicit immune responses that can result in allergic reactions. For example, asthma and other hypersensitivity reactions are caused by exposure to specific fungi antigens in the environment. A second fungal disease-causing mechanism involves toxins generated by fungi. For example, afatoxin produced by *Aspergillus flavus* is highly toxic and induces tumors in some animals. The third mechanism of fungal related disease is through infection. The growth of a fungus on or in the body can cause symptoms that range in severity from relatively innocuous, superficial diseases to serious, life-threatening diseases. In 1996 there were over a million serious fungal infections in the United States. The problem of serious invasive fungal infections has increased with advances in transplantation technology and the growing numbers of patients with immunosuppressive viral infections. *Aspergillus*

infections are common in up to 5% of immunosuppressed patients with mortality rates approaching 90%.

Conventional anti-fungal agents include topical antiseptic chemicals used for non-invasive infections and polyene and azole antibiotics which inhibit fungal cell wall sterol biosynthesis. Polyenes produced by streptomyces species bind to ergosterol (euqivalent of cholesterol in higher eukaryotic cell membranes), which disrupts the normal membrane function and eventually causes membrane permeability and cell death. Azole is a group of antibiotics that selectively inhibit ergosterol biosynthesis. The treatment with azoles results in the inability of fungi to produce a normal membrane, leading to membrane damage and alteration of critical membrane activities. Polyenes and azoles have satisfactory effects on common species of fungi such as *Candida, Histoplasma* and *Coccidiodes*, but they exhibit little activity against the invasive *Aspergillus* which are a common cause of death in the immunosuppressed population. In addition, treatment of fugal infection based on polyenes and azoles is often toxic and has many undesirable side effects. The use of the existing anti-fungal agents has also resulted in the emergency of populations of resistant fungi and the emergency of new pathogenic fungi strains.

Thus, it is advantageous to provide novel antibiotics with different mechanisms of action from those in the prior art. These novel antibiotics may be used to broaden the scope of anti-fungal treatment and complement the activities of known anti-fungal agents. They may also be used to treat fungal infections in humans and animals which are resistant to conventional drugs. The use of lytic enzymes to degrade fugal cell walls as the basis for anti-fugal treatment in animals and humans is the focus of the instant invention.

Many fungi secrete lytic enzymes into their environment as a means to generate food sources from polysaccharides or to gain competitive advantage in their microenvironment by inhibiting the growth of other fungi or parasitizing their neighbors. Haran *et al.*, Microbiology 142:2321-2331 (1996). Archer *et al., Crit. Rev. Biotechnol.* 17(4):273-306 (1997). The inhibition of fungal growth is attributed to lytic enzymes that degrade fugal cell walls and eventually lead to fungal cell lysis. Fungal lytic enzymes include glucanase, chitinases, proteases, lipase, and other hydrolytic enzymes (glucanase and chitinase are glucan-degrading and chitin-degrading enzymes respectively). These lytic enzymes can be further divided into subcategories according to their modes of degradation reaction and type of linkage(s) they degrade, such as, endo- or exo- enzymes

and (1,3)-β- or (1,4)-β- enzymes. Lytic enzymes function in an endo fashion cleave the polymeric linkage at random sites along the polysacchride chain. Those that function in an exo- fashion cleave subsequent polymeric unit from the end. Glucanase and chitinase such as endo and exo (1,3)-β-glucanases, endo and exo (1,4)-β-glucanases, endo and exo (1,6)-β-glucanases, endochitinases, exochitinases, chitobiohydrolases, endochitosanases, exochitosanases, 1,4-β-poly-N-acetyl-D-glucosaminidase, and endo and exo 1,4-β-poly-D-glucosaminidase have been detected in a wide range of fungi species.

Considerable research effort has focused on the studies of lytic enzymes produced by *Trichoderms*. These are common fungi found in almost any soil. They are strongly antagonistic to other fungi. The antagonism is in part due to their secretion of lytic enzymes, such as glucanases, chitinases and proteases, to degrade cell walls of other fungi and in turn utilize their nutrients. 1,3- -glucan is one of the main structural components of the fungal cell wall, and 1,3- -glucanases are secreted by a number of *Trichoderma* species. Kitamoto *et al., Agric. Biol. Chem.* 51:3385-3385 (1987); Dubourdieu *et al., Carbohydr. Res.* 144:277-287(1985); Lorito *et al., Phytopathology* 84:398-405 (1994); Del Rey *et al., J. Gen. Microbiol.* 110:83-89 (1979).

Many of the 1,3- -glucanases have been extensively characterized and studied, and many of their encoding genes have been identified and cloned. Their involvement in biological control and plant defense mechanisms against fungi have also been well documented. Haran *et al., Microbiology* 142:2321-2331 (1996). 1,6- -glucanases have been shown to lyse yeast and fungal cell walls. Relatively little information is reported with respect to their purification, characterization, and anti-fungal activities. Haran *et al., Microbiology* 142:2321-2331 (1996). *Trichoderma harzianum* was shown to produce at least two extracellular 1,6- glucanase. De la Cruz *et al.* were the first to purify one of the two 1,6- glucanases to homegeneity and to study their hydrolytic activity against fungal cell walls. De la Cruz *et al., J. Bateriol.* 177:6937-6945 (1995). Several chitinases are secreted by *Trichoderma harzianum*. Many of the chitinases have been identified and purified to homogeneity or near homogeneity. Haran *et al., Microbiology* 142:2321-2331 (1996) Several chitinase encoding genes have also been cloned and overexpressed. De La Cruz *et al.*, Eur. J. Biochem. 206:859-867 (1992).

A variety of glucases, chitinases, proteases and other hydrolytic enzymes produced by *Trichoderma* species have been implicated in the biological control of plant fungal pathogens. These lytic enzymes have not been used for treating fungal infections

in vivo. The treatment based on these lytic enzymes disclosed herein offers a new approach to fighting fungal infections, especially against the more invasive and resistant fungal infections. Because humans and animals are not known to have glucan or chitin structures like those of lower animals and microbes, glucanases, chitinases and proteases should not display significant toxicity or undesirable biologic effects in humans or animals.

SUMMARY OF THE INVENTION

In general, the present invention features a new method for isolating and purifying lytic enzymes useful for treating fungal infections from *Trichoderms*. The present invention further features methods of treating fungal infections in mammals including humans by administering one or more lytic enzymes and compositions comprising the same.

In one embodiment, novel methods for isolating and purifying lytic enzymes from *Trichoderms* is disclosed. Specifically, the novel methods comprise the steps of (i) precipitating the cellular material from a *Trichoderma* species, (ii) isolating the proteins therefrom, (iii) precipitating the lytic enzyme by additing its substrate(s), and (iv) purifying the enzyme by isoelectric focusing. The method disclosed herein is applicable to a variety of lytic enzymes from *Trichoderma* species, and may be practiced on a variety of species to isolate a variety of lytic enzymes without undue experimentation. Exemplary lytic enzymes according to the present invention include glucanases, chitinases, chitosanases and proteases. In preferred embodiments, the present method is applied to isolating and purifying 1-6 glucanase from *Trichoderma harzianum*.

In a second embodiment, the present invention features methods for treating mammals including humans suffering from fungal or mycoparasitic infections by administering a pharmaceutically effective amount of one or more lytic enzymes such as those obtained from a *Trichoderm*. Lytic enzymes produced by *Trichoderms* useful for treating fungal and mycoparasitic infections include glucanases, chitinases, chitosanases and proteases. They may be administered *in vivo* to organisms in order to treat, eliminate or prevent infection by organisms possessing a cell wall such as fungi. Exemplary fungal species that may be treated by the compounds of the present invention include, for example, *Aspergillus* infections.

