CELL WALL DEGRADING ENZYMES: AN APPROACH TO THE CONTROL OF FUNGAL INFECTION OF HUMAN BURNS

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CELL WALL DEGRADING ENZYMES: AN APPROACH TO THE CONTROL OF FUNGAL INFECTION OF HUMAN BURNS. (Project no. 2N061102B71D)
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I. Objectives

To produce enzymes that lyse the cell walls of pathogenic fungi, and to evaluate these enzymes as selective agents in the control of fungal infection of burn tissue.

II. Background Synopsis

The increased occurence of invasive fungal infections in burn wounds is probably a direct result of the more effective bacterial control measures recently introduced. Systemic and topical chemotherapy have failed to control zygomycetes* in burns, and frequently these infections lead to major amputation or death. Since zygomycetes have biochemically distinctive cell walls, potential methods for their selective control are possible including the application of cell wall degrading enzymes or inhibitors of cell wall synthesis. The results presented cover the former approach: the isolation and application of cell wall degrading enzymes towards the control of fungal (Zygomycete) infection of burn wounds. The possibility of testing inhibitors of fungal growth is also discussed.

* Zygomycetes are specifically referred to, though these organisms are loosely classified as Phycomycetes in pathogenic mycology texts.

III. Results

The initial aims of the proposal were:

1. To isolate the microorganisms having the lytic properties against Zygomycetes.
2. To isolate lytic enzymes from microorganisms effective against cell walls of pathogenic Zygomycetes and to characterize the physico-chemical parameters of the lytic complex.

The results obtained to date are grouped under these headings:

1. Isolation of microorganisms capable of lysing Zygomycete cell walls:

   a) General lytic microorganisms

      Our screening procedures to find microorganisms with lytic activity towards Zygomycetes have emphasized two points:

      i) Ability to degrade unmodified walls; for example, ability to degrade natural polysaccharide-protein complexes and crystalline polymeric components that occur in cell walls.

      ii) Ability to degrade the live pathogen.

In our initial broad screening program, we used the "agar plate clearing assay"; i.e., microbes were grown on a mineral-salts agar medium (Table I TT) containing fungal hyphal walls (0.2%) as the sole carbon source. Clear zones (lysis) around the colonies growing on the translucent agar medium indicated production of extra-

cellular lytic enzymes by the organism. Rhizopus rhizopodiformis OM 9395, a representative Zygomycete, was used for the preparation of the cell wall substrate. This is a pathogenic strain received from Commander Pruitt at the U.S. Army Burn Unit, Fort Sam Houston, Texas, and was isolated from human burn tissue of a patient. Cell wall preparations were obtained from R. rhizopodiformis grown in liquid medium (Table I - TT medium - 500 ml/2.1 Fernbach flask at 200 rpm, 37°C, 3 days). The mycelium was harvested by filtration through cheesecloth, washed with water and then the organism was killed by suspension in 50% ethanol overnight. The hyphal mass was ball milled in 50% EtOH for 1-2 days, the macerated walls collected by centrifugation, washed with water and finally collected by centrifugation. The preparation was dried by successive extractions in ethanol, acetone and ether. This was termed "crude cell wall." This method was used in order to minimize major structural or physical changes in the wall structure. On autoclaving in liquid medium, the cell walls tend to aggregate and agar plates of uniform translucency are thus difficult to prepare. Hence cell walls and the growth medium were prepared separately at double strength. After autoclaving, the wall preparations in water were rehomogenized in a sterile Waring blender and then mixed with the agar medium. This medium was then distributed to Petri dishes using an automatic pipetting device (7 ml/glass dish or 5 ml/plastic dish).

The "agar plate clearing assay" allowed us to rapidly screen a large number of cultures (over 200) for lytic activity. The cultures (180 fungi, 46 bacteria) were obtained from the Natick Culture Collection, Rutgers University Culture Collection (16) and also by enrichment from six soils (18).

Direct enrichment of lytic organisms was obtained by adding soil to a liquid culture medium (TT) containing 0.2% Rhizopus cell walls, and then performing serial transfers to similar flasks after a few days incubation.