In a third embodiment, the present invention encompasses cellular transformation vectors containing nucleic acid sequences encoding therapeutic lytic enzymes from *Trichoderma* species. The enzymes according to the present invention may be administered by genetic therapy techniques wherein a nucleotide encoding the therapeutic compound is administered to a cell or to an organism in order to produce the therapeutic compound endogenously. Those of skill in the art will appreciate many methods for administering transformation vectors containing nucleic acid sequences encoding therapeutic lytic enzymes.

In a fourth embodiment, the present invention features pharmaceutical compositions containing lytic enzyme(s) useful for treating fungal infections. The lytic enzymes of the present invention may be administered alone or in pharmaceutically acceptable compositions to treat infections caused by organisms sensitive to their activities, such as fungi possessing a cell wall. In preferred embodiments, lytic enzymes isolated from *Trichoderma harzianum* are particularly effective against fugal infection. In particularly preferred embodiments, a β-1,6-glucanase isolated from *Trichoderma harzianum* has demonstrated anti-fungal activity in mammals against *Aspergillus* infection.

Such compositions may be formulated so as to be adapted to the specific method of administration. Such compositions may be optimized for administration of the enzyme by parenteral, topical or oral administration. Additionally, the enzyme may be administered by cellular transformation vectors containing nucleic acid sequences encoding therapeutic lytic enzymes.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the survival rate after glucanase treatment of *Aspergillus* infection in mice.

Figure 2 illustrates the survival statistics for up to 18 days post inoculation among subjects treated with PBS intraperitoneal, chitinase intraperitoneal at a dosage of 100 mg/kg, chitinase intravenous at a dosage of 100 mg/kg, chitinase intraperitoneal at a dosage of 25 mg/kg, and amphotericin B intraperitoneal at a dosage of 1 mg/kg.

DETAILED DESCRIPTION OF THE INVENTION

The present invention features novel methods for isolating and purifying lytic enzymes from *Trichoderms*. Moreover, the present invention features methods of treating fungal infections in mammals including humans by administering lytic enzymes and compositions comprising the same.

The novel methods for isolating and purifying lytic enzymes from *Trichoderms* comprise the steps of (i) precipitating the cellular material from a *Trichoderma* species, (ii) isolating the proteins therefrom, (iii) precipitating the enzyme by addition of its substrate(s), and (iv) purifying the enzyme by isoelectric focusing. The lytic enzymes that may be isolated and purified by the present invention include glucanases, chitinases, chitosanases and proteases. Exemplary lytic enzymes include, but are not limited to endo and exo (1,3)-β-glucanases, endo and exo (1,4)-β-glucanases, endo and exo (1,2)-β-glucanases, endo and exo (1,6)-β-glucanases, endochitinases, exochitinases, chitobiohydrolases, endochitosanases, exochitosanases, 1,4-β-poly-N-acetyl-D-glucosaminidase, and endo and exo 1,4-β-poly-D-glucosaminidase. The invention is also specifically intended to encompass lytic enzymes from *Trichoderms* such as, but not limited to, *T. atroviride, T. cirtinoviride, T. hamatum, T. harzianum, T. koningii, T. lignorum, T. longibrachiatum, T. polysporum, T. pseudokoningii, T. reesei, T. saturnisporum, T. todica, T. virgatum* and *T. viride*. In some preferred embodiments, the present method has been successfully applied to isolating and purifying a 1-6 glucanase enzyme from *Trichoderma harzianum*. This particular enzyme has a molecular weight of about 43,000 daltons and an isoelectric point of about 5.8.

The present invention also provides novel methods and compositions for treating a variety of fungal and other microbial diseases in mammals including humans by administering one or more lytic enzymes according to the present invention. The present invention differs substantially from many other forms of medical therapy for fungal infections because conventional therapy methods use small molecules that inhibit fungal cell wall and membrane sterol synthesis. Because of the mechanisms of action, the existing methods have significant toxicity and side effects in the recipients. The instant invention features a new mechanism for treating fungal infections by administering a pharmaceutically effective amount of one or more lytic enzymes useful to degrade fungal cell walls and to eventually cause fungal cell lysis and death. The instant method is especially designed to target invasive forms of fungal infection for which the existing

methods are not optimally effective. In addition, the instant method is also effective in treating fungal infections that are resistant to the existing methods of treatment. In preferred embodiments, a 1-6 glucanase enzyme isolated from *Tricoderma harzianum,* shown to possess potent anti-fungal activity in in vitro assays, is administered. In some preferred embodiments, a lytic enzyme according to the invention is administered to effectively treat *Aspergillus* infection.

In another aspect, the present invention also provides pharmaceutical compositions comprising one or more lytic enzyme(s). Therapeutic enzymes may be administered in a number of ways such as parenteral, topical, intranasal, inhalation or oral administration. In some embodiments, the invention provides for administering the enzyme in a pharmaceutical composition together with a pharmaceutically-acceptable carrier which may be solid, semi-solid or liquid or an ingestible capsule. Examples of pharmaceutical compositions useful in the present invention include tablets and drops, such as nasal drops. Compositions for topical application include, but are not limited to ointments, jellies, creams and suspensions, aerosols for inhalation, nasal spray, and liposomes. One or more lytic enzyme will comprise between 0.05 and 99% or between 0.5 and 99% by weight of the composition. In preferred embodiments, the enzyme content may be between 0.5 and 20% for injection and between 0.1 and 50% for oral administration.

To produce pharmaceutical compositions for oral application containing the therapeutic lytic enzyme(s), the enzyme(s) may be mixed with a solid, pulverulent carrier. The carrier may include, but is not limited to lactose, saccharose, sorbitol, mannitol, a starch (for example, a potato starch or a corn starch), amylopectin, laminaria powder, citrus pulp powder, a cellulose derivative and gelatine. The pharmaceutical compositions may also include lubricants such as magnesium or calcium stearate or a Carbowax or other polyethylene glycol waxes, and they may be compressed to form tablets or cores for dragees. If dragees are required, the cores may be coated with, for example, a concentrated sugar solution. The sugar solutions may contain gum arabic, talc and/or titanium dioxide, or alternatively a film forming agent dissolved in easily volatile organic solvents or mixtures of organic solvents. Dyestuffs may be added to such coatings, for example, to distinguish between different contents of active substance. For a composition of soft gelatine capsules consisting of gelatine, or glycerol as a plasticizer, or similar closed capsules, the active substance may be admixed with a Carbowax® or a suitable oil such as sesame oil, olive oil, or arachis oil. Hard gelatine capsules may contain

granulates of the active substance with solid, pulverulent carriers such as lactose, saccharose, sorbitol, mannitol, starches (for example, potato starch, corn starch or amylopectin), cellulose derivatives or gelatine, and they may also include magnesium stearate or stearic acid as lubricants.

Therapeutic lytic enzymes of the present invention may also be administered parenterally such as by subcutaneous, intramuscular or intravenous injection or by sustained release subcutaneous implant. In subcutaneous, intramuscular and intravenous injection, a therapeutic enzyme or other active ingredient may be dissolved or dispersed in a liquid carrier vehicle. For parenteral administration, the active material may be suitably admixed with an acceptable vehicle, preferably of the vegetable oil variety such as peanut oil, cottonseed oil and the like. Other parenteral vehicles such as organic compositions using solketal, glycerol, formal, and aqueous parenteral formulations may also be used. For parenteral application by injection, compositions may comprise an aqueous solution of a water soluble pharmaceutically acceptable salt of the active acids according to the invention, desirably in a concentration of 0.5 -10%, and optionally also a stabilizing agent and/or buffer substances in aqueous solution. Dosage units of the solution may advantageously be enclosed in ampoules. When therapeutic enzymes are administered in the form of a subcutaneous implant, the compound may be suspended or dissolved in a slowly dispersed material known to those skilled in the art or administered in a device which slowly releases the active material through the use of a constant driving force such as an osmotic pump. In such cases, administration over an extended period of time may be possible.

For topical application, the pharmaceutical compositions are suitably in the form of an ointment, gel, suspension, cream or the like. The amount of active substance may vary, for example between 0.05- 20% by weight of the active substance. Such pharmaceutical compositions for topical application may be prepared in known manners by mixing the active substance with known carrier materials including but not limited to isopropanol, glycerol, paraffin, stearyl alcohol, polyethylene glycol. The pharmaceutically acceptable carrier may also include a known chemical absorption promoter. Examples of absorption promoters are dimethylacetamide (U.S. Patent No. 3,472,931), trichloro ethanol or trifluoroethanol (U.S. Patent No. 3,891,757), certain alcohols and mixtures thereof (British Patent No. 1,001,949). A carrier material for topical application to unbroken skin is also described in the British patent specification No. 1,464,975, which discloses a carrier material consisting of a solvent comprising 40

70% (v/v) isopropanol and 0 60% (v/v) glycerol, the balance, if any, being an inert constituent of a diluent not exceeding 40% of the total volume of solvent.

The dosage at which pharmaceutical compositions containing one or more lytic enzymes are administered may vary within a wide range and depends on various factors, such as the severity of the infection and the age of the patient. The dosage may have to be individually adjusted. In preferred embodiments, the amount of therapeutic enzyme is from about 0.1 mg to about 2000 mg or from about 1 mg to about 2000 mg per day. The pharmaceutical compositions containing a therapeutic lytic enzyme may suitably be formulated so that they provide doses within these ranges either as single dosage units or as multiple dosage units. In addition to containing a therapeutic lytic enzyme (or therapeutic lytic enzymes), the pharmaceutical compositions may contain one or more substrates or cofactors for the reaction catalyzed by the therapeutic enzyme in the compositions.

The therapeutic lytic enzymes according to the present invention may be administered by means of transforming patient cells with nucleic acids encoding a therapeutic enzyme when the therapeutic enzyme is a protein or ribonucleic acid sequence. A nucleic acid sequence encoding a therapeutic lytic enzyme may be incorporated into a vector for transformation into cells of a subject to be treated. A vector may be designed to integrate into the chromosomes of the subject, for example, retroviral vectors, or to replicate autonomously in the host cells. Vectors containing nucleotide sequences encoding a therapeutic lytic enzyme may be designed to provide for continuous or regulated expression of the enzyme. Additionally, the genetic vector encoding the therapeutic enzymes may be designed to stably integrate into the cell genome or to only be present transiently. The general methodology of conventional genetic therapy may be applied to polynucleotide sequences encoding therapeutic enzymes. Reviews of conventional genetic therapy techniques can be found in Friedman, *Science 244*:1275-1281 (1989); Ledley, *J. Inherit. Metab. Dis.* 13:587-616 (1990); and Tososhev *et al.*, *Curr Opinions Biotech.* 1:55-61 (1990).

EXAMPLES OF THE PREFERRED EMBODIMENTS

The following examples further illustrate the present invention. These examples are intended merely to be illustrative and are not to be construed as limiting.

EXAMPLE 1

Isolation of 1-6 Glucanase from *Trichoderma harzianum*

Procedure:

Culture of *Trichoderma harzianum* for enzyme isolation was accomplished under the following growth conditions. *T. harzianum* (ATCC 52324) was obtained from the American Type Culture Collection (Rockville, MD). The lyophilized pellet was resuspended in modified Czapek medium (250 ml containing 0.2 mg/L $MgSO_4 \bullet 7H_20$, 0.9 mg/L KH_2PO_4, 0.2 mg/L KCl, 1.0 mg/L NH_4NO_3, and 0.002 mg/L Zn^{++}) supplemented with 10% glucose. The culture was allowed to grow for 48 hours at 24°C with aeration. The culture was filtered, and the cells were resuspended in the media described above (1.0 L) except that 1.5% chitin was substituted for the 10% glucose. The culture was incubated for four days at 24°C with aeration.

Isolation of lytic enzymes was performed at 4 °C. Following the incubation, the cells were filtered through a filter paper (Whatman no. 1) and the filtrate was centrifuged at 6,000 x g for 10 minutes. The supernatant was precipitated with ammonium sulfate to 80% saturation. The precipitate was recovered by centrifugation at 12,000 x g for 20 minutes and resuspended in distilled water. The mixture was then dialyzed against 50 mM potassium acetate buffer, pH 5.5. The dialyzed fraction contained lytic enzymes.

The crude enzyme (10 g) was dissolved in water (100 ml) and dialyzed against sodium acetate buffer, pH 5.0. The dialyzed enzyme was adsorbed on alcohol precipitated pustulan (1-6 glucan, 5.0 g) at 4 °C for 20 minutes. The supernatant containing non-adsorbed enzyme was collected by centrifugation and readsorbed on fresh pustulan. The process was repeated for three times. All pustulan-enzyme precipitates were pooled and washed three times with sodium acetate buffer (pH 5.0, 100 ml) containing 1M sodium chloride. The pustulan-enzyme complex was then resuspended in phenylmethylsulfonyl fluoride (1mM) with 0.02% sodium azide and incubated overnight at 37 °C to digest and release the enzyme. All clarified supernatants obtained after pustulan-enzyme incubation treatment were pooled and centrifuged at 12,000 x g for 10 minutes. The supernatant was dialyzed against sodium acetate buffer, pH 5.0. This final preparation was then subjected to preparative isoelectric focusing using ampholytes from pH 5-7 in a Rotofor® unit. Isolecectric focusing was run at 12 watts at 4 °C for 3 hours. Fractions were collected, pooled, concentrated and dialyzed. Purified enzyme was used for physicochemical characterization and anti-fungal testing.

Results:

The final preparation product, 1-6 Glucanase, appeared as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular weight of 43,000 daltons and an isolelectric point of 5.8. The purified enzyme had a pH optima of 5.0 and temperature optima of 40-50 °C. The enzyme was highly specific in cleaving only 1-6 linked polymers of glucose as demonstrated in Example 2 below producing 1-6 polymers of glucose of greater than 2 units from pustulan.

EXAMPLE 2

Characterization of Specific 1-6 Glucanase Enzyme Activity

Procedure:

The activity of 1-6 glucanase was determined by incubating the enzyme (0.3 ml) in 1% pustulan in 50 mM sodium acetate buffer (pH 5.0, 0.1 ml) for 10 minutes at 37 °C. The reaction was stopped by adding dinitrosalicylic acid (0.75 ml). The reducing power of the digest was measured. One unit of enzyme is the amount of enzyme that forms an increase of reductive power equivalent to 1 m of glucose per minute.

Results:

The crude enzyme was found to contain 2 units per milligram of protein. The final purified enzyme was found to contain 20 units per milligram of protein, or approximately 100-fold purification.

EXAMPLE 3

Characterization of Anti-Fungal Activity

Procedure:

The anti-fungal activity of the 1-6 glucanase was determined in 96-well microtiter plates using RPMI-1640 medium with glutamine (150 ml) and a spore suspension of approximately 1000 spores of *Aspergillus fumigatus* in the same RPMI-1640-glutamine medium (50 ml). 1-6 glucanase enzyme (0.008-0.08 units) in 50 mM sodium acetate buffer (pH 5.0) was added. Control buffer without enzyme was also used.

The microtiter plate was incubated at room temperature for 18 hours and then transferred to a 37 °C incubator. Growth of fungal hyphae was monitored using an inverted microscope.

Results:

The control wells in the plate had good growth of hyphae with over 50 hyphal colonies per well. The crude enzyme preparation was similar to the control, but the purified enzyme showed only minimal hyphal growth with fewer than 5 hyphal colonies per well.