A second procedure was used to assess the gross utilization of R. rhizopodiformis cell walls as a substrate for growth. Cultures were grown in liquid salts medium (Table I (TT)) with Rhizopus cell walls as the sole carbon source. The percentage loss of substrate was used as a semi-quantitative parameter of cell wall utilization, confirmation being made by microscopic observation. Several fungi that grew rapidly on the Rhizopus cell wall agar medium and thus obscured any lytic zones, were also included in this second screening procedure. These two screening methods have yielded 47 fungi, 11 streptomycetes, and 5 bacteria of potential use. Representative results are given in Table II.

The ability of all potentially useful cultures to produce extracellular lytic enzymes that were active against "native" Rhizopus walls was determined. Cultures were grown with R. rhizopodiformis walls as the carbohydrate source. The culture fluids were assayed for extracellular lytic enzymes by measuring the release of reducing sugars as a result of enzyme action against

ball milled Rhizopus or Mucor cell walls (Table II) It should be noted that these enzyme assays were carried out at 37°C for 24 hours. Such conditions were necessary to show activity against this "unmodified" insoluble wall material. However, this long incubation period actually aids us in our search for particularly stable lytic enzymes. The enzyme complexes do appear amenable to concentration quite readily (see Table III).

1 b) Lysis of live Zygomycetes

Screening to determine if cultures can lyse living cultures of Rhizopus are being studied by two methods:

(i) Lysis of mature hyphae

(ii) Lysis of sporelings

In the former method live cultures of Rhizopus were inoculated with the lytic test organism and the incubated for several days. Pure Rhizopus cultures were used as controls to check any degree of autolysis but none was recorded. Direct visual and microscopic observation was used to observe lysis. This test has shown that only 6 strains can lyse the R. rhizopodiformis under these experimental conditions (Table IV)

However. in the proposed model for using cell wall lytic enzymes, it was emphasized that the most susceptible zone to lysis was the hyphal tip of the germinating spore. Hence we have been attempting to develop an assay based on the lysis of the germinating spore. A standardized spore germination (30% or

greater) in four hours using TT medium lacking peptone has been obtained. The initial spore preparations are obtained from cultures grown on malt agar (BBL) for 3 days at 37°C in the presence of intermittent fluorescent light. It should be noted that members of the Mucorales sporulate under quite different conditions. For example we grew Mucor genevensis, Mucor rouxii and R. arrhizus on five different media (Table I (b,c,d,e,f)) in the dark or in constant fluorescent light. R. arrhizus sporulated best on YPG medium in the presence of light. M. genevensis sporulated best on Cove's medium and potato dextrose agar in the light. Darkness tended to induce heavy zygospore formation in all media. M. rouxii grew best on YPG medium in light or dark, but the latter heavily favored sporangiophore formation.

To date we have only tested one concentrated extracellular enzyme system (Penicillium islandicum) for its effect against R. rhizopodiformis sporelings. This enzyme preparation inhibited the growth of the spore germ tubes, promoting much vacuolation as seen by light microscopy. An enzyme preparation of Streptomyces satsumaensis similarly caused marked vacuolation of the Rhizopus hyphae. We feel this assay will be fundamental in our study and are continuing to refine it. The objective is to make it fast and accurate by utilizing it in conjunction with a spectrophotometer. Sporelings will be incubated with lytic enzyme preparations in the presence of an osmotic stabilizer (KCl is presently being used). The assay will be terminated by addition of water causing lysis of any spheroplasts with concomitant decrease in optical density.

Release of protein may also be measured.

In summary of this section, we have found several potentially useful lytic microorganisms, but are presently determining which will yield the most effective lytic system. Part of this approach is to develop the lytic assay of the live Rhizopus spore system. We must also consider using mixtures of enzymes from two or three different cultures in order to gain the most effective lytic system. Hence characterization of certain of the enzymes systems has been initiated.

2. Isolation and characterization of enzymes showing lytic activity towards Zygomycete cell walls.

(a) Cell wall polysaccharides

A prerequisite for this stage of the proposal is some understanding of the composition of the Zygomycete cell wall. Unfortunately, Zygomycete walls have not been widely studied. Mucor rouxii walls have been studied in detail, while qualitative studies of Zygorhynchus vuilleminii and Phycomyces blakesleeanus has been performed (1). The wall composition of the Zygomycetes is thus derived from limited but consistent evidence from examination of the Mucoraceae. The main polysaccharides of M. rouxii are chitin, chitosan (a poorly or non-acetylated form of chitin), mucoran (a heteropolymer of fucose, mannose and glucuronic acid the principal repeating sequence being D-Man (1→4) - α-D - GlcU - (1→3) -D-Man (2) and mucoric acid (a glucuronic acid polymer). Other components include protein (6%), lipid (3%) and phosphate (23%). Examination of our initial chemical and enzymatic hydro-