EXAMPLE 4

Characterization of Anti-Fungal Activity in Microorganisms and Animals

Procedure:

Fungal Organisms. *A. fumigatus* phialoconidia (conidia) were used as infectious particles throughout this study. Isolates of A. *fumigatus* originally obtained from patients were maintained on potato dextrose agar for spore and conidia harvesting. Spores or conidia were harvested in saline and vortex-mixed to break up clumps. The mixture was filtered through eight layers of cheesecloth and washed three times in saline. The concentrate was examined by light microscopy. Spore suspensions were free of hyphal fragments. Viability counts for the production of inocula were determined on Sabouraud's agar. The viability of spores or conidia was always >95%.

Induction of Immunodeficiency and Cortisone Acetate Treatment. Four- to six-week-old female pathogen-free mice (CD-1 strain) were obtained from Charles River Breeding Laboratories (Kingston, NY). The mice were given free access to water and a standard laboratory diet until 8 hr before cyclophosphamide or buffer injection, when food was withdrawn. Cyclophosphamide was used to induce immune suppression. Briefly, cyclophosphamide was dissolved in ice-cold citrate buffer (pH 4.2). A dose of 250 mg/kg (0.2 ml) was injected intraperitoneally within 10 min of dissolution. Control animals received buffer (0.2 ml). Mice were used in the experiment 7-14 days after cyclophosphamide or buffer injection. Cortisone acetate was injected subcutaneously in a daily dose of 125 mg/kg in 0.15 M NaCl solution (0.1 ml) for six consecutive days just before challenge. Control animals received NaCl solution alone (0.1 ml).

Animal Models. Graded doses (100 to 10 million) of spores, conidia, or sterile aqueous inocula were administered intravenously seven days after the injection of cyclophosphamide or buffer or on the day after completion of the cortisone acetate or buffer treatment. Animals were observed for 15 days and the LD 50 determined. When the animals died or were killed, the organ distribution of viable fungi was determined. Portions of lung tissue were processed and stained with Grocott methenamine silver and hematoxylin and eosin for histological evaluation (Waldorf *et al.*, *J Infectious Disease* 150:752-760, 1984).

Enzyme Treatment. Thirty minutes after intravenous inoculation with spores or conidia, animals were administered intravenously either normal saline (0.1 ml) as controls or a 1-6 glucanase solution (0.15 ml, 2 units of activity). Treatment continued every 24 hours for 5 days.

Results:

There were 8 control animals and 5 enzyme-treated animals. The survival curve is illustrated in Figure 1. The test of significance for the result was a p value of <0.05 (2-tailed Wilcoxon Rank-Sum analysis). The results showed 60% of mice treated with 1-6 glucanase were surviving 15 to 20 days post inoculation. None of the untreated mice were surviving at the same time.

EXAMPLE 5

Isolation of Chitinase from *Trichoderma harzianum*

Procedure:

Fungal organisms. *Trichoderma harzianum* phialoconidia (conidia) were used as infectious particles. Isolates of A. *fumigatus* originally obtained from patients were maintained on potato dextrose agar for spore and conidia harvesting. Spores of conidia were harvested in saline, vortex mixed to break up clumps, filtered through eight layers of cheesecloth, washed three times in saline, and examined by light microscopy. Spore suspensions were free of hyphal fragments. Viability counts for the production of inocula were determined on Sabouraud's agar. The viability of spores or conidia was always >95%.

Enzyme production and purification

Enzymes were produced using strain PI of *Trichoderma harzianum* (ATCC 74058). The strain was grown for 4 days on a rotary shaker in Richard's modified medium, which contained 10 g of KNO_3, 5 g of KH_2PO_4, 2.5 g of $MgSO_4$-$7H_2O$ 2 mg of $FcCl_3$, 1% (w/v) crab shell chitin (Sigma), 1% polyvinylpyrrolidone (Polyclar AT, GAF Corp., Wayne, NJ), 150 ml of V8 juice, and 1,000 ml of H_2O at pH 6.0. The biomass was removed by filtration, the supernatant dialyzed against 50 mM potassium phosphate buffer (pH 6.7), and enzymes separated by gel filtration chromatography in a chromatography column packed with Sephacryl S-300 (Pharmacia LKB Biotechnology, Upsala, Sweden), followed by chromatofocusing. A single protein with endochitinase activity was obtained. Purity was confirmed by using native and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophorcsis (PAGE) (PhastSystem, Pharmacia) and by isoelectric focusing (IEF). The fractions containing chitobiosidase activity were further separated in a Rotofor IEF cell (Bio-Rad, Richmond, CA). Peak fractions containing only chitobiosidase activity were collected, dialyzed against distilled water, and concentrated to dryness in a SpeedVac apparatus (Savant Instruments, Farmingdale, NY). PAGE, followed by staining, with Coomassie blue, indicated a single protein band. Protein concentration in the enzyme preparations was determined using the Micro BCA protein assay (Pierce, Rockford, Il.,) with trypsin inhibitor from soybean (Sigma) as the standard protein. Enzyme solutions were kept at 4° C and utilized for the biossays within 2 weeks or dried in a SpeedVac apparatus and stored at –20° C until used.

Induction of immunedeficiency and corisome acetate treatment. Four- to six-week-old female pathogen-free mice (CD-1 strain) were obtained from Charles river Breeding Laboratories (Kingston, NY). The mice were given free access to water and standard laboratory diet until 8 hours before cyclophosphamide or buffer injection, when food was withdrawn. Cyclophosphamide was used to induce immune suppression. Cyclophosphamide was dissolved in ice-cold citrate buffer (pH 4.2), and a dose of 250 mg/kg in 0.2 ml was injected intraperitoneally within 10 minutes of dissolution. Control animals received 0.2 ml of buffer. Mice were used in the experiment 7-14 days after cyclophosphamide or buffer injection. Cortisone acetate was injected subcutaneously in a daily dose of 125 mg/kg in 0.1 ml of 0.15 M NaCl solution for six consecutive days just before challenge. Control animals received 0.1 ml of NaCl solution alone.

Animal Model. Graded doses (100 to 10 million) of spores, conidia, or sterile aqueous inocula were administered intravenously seven days after the injection of cyclophosphamide or buffer or on the day after completion of the cortisone acetate or buffer treatment. Animals were observed for 15 days, and the LD 50 was determined. When the animals died or were killed, the organ distribution of viable fungi was determined. Portions of lung tissue were processed and stained with Grocott methenamine silver and hematoxylin and eosin for histological evaluation (Waldorf et al., *J Infectious Disease* 150:752-760, 1984).

Enzyme Treatment. Thirty minutes after intravenous inoculation with spores or conidia, animals were administered intravenously either 0.1 ml of normal saline (controls) or 0.15 ml containing 2 units of chitinase. Treatment continued every 24 hours for 5 days. In all experiments, treatment starts 30 min after infection and daily thereafter until mice are dead or moribund (or until untreated controls are all dead or moribund).

Results:

The survival statistics for 18 days post inoculation among subjects treated with PBS intraperitoneal, chitinase intraperitoneal at a dosage of 100 mg/kg, chitinase intravenous at a dosage of 100 mg/kg, chitinase intraperitoneal at a dosage of 25 mg/kg, and amphotericin B intraperitoneal at a dosage of 1 mg/kg are provided below in Table I. These data are presented graphically in Figure 2.

EXAMPLE 6

A chitinase molecule of 389 amino acids and a molecular weight of 42,393 having an isoelectric point of 6.3 was produced in an *Escherichia coli* cell line as describedin Invitrogen's catalog (1999 edition, page 191). The expression vector pBAD/His A with cloning sites Nco-Pst having a gene encoding a chitinase was inserted into the *E. coli* cells.

Fermentation

The chitinase strain is transferred from a previous LB positive ampicillin (100 μg/ml) plate or a frozen stock tube to a new LB positive ampicillin plate. The plate is incubated at 37° C for 48 hours. Inoculation flasks containing 200 ml of 25 g/l LB media and 0.2% w/v glucose are prepared. Ampicillin (100 μg/ml) is filter sterilized into the flask before inoculation. The shake flask is inoculated with a single colony and incubated for 12 hours. 375 grams of LB media and 30 grams of glucose (dextrose) are dissolved into 4 liters of water. The media is added to a fermentor and brought up to 14 liters. One ml of antifoam is added. The media is sterilized for 35 minutes at 121° C. Once the temperature is stabilized at 37° C, ampicillin is filtered in to achieve 100 μg/ml. The fermentor conditions are set to a pH of 7.0, agitation to 500 rpm, air flow to 15 lpm, temperature at 37° C, back pressure to 2.5 psig, and dissolved oxygen cascade set to 30% before inoculation. The fermentor is inoculated with 200 ml of shake flask culture. The culture is allowed to grow until the glucose is exhausted (about 5 hours). The cultrue is induced by filter sterilizing in 3 grams of (L) arabinose and maintained at 37° C for three hours. The temperature is changed to 28° C overnight. The fermentor is harvested the next morning for a total induction time of 18 hours and a total run time of about 24 hours. The culture is harvested and run throught a continuous tubular bowl centrifuge at 230 ml/min to pellet the cells. The cell pellet is stored in a -45° C freezer. A cell pellet is broken by vortexing the cells with silica and creating shear. The slurry is centrifuged and the supernatant assayed. The average chitinase activity is 4 units per ml.

Purification

Frozen cells are thawed overnight at 4° C. For every gram of cell paste an equal volume of lysis buffer which contains sodium phosphate, pH of 7.4., 100 mM EDTA and 1 μM PMSF is used to resuspend the cell mass. The suspension is passed twice through a homogenizer at 12,000 to 15,000 PSI. Lysed cells are centrifuged for one hour at 19,000 X g. This removes cell debris and retains greater than 95% of enzyme activity. The lysate is readjusted to a pH of 7.4. A Q-Sepharose Fast Flow, 1.5 L column is used as a flow through step. The column is equilibrated with sodium phosphate, pH of 7.4. The flow rate for Q-Sepharose is done at 100 cm/hour. Conductivity of the cell lysate should be maintained as low as possible, less than 10 mS/cm. Chitinase does not bind to the media if the pH is controlled properly. Material from only the first flow through peak is

collected and used for the next step. A Phenyl-Sepharose Fast Flow, 3-4 L coulumn is used to capture chitinase. The flow rate for Phenyl-Sepharose is set at 100 cm/hour. Add enough NaCl to the flow through material from the previous step to make the final concentration 3M. Bound chitinase is eluted from the Phenyl-Sepharose column by applying a descending linear salt gradient to 0 M NaCl using 10 column volumes. The fractions comprising the activity are pooled and diafiltered into 25 mM sodium borate, pH 9.0 using a Sartorius Sartocon 10 Kda NMWCO filtration system. The volume of borate buffer used for the diafiltration is about 5 volumes. A DEAE-Sepharose Fast Flow (50 mm diameter x 100 mm height) column is used to bind and concentrate chitinase. The flow rate for DEAE-Sepharose is performed at 100 cm/hour. The bound chitinase is eluted off the DEAE-Sepharose column by applying an ascending linerar salt gradient to 2 M NaCl. The cleanest fraction is analyzed by SDS-PAGE which generally comprise the largest amount of activity, are pooled, diafiltered into phosphate buffered saline at pH of 7.4. The usable fractions are pooled and diafiltered into PBS at pH of 7.4 using a sartorius Sartocon 10 Kda NMWCO filtration system. The volume of PBS used for the diafiltration is more than five volumes. The resulting material is assayed for activity, protein, purity and entotoxin.

Fluorescence Assay to measure Chitinase Activity

Chitinase samples reacts with the substrate, 4-Methylumbellifery-N,N',N''-triacetyl-β-chitotrioside, by cutting off the 4-methyllumbelliferone. This release of 4-methylumbelliferone is measured in microplates in a fluorescence microplate reader. The wavelength of the light for excitation is 355 nm and for emission is 460 nm.

Reagents Preparation

Prepare 0.5M Sodium Phosphate (500mL): Add 35.49g of Sodium Phosphate into 500mL of water. Mix until clear. (Various volumes can be made according to need.). Prepare 0.5M Sodium Phosphate Monobasic (500mL). Add 34.50g of Sodium monobasic into 500mL of water. Mix until clear. (Various volumes can be made according to need.).

Prepare 50mM Phosphate buffer, pH 7.2: Make 1:10 dilution of 0.5M Sodium Phosphate solution to get a final concentration of 50mM. Make 1:10 dilution of 0.5M

Sodium Phosphate Monobasic solution to get a final concentration of 50mM. Use the following ratio to get pH 7.2, approximately 68.4% Na_2HPO_4 + 31.6% NaH_2PO_4. Use the pH meter to read pH and adjust until pH is at 7.2.

Prepare 1mM 4-Methylumbellifery-N,N',N''-triacetyl-β-chitotrioside: Pipette 800µl of DMSO into vial and mix until clear. Add the 800µl of 4-Methylumbellifery-N,N',N''-triacetyl-β-chitotrioside to 5560µl of 50mM Phosphate buffer, pH 7.2 to obtain a final volume of 6360µl.

Assay method

Cracking the cells (if necessary): Pipette 1 ml of sample into 1.5mL microcentrifuge tube. Centrifuge for 2 min. at 14,000 rpm (revolution per minute). Pour out supernatant from tube. Add 100µl 50mM Phosphate buffer, pH 7.2 into tube. Vortex to resuspend pellet into solution. Add approximately 100µl of glass beads and vortex for 30 sec. and ice for 30 sec. Repeat 6 times. Centrifuge tube for 2 min. at 14,000 rpm. Pipette out supernatant into separate tube.

Prepare 1mM 4-Methylumbelliferone stock solution: Weigh out .0881g 4-Methylumbelliferone into a tube. Add 25 ml of 50mM Phosphate buffer, pH 7.2. Mix. Add 6N NaOH into solution until all of the 4-Methylumbelliferone is dissolved. Q.S. solution to 500mL with 50mM phosphate buffer, pH 7.2.

Prepare 4-Methylumbelliferone standard curve: This solution can be kept up to twelve months at -20°C.

4-Methylumbelliferone Concentration µM	**4-Methylumbelliferone Stock Solution 1mM**	**50mM Phosphate buffer, pH 7.2**
0	0µl	10000µl
1	10µl	9990µl
5	50µl	9950µl
10	100µl	9900µl
15	150µl	9850µl

Reaction Method: Pre-cool plate and all reagents on ice to minimize variablility. Pipette 100µl of each 4-Methylumbelliferone standard (0, 1, 5, 10, 15µM) into each well. Pipette 95µl of 50mM Phosphate buffer, pH 7.2. This will serve as the blank. Pipette 90µl of 50mM Phosphate buffer, pH 7.2 into each well for each sample. Pipette 5µl of sample into each sample well. Sample(s) may need to be diluted to give a value (µM) between 0-15 µM. Pipette 5µl of 1mM 4-Methylumbellifery-N,N',N''-triacetyl-β-chitotrioside into each sample and the blank. Incubate plate for 10 min. at 37°C. Shake to ensure mixing.

Absorbance reading: Prepare the Fluorescence microplate reader for measurement at: Excitation: 355 nm, Emission: 460 nm. Allow for machine to warm up for at least 30 min. Set up the template to assign standards, samples, and dilution factors as needed. Read plate.