lysates of the crude R. rhizopodiformis cell walls by paper chromatography showed similar overall components. A more detailed study of non-pathogenic R. arrhizus and M. genevensis (Table V) generally supports the basic wall composition found by prior workers. Glucose was present only as trace amounts. Chitosan is thought to be characteristic of the Zygomycete wall though an extremely limited number of species have been tested (Mucor rouxii, Phycomycetes blakesleeanus and Rhizopus arrhizus). In view of this limited data and also in that we found specific activity towards chitosan in our lytic enzyme preparations (see below). we surveyed a range of fungal classes for the presence of chitosan in the cell wall. Wall preparations were obtained by ball milling and extensive washing. They were extracted with 0.4N HCl for 30 minutes at 100°C and rinsed hot. Chitosan (HCl soluble) and chitin (HCl insoluble) were then measured by determining the amount of glucosamine present following hydrolysis in 6N HCl at 100°C for 12 hours (Table VI). The generalization previously assumed of chitosan occurring in the Zygomycetes is confirmed. Karlingia sp., a chytrid, is worthy of further study. Chitosan is an ill-defined polysaccharide, ranging from a completely to a partially acetylated polymer of glucosamine residues. In M. rouxii, it is 7% acetylated, while commercial preparations obtained by treating chitin with concentrated alkali, have a 20 - 30% acetyl content. Our commercial samples range from 17 - 30% (Table VII).

These results were obtained by titrating the acetic acid released following hydrolysis of the chitosan, a rather gross method (3). We are presently trying to estimate the acetyl content of the Zygomycete chitosans using n.m.r. spectroscopy by comparing the size of the acetyl signal with that of other protons.

We have thus established that the walls of the Zygomycetes used in our study have the same basic composition to that reported by prior workers and have extended the observations on the presence of chitosan in fungi.

b) Lytic enzymes

Characterization of the lytic enzyme complexes has been initiated. Lytic enzymes were induced by growing cultures with R. rhizopodiformis cell walls as sole carbon source in liquid medium. Culture broths were clarified by centrifugation and then dialyzed. These preparations were applied to walls for 16 hours at 50°C in dilute buffered solution (fungi pH 5.5, bacteria pH 6.5). 28 preparations were tested, all showing endo-glycosidase activity for paper chromatographic analysis of the hydrolytic products (n butanol: acetic acid:water 12/3/5 - p - anisidine spray). Chromatographic patterns obtained indicated that the different organisms have a range enzymes with different specifities and action patterns. As such the most effective lytic system to be obtained will probably be a mixture of different enzymes from

individual cultures; e.g., we find that several of these crude enzymes with no apparent activity against Rhizopus or Mucor ball milled cell walls, do indeed have significant activity against ball milled shrimp chitin or chitosan. This could result from shielding of the chitin/chitosan in cell wall from enzyme action by other polymers.

While screening to determine which enzymatic activities were present in the crude lytic enzyme preparations, we noted that those organisms that utilized Rhizopus cell walls also had chitosanase activity in the lytic preparation. Such an enzymatic specificity has not previously been recorded. Hence we more intensly surveyed for chitosanase producers and made a comparison with chitinase production. It was apparent that the crude lytic preparations showed considerable specificity to either chitin or chitosan (Table VIIIa, VIIIb). We consider chitosanases as a new class of enzymes (Manuscript in preparation - 5).

Two of these lytic enzyme systems showing high chitosanase activity have been explored further - Penicillium islandicum QM7571 and Bacterium sp.S6E. In our initial screening, P. islandicum but not S6E was able to lyse the live Rhizopus culture. By varying the cultural conditions we have raised the enzyme levels in the culture broths 20-fold, e.g. Table IX; with regard to carbohydrate source for the bacterial isolate S6E, reprecipitated chitosan was the best substrate. Mucor walls and mushroom were

fairly good, but Mucor walls were superior at the 1.0% level. For P. islandicum the reprecipitated chitosan was quite poor and Mucor walls are the best substrate, giving the highest enzyme levels when used at 1.0% (Table IX, E.T. Reese data).