CALCULATIONS

Use the linear regression method available on Microsoft Excel (or equivalent statistical software program) to generate an equation by plotting the absorbance values for each 4-Methylumbelliferone standard (ordinate scale) versus the amount of 4-Methylumbelliferone, i.e., 0, 1, 5, 10, 15 µM (abscissa scale). Record the slope (m), intercept (b), and correlation coefficient (r^2) of the linear regression equation ($y = mx + b$), as determined by the Microsoft Excel software (or equivalent statistical software program).

Calculate and report the amount of each sample, µM, by substituting the absorbance value of each sample into the linear regression equation.

$$\text{Protein Amount } (\mu M) = \frac{\text{Absorbance Value} - \text{Intercept (b)}}{} \times \text{Dilution factor}$$

Record all information and calculations for sample(s). Most microplate reader software should automatically do the above calculations. Use the above steps if the microplate reader does not automatically generate the desired values.

Calculate activity units using the following calculation:

$$\text{Activity} = \frac{\text{(Protein Amount (µM)) x (volume of standard (L))}}{\text{(Enzyme vol in rxn (mL)) x (rxn time (min))}} = \text{units/mL}$$

One unit is defined as the amount of enzyme required to release 1 μmole of methylumbelliferone from MUF-$(GlcNAc)_3$ per min. at 37°C, pH 7.2. If the Protein Amount (μM) is < 1 μM, print result as " < 0.002 units/mL "

The acceptance criteria is generally that the coefficient of correlation, r, for the individual absorbance of the standards vs. their respective 4-Methylumbelliferone concentration should be ≥ 0.980.

EXAMPLE 7

The glucanase molecule is 452 amino acids in length with a molecular weight of about 38,000 and an isoelectric point of about 5.8. A gene encoding glucanase was transferred into a Pichia Pastoris cell line mk71 using a pPICZb-Glucl expression vector obtained from Invitrogen.

Inoculum Preparation

About 100 μl of a Gluc 16 glycerol stock was inoculated into 10 ml of BMGY medium. This culture was grown 24 hours and became visibly dense. The 10 ml culture was used to inoculate 200 ml of a batch medium containing 25 g/l sodium hexametaphosphate (EM Science), 34 g/l fermentation basal salts (Invitrogen), 9 g/l ammonium sulfate, 40 g/l glycerol, 4.35 ml/l PTM_1 trace metals (Invitrogen) and histidine (Sigma) supplemented to 0.1% final concentration as needed and grown overnight (16 hours). A three liter fermenter was inoculated with the entire 200 ml flask culture and grown dense with glycerol fed-batch. This three liter fermenter culture was used to seed a 100 liter bioreactor.

Fermenter Preparation

The fermenter was sterilized with 60 liters containing the fermentation basal salts, ammonium sulfate, and glycerol. Sodium hexametaphosphate and PTM1 trace metals were made us as a 10X stock solution, filter sterilized and added to the ferementer after it had been cooled to 30° C. The pH of the medium was adjusted to 5.0 with concentrated ammonium hydroxide for the initial batch culture. A 2500 ml stock solution of 12% (w/v) histidine was prepared and sterilized. 500 ml of histidine stock solution (0.1% final concentration) was added to the fermenter before inoculation and four more times during the fermentation. The dO_2 and pH probes were calibrated and checked for proper

operation. Dissolved oxygen concentration was maintained by varying the agitation between 150 and 500 rpm. When the agitation neared its maximum value, back pressure on the fermenter was increased up to 12 psig to achieve higher oxygen transfer rates. To maintain dO_2 levels at maximum agitation and back pressure, oxygen wa supplemented into the air sparge using a mass flow controller. Control of the pH was achieved by ammonium hydroxide addition. Foam control was achieved by automatic addition of KFO 673 (50% solution in methanol) antifoam.

Fermentation and Sampling

Standard *Pichia pastoris* fermentation protocols were followed for the fermentation. After an initial batch phase growth on glycerol, a glycerol fed-batch was started. The fermentation was fed 20 liters of 50% glycerol before induction. dO_2 spike tests were performed throughout the glycerol fed-batch and each gave a spike time of less than 60 seconds. During the glycerol fed-batch, the pH setpoint was changed to reach the desired induction pH (3.0) by the cultures own natural acidification. Mixed feed (0.2% methanol, 9 ml/l/hr 50% glycerol) Muts induction was started immediately after the end of the glycerol fed-batch. The methanol concentration was controlled using a Raven Biotech controller.

The fermentation trend graphs included with this report show the dissolved oxygen, pH, temperature, and agitation rate. Five samples of the fermentation were taken throughout the time course and numbered consecutively. Three samples were collected pre-induction and two during methanol fed-batch (post-induction). The wet cell weights for samples 1 though 5, respectively, were: 146, 200, 280, and 284 g/l. Sample one and two were taken in the moddle of glycerol fed-batch, sample three was taken at the end of glycerol fed-batch, sample four was taken 12 hours post induction, and sample five was taken 20 hours post induction. The cell pellets from the samples were frozen at -20° C.

Histidine additions were made approximately every 100 g/l. The additions were made when the culture WCW was 54 (glycerol batch phase), 200 (glycerol fed-batch phase), and 280 (glycerol fed-batch phase). Finally, a histidine addition was don 12 hours post induction (again, 280 g/l WCW).

The fermentation was harvested by tangential flow microfiltration (0.2 μm PES membrane). The cell suspension was concentrated to 514 g/l (sample 6), about 50 liters, then a constant volume wasing technique was used to remove the spent medium from the

cell product. The cell suspension was then concentrated to about 40 liters of cell suspension (550 g/l WCW) before snap freezing with liquid nitrogen.

Purification

Cells from 100 L fermentation grown by Invitrogen were concentrated and diafiltered by tangential flow filtration into 25 mM NaOAc, pH 4.5, 1 mM EDTA. The final volume was 40 L with a density of 550 g/l (wet cell weight). The cells were exuded directly into liquid nitrogen and stored frozen at -80° C. Approximately 20 L of cells were passed two times through an Avestin C-50 homogenizer at 30,000 psi at 4° C and the homogenate was frozen on dry ice, then stored at 80° C. The homogenate was cleared by centrifugation (10,000 rpm, 60 minutes, 4° C), yielding 13.5 L of cleared lysate containing approximately 35 g of total protein.

The cleared homogenate was adjusted to 1M $(NH_4)_2SO_4$ by adding one volume of 25 mM NaOAc, pH 4.5, 3M $(NH_4)_2SO_4$, 1 mM EDTA per two volumes of lysate. The homogenate was run on a 1.3 L Phenyl Sepharose Fast Flow (Parmacia Catalog # 17-0965-03) 14 cm diameter x 8.5 cm height column at a flow rate of 300 ml/min. The column was preconditioned with 5 column volumes of 0.1 N NaOK, 5 column volumes of water, and 5 column volumes of EQ buffer (25 mM NaOAc, pH 4.5, 1M $(NH_4)_2SO_4$, 1 mM EDTA. A 10 column volume wash of EQ buffer and a ten column volume wash of 25 mM NaOAc, pH 4.5, 0.5 M $(NH_4)_2SO_4$, 1 mM EDTA was performed. Elution was done with ten column volumes of 25 mM NaOAc, pH 4.5, 1 mM EDTA.

Glucanase assay

Protein concentrations were determined by Bradford assay using Bovine Gamma Globulin as a standard. Activity was determined by reacting glucanase contaiing solutions with the fluorogenic substrate GT-4-mu (4-methylumbelliferyl β D-gentotrioside; Toronto Research Chemicals Inc. Catalog # M33449). The activity assay was performed as follows: Made two fold serial dilution of protein in eight wells of a 96 well microtiter plate (10 µl of Elution Buffer). Initiate the reaction by adding 100 µl of prewarmed (about 37° C) 40 µM GT-4-Mu (in 50 mM NaOAc, pH 5.5). Determine the RFU's in 5 minute rate assay at 37° C, λ_{EX}= 355, λ_{EM}= 460. Calculate RFU's/ml from RFU readings in the linear range of the dilution series (RFU x dilution factor/0.01 ml).

Total reactivity (RFU) = RFU/ml x volume. Values greater than 10,000 are considered good.

EXAMPLE 8

The glucanase molecule is 452 amino acids in length with a molecular weight of about 38,000 and an isoelectric point of about 5.8. A gene encoding glucanase was transferred into a *Saccharomyces cerevisiae* using a pYES2 expression vector having cloning sites sac-xho.

The *Saccharomyces cerevisiae* strain is transferred from a previous yeast nitrogen base (YNB) without amino acids plus URS drop out plate to a new plate and incubated at 30° C for 48 hours. Inoculation flasks containing 200 ml of 6.7 g/l YNB without amino acids and 0.77 g/l URA drop out (Clontech) media are prepared. Shake flasks are inoculated with a single colony and incubated for 12 hours. 94 g of YNB without amino acids and 11 g of URA drop out powder was dissolved into 4 L of water. Media ws added to the fermentor and brought up to 14 L in the fermentor. One ml of antifoam was added. The media was sterilized for 35 minutes at 121° C. Once the temperature was stabilized at 30° C, 14 g of sterile glucose was added. Fermentor conditions were controlled at pH 6.5, agitation 500 rpm, air flow 15 lpm, temperature 30° C, back pressure 2.5 psig. The fermentor was inoculated with 200 mls of shake flask culture. Once the glucose concentration is exhausted (around 32 hours), the culture is induced with 300 grams of Galactose. The culture remains at 30° C and 1 vvm of air for another 36 hours. The cell broth is run through a continuous centrifuge to pellet the cells. The cells are stored in a -80° C freezer. The Glucanase assay is read by fluorescent spectrophotometry and good levels of expressareion are considered to be greater than 10,000 RFU/microgram of protein. The activity of the glucanase may be assessed as per the protocol set forth in the preceding example.

EXAMPLE 6

Chondroitinase B Standard Operating Procedure

Source

The chondroitinase B sequence was retrieved from *Flavobacterium heparinum* (ATCC 13125) DNA by PCR amplification. Primers were designed based on a sequence

previously deposited in Genbank (Accession U27584). The signal peptide was not included within the amplified coding sequence. Primer 5'-terminal restriction sites allowed direct cloning into the thioredoxin-fusion plasmid pThioHis (Invitrogen) cut with NcoI and EcoRI. Expression was optimized in *E. Coli* strain BL21.

Materials Needed

Cell Culture	LB/carbenicillin plate with pThioHisChondB in BL21 cells
	50 ml sterile LB/carbenicillin (60 g/mL) in a 250 ml flask
	4 x 1.0 L sterile LB/carbenicillin in 2.8 L flasks with >4 cm stir bars
	20 mL 200 mM IPTG
	4 stir-plates, 4 large trays (for ice), 4 styrofoam platforms
	2 x 500 mL centrifuge bottles
Lysis	50 mM Tis-HCl, pH 8.0, 5 mM imidazole, 1 mM EDTA, 1 mM Pefabloc SC, 2 g/mL Leupeptin
	Dry ice-ethanol bath
	2 x 35 mL Oak Ridge centrifuge tubes
Column	Heparin-acrylic bead column (10-20 mL/L culture media)
	500 mL 50 mM Tris-HCl, pH 8.0
	40 mL 50 mM Tris-HCl, pH 8.0, 150 mM NaCl

Standard Operating Procedure

Preparation

Autoclave 2.8 L flasks with LB media and stir bars. Add carbenicillin after cooling. Media can be stored at room temperature for a day or two. Pour a heparin bead column and wash with 2 column volumes of >1 M NaCl and at least 3 column volumes of 50 mM Tris-HCl, pH 8.0. Inoculate a single-colony of pThioHisChondB in BL21 cells into 100 mL of LB/carbenicillin. Incubate at 37°C overnight with 250 rpm shaking. Subculture if stationary.

Cell Culture

Seed 20 mL of overnight culture into each 2.8 L flask. Shake at 37°C with 250 rpm shaking until $0.5<OD_{550}<0.7$. Immediately transfer bottles to ice-filled trays on stir-plates and stir at moderate speed until culture temperature is <10°C (should take about an hour). Add a few drops of Antifoam 289 at this stage to prevent frothing of cultures. Add 5 mL 200 mM IPTG to each culture. Allow 5 minutes for inhibitor to soak in, then remove ice-trays. Allow cultures to stir at room temperature for three hours. Place styrofoam between bottles and stir-plates as the latter will warm up and heat the cultures. Pellet cultures in 2 x 500 mL bottles (8000 rpm for 10 minutes). Weigh the two pellets (combined weight is usually between 5-7 grams).

Cell Lysis

Resuspend the pellets in a total of 80 mL lysis buffer (protease inhibitors added fresh) and transfer to 2 x 50 mL polypropylene Falcon tubes. Vortex to insure that cells are completely resuspended and that no clumps remain. Except for column chromatography and where otherwise stated, all subsequent steps should be performed at 4°C. Immediately freeze the cells by immersing in a dry ice-ethanol bath. Allow at least 10 minutes for complete freezing to occur. Quickly thaw the cell solution by immersing the tubes in a 37°C bath and shaking. Place thawed cells on ice and sonicate with Branson 450 microtip using an output of 3-4 and a duty cycle of 100% for at least 3 x 15 seconds. Transfer the lysate to 2 x 35 mL Oak Ridge tubes and clarify by spinning at >10,000 rpm for at least 30 minutes (11,000 rpm seems to be sufficient). Transfer the supernatant back to a single 50 mL Falcon tube and supplement with $MgCl_2$ to 10 mM, RNase A to 10 g/mL, and DNase I to 10 g/mL. Rock the tube at 4°C for 30 minutes. The weight of the pellet after lysis is usually about 1/3 that of the original cell pellet. Assay 5 L and 1 L supernatant for activity against dermatan sulfate.

Column Chromatography

Load heparin column with entire nucleic acid-depleted supernatant. Allow the sample to flow through the column at a rate of <2.0 mL/minute. Assay 5 l of the flow-through to ensure capture of the chondroitinase. Wash the column with 50-100 mL 50 mM Tris-HCl, pH 8.0. Check the wash for activity to confirm that the majority of the chondroitinase remains bound. Elute the chondroitinase with 40 mL (or less) of 50 mM

Tris-HCl, pH 8.0, 150 mM NaCl. Determine the activity of the eluate. Supplement with a stabilizing agent. Successful additives to the column media that have so far been determined are BSA or ovalbumin at 0.05%. Run a 10% protein gel of the pre-column supernatant, flow-through, wash, and eluate fractions for SDS-PAGE. The molecular weight of thiochondroitinase B is about 66 kD. The column can be regenerated by washing with >1 M NaCl and reequilibrating with the 50 mM Tris-HCl, pH 8.0. The matrix can be stored in Tris buffer containing 0.02% thimerosal.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, modifications of the modes described for carrying out the invention which are obvious to those skilled in the pharmaceutical arts or related fields are intended to be within the scope of the following claims.

WE CLAIM:

1. A method for purifying a lytic enzyme from a fungus comprising, the steps of-.
   (a) precipitating cellular material of the fungus;
   (b) isolating protein components from the cellular material;
   (c) precipitating the lytic enzyme using its substrate; and
   (d) purifying the lytic enzyme using isoelectric focusing.