The Penicillium enzyme is released late in the culture cycle (9 days). Initial purification steps have been performed. Dialysis of the crude broth increases the apparent activity of the enzyme, while both ultrafiltration and ammonium sulfate fractionation are convenient high yielding concentration steps (Table III). This preparation shows slight activity towards p-nitrophenyl-β-D-N-acetyl glucosamine. (The enzyme is not active against Azure chitin or Azure chitosan (Calbiochem Co.). However, we have previously experienced difficulty with similar products (Azure cellulose), which the company noted. A positive control is needed, but we have not been able to demonstrate one yet!)

The Penicillium enzyme and bacterial S6E enzyme differ in their substrate specificities. The bacterial enzyme has more specific requirements, it being active against chitosan but inactive towards colloidal or γchitin. The fungal enzyme though showing greatest activity against chitosan substrates does however show slight activity towards shrimp colloidal chitin and alightly greater activity towards γ chitin. In comparison Serratia marcescens (QM B1466) chitinase is not active against chitosans. It should be noted that P. islandicum can lyse the pathogen rapidly in culture, while bacterium S6E takes around 30 days (Table IV).

Our experience with the enzymes indicates that both chitosanase and chitinase will be necessary for lysis of the pathogen. However, the P. islandicum chitosanase has a pH optimum of 4.5, which may restrict its use for application in the burn wound model. The bacterial S6E chitosanase and Serratia chitinase have optimal pH's around 6.0 and thus this combination of enzymes may be more practical.

Summary

Studies on the control of pathogenic fungi infecting burn tissue by therapeutic use of cell wall-degrading enzymes has been initiated. Two hundred strains of fungi and bacteria have been screened for their ability to degrade the cell wall of Rhizopus rhizopodiformis, the test pathogen having been isolated from a burn patient. The microorganisms screened were obtained by enrichment techniques and from the Natick Culture Collection. Screening was carried out on the basis of "plate clearing" assays, direct utilization of the walls and production of hydrolases lysing the Zygomycete cell wall and also chitosan. Potential lytic organisms include 47 fungi, 11 streptomycetes and 6 bacteria. Six of these microorganisms have been found that will lyse live cultures of the pathogen Rhizopus rhizopodiformis. Each of these organisms were found to produce enzymes that degraded chitosan. Chitinase enzymes were not active against this substrate. A new class of enzymes, chitosanases, has been proposed on the basis of specificity of the lytic enzymes. The best producers of chitosanase to date are Penicillium islandicum and an unidentified bacterial isolate, S6E. The chitosanases studied are endo-splitting enzymes, fungal preparations showing optimal activity at pH 4.5 and bacterial ones at pH 6.0. A survey for the presence of chitosan in fungi has shown that this polymer occurs widely in Zygomycetes and in one chytrid but not in other fungal classes.

IV. Proposed Research

Several major areas will be explored:

(i) Continued testing and characterization of the cell wall lytic complexes to evaluate the most effective ones for lysis of the germinating spore.

Prior to an assessment of the therapeutic value of a lytic complex, it would be desirable to know of several of its physico-chemical parameters. Optimal conditions with regard to its stability to temperature, pH and chemical reagents will be determined; e.g., it will be essential to have enzymes that are stable to 37°C with an optimum pH around 7.0 and are not inactivated by other therapeutic treatments (stability to the antibacterial agent, sulfamylon (mafenide acetate)). With regard to pH, the streptomycete enzymes appear to be of most potential use. We are evaluating such systems including a species known to form spheroplasts from Mucor (6). More definitive studies, following purification of the lytic complex, may further aid in its application.

(ii) Evaluation of determinants of models for invasion of surgical wounds by Zygomycetes and protection afforded by lytic enzymes.