2. The method according to claim 1 wherein the lytic enzyme is a β1-6 glucanase.

3. The method according to claim 1 wherein the fungus is a *Trichoderm.*

4. The method according to claim 1 wherein the furious is *Trichoderma harzianum.*

5. The method according to claim 1 wherein the lytic enzyme is selected from the group consisting of Endo-(1-3)-β-N-glucanase, Exo-(l-3)-β-N-glucanase, Endo-(1 -6)-β-N-glucanase, Exo-(1-6)-β-N--glucanase, Endo-(1-4)-p-β-glucanase, Endo-(1-4)-β-N-glucanase, Endo-(1-2)-β-N-glucanase, Endo-(1-2)-β-N-glucanase, 1-4-β-poly-N-acetyl-D-glucosaminidase, a chitinase, a chitobiosidase, a chitobiohydrolase, endo- 1 -4-β-poly-D-glucosaminidase, exo-1-4-β-poly-D-glucosaminidase, and a protease.

6. The method according to claim 1 wherein the lytic enzyme is β1-6 glucanase.

7. The method according to claim 1 wherein the lytic enzyme is a chitinase.

8. The method according to claim 1 wherein the fungus is selected from the group of *Trichoderm* species consisting of *T. atroviride, T. cirtinoviride, T. hamatum, T. harzianum, T. koningii, T. lignorum, T. longibrachiatum, T polysporum, T. pseudokoningii, T reesei, T saturnisporum, T. todica, T virgatum,* and *T. viride.*

9. A pharmaceutical composition comprising one or more lytic enzymes having the ability to lyse fungal cell walls and a pharmaceutically acceptable carrier or diluent.

10. The composition according to claim 9 wherein the lytic enzyme is purified from a fungus.

11. A pharmaceutical composition according to claim 9 wherein the lytic enzyme is selected from the group consisting of Endo-(1-3)-β-N-glucanase, Exo-(1-3)-β-N-

glucanase, Endo-(1-6)-β-N-glucanase, Exo-(1-6)-β-N-glucanase, Endo-(1-4)-β-N-glucanase, Endo-(1-4)-β-N-glucanase, Endo-(1-2)-β-N-glucanase, Endo-(1-2)-β-N-glucanase, 1-4-β-poly-N-acetyl-D-glucosaminidase, a chitinase, a chitobiosidase, a chitobiohydrolase, endo-1-4-β-poly-D-glucosaminidase, exo-1-4-β-poly-D-glucosaminidase, and a protease.

12. The composition according to claim 9 wherein the lytic enzyme is β1-6 glucanase.

13. The composition according to claim 9 wherein the lytic enzyme is a chitinase.

14. A method for treating fungal infections comprising the step of administering a pharmaceutically effective dose of a pharmaceutical composition according to claim 9.

15. The method according to claim 14 wherein the pharmaceutical composition comprises a β1-6 glucanase.

16. The method according to claim 14 wherein the pharmaceutical composition comprises a chitinase.

17. The method according to claim 14 wherein the fungal infection is caused by a species of *Aspergillus*.

18. The method according to claim 14 wherein the fungal infection is caused by a species having a cell wall comprising a β-1,6-glucan.

19. The method according to claim 14 wherein the fungal infection is caused by a species having a cell wall comprising a β-1,4-chitin.

20. A recombinant plasmid comprising a nucleic acid sequence encoding a lytic enzyme having the ability to lyse fungal cell walls or a biologically active fragment thereof.

21. A recombinant plasmid according to claim 20 wherein the lytic enzyme is selected from the -group consisting of a βl-6 -glucanase and a chitinase.

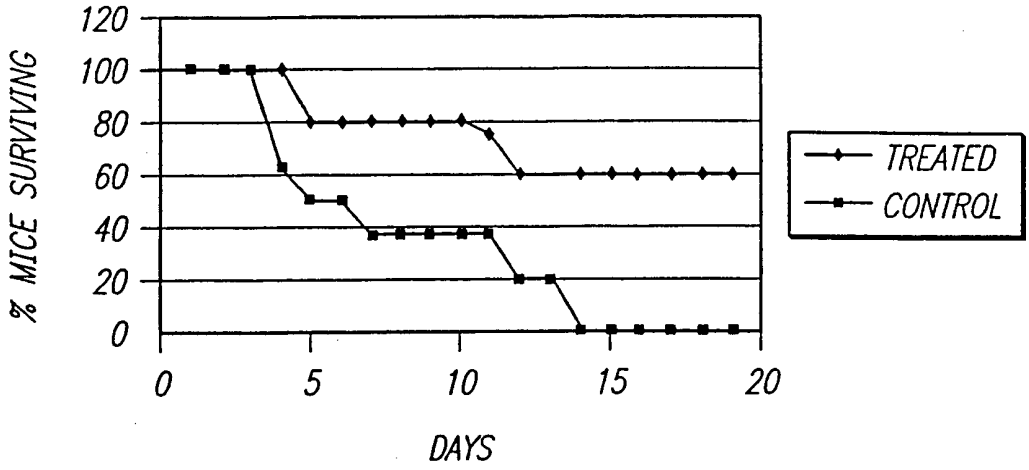
% MICE SURVIVING
120
100
80
60
40
20
0
0
5
10
15
20
DAYS
TREATED
CONTROL

FIG. 1

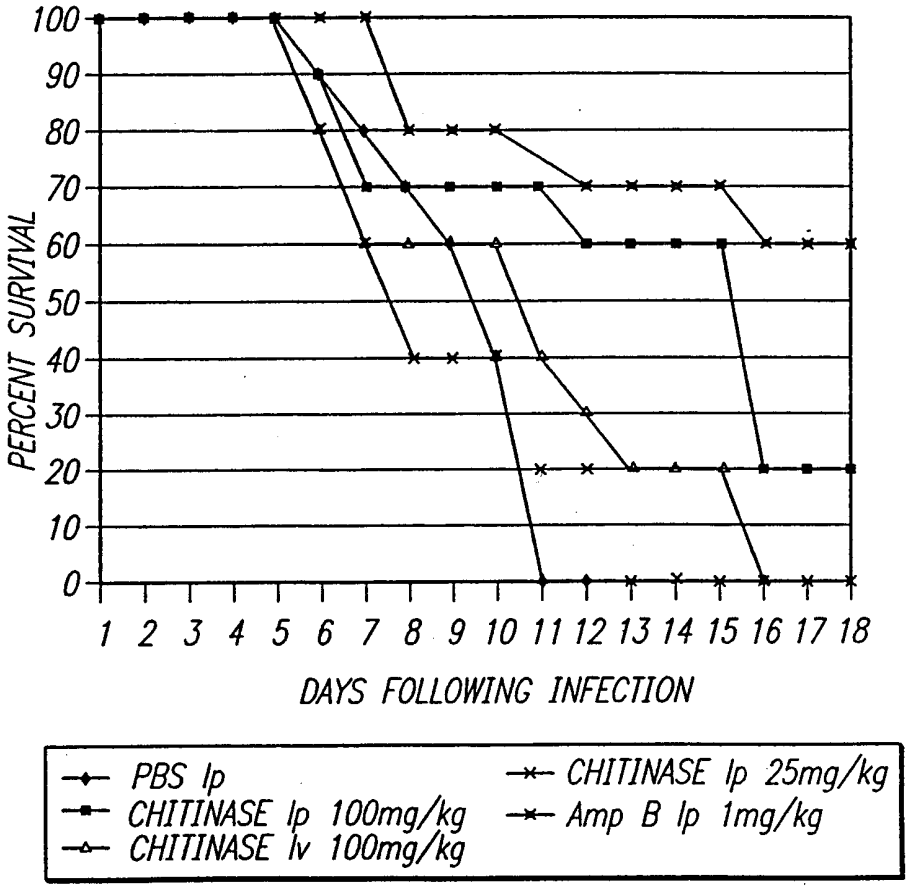
PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
DAYS FOLLOWING INFECTION
PBS Ip
CHITINASE Ip 100mg/kg
CHITINASE Iv 100mg/kg
CHITINASE Ip 25mg/kg
Amp B Ip 1mg/kg

FIG. 2