The efficacy of crude and purified enzymes will be tested to control fungal infection of wounds using alloxan diabetic rabbits as the experimental animal. The diabetes in rabbits will be produced by a single intravenous injection

of 200 mg/kg body weight of freshly prepared solution of alloxan in saline according to the procedure of Schauble and Baker (7). Paired surgical wounds will be produced on the back of anaesthetized rabbits. These wounds will be infected with a standardized spore suspension of Rhizopus or Mucor. One set will be treated with lytic enzymes while the other will serve as the infected control. At varying intervals animals from treated and untreated groups will be sacrificed and the efficacy of treatment will be evaluated by the gross appearance, histopathology and the presence of viable organisms by culture. In case an adverse allergic reaction is encountered, further purification of the active lytic enzymes will be attempted with the hope of eliminating the harmful components. An effective "purified lytic complex" has been prepared by purifying the specific enzymes and then remixing the desired components (Sietsma, Eveleigh and Haskin, (8)).

(iii) Evaluation of lytic enzymes in the infected burn model system.

The efficacy of crude and purified enzymes will be tested to control fungal infection of burn wounds by Zygomycetes using diabetic rats as the experimental animal. The alloxan diabetic rats will be burned with steam by a standard method (McRipley and Garrison, (9)). The burn wounds will be infected with a standardized spore suspension of Rhizopus or Mucor and the efficacy of enzyme preparations will

be tested as described above for surgical wounds. The efficacy of the lytic enzymes will also be tested on mixed bacterial and fungal infections of experimental wounds with and without associated antibacterial therapy.

(iv) Evaluation of polyene antibiotics (water soluble methyl ester of amphotericin B.)

Polyene antibiotics have not elicited their initial expectations as antifungal agents due to their antagonistic effects towards the renal system and also their poor solubility characteristics. Commander Pruitt has informed us that limited testing of nystatin and amphotericin B in the burn model has been carried out at Fort Sam Houston. However, both Commander Pruitt and Dr. Bruck (now at Columbia Presbyterian Hospital) have not found any published references to use of polyenes for protection of burn wounds from fungal attack. As amphotericin B is presently recommended for use only in potential terminal cases of systemic fungal infection, the lack of data with regard to the burn system is not too surprising.

Our laboratories have recently described a new water soluble polyene antibiotic the methyl ester of amphotericin B (10). It attacks a wide range of fungi at dramatically low concentrations, the latter aspect removing the untoward renal effects. Thus this methyl ester of amphotericin B is a prime candidate for testing in clinical mycology and in the burn model system. We wish to test it by similar protocols to

those described above.

Polyoxin D, a chitin synthetase inhibitor (11) has been shown to inhibit a range of chitin containing fungi. Our preliminary trials (Table X) have not supported these results, but further trials are necessary.

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TABLE I. CULTURE MEDIA

(a) Streptomyces Lytic Enzyme Medium (TT) - S. Tabata and G. Terui, J. Ferm. Technol. 40, (1962) 336

Modifications - has $(NH_4)_2SO_4$ in place of $NaNO_3$. For solid, add 17 g. agar.

Crude Yeast Cell Wall	3.0 g
K_2HPO_4	2.0 g
Yeast Extract (Difco)	0.5 g
KCl	0.5 g
Glucose	0.5 g
$MgSo_4.7H_2O$	1.0 g
Peptone (Bacto)	1.0 g
NaCl	0.5 g
$(NH_4)_2SO_4$	1.0 g
distilled H_2O	to 1000 ml

(b) YPG Medium - S. Bartnicki-Garcia, W. J. Nickerson, Biochim. et Biophys. Acta 58 (1962) 102-119

Modifications - pH to 4.5 (H_2SO_4). For solid medium, add 17 g. agar.

Yeast Extract (Difco)	3.0 g
Peptone (Bacto)	10.0 g
Glucose	20.0 g
distilled H_2O	to 1000 ml

Procedure - After dissolving, adjust pH to 4.5 with 1.0 N H_2SO_4 (8.3 ml/l medium)

(c) Cove Salts Medium - D. J. Cove, Biochim. Biophys. Acta 113 (1966) 51-56

Modifications - organic Nitrogen has been replaced with $NaNO_3$

Table I. continued

Stock Solution I (Salts)		Conc. in Final Medium
KCl	26.0 g	0.52 g/l
$MgSO_4$	26.0 g	0.52 g/l
KH_2PO_4	76.0 g	1.52 g/l
$NaNO_3$	300.0 g	6.00 g/l
$CHCl_3$ (as preservative)	2.0 ml	
distilled H_2O	to 1000 ml	

Stock Solution II (Trace Elements)		
Citric Acid	5.00 g	5.0×10^{-3} g/l
$FeCl_3.6H_2O$	0.97 g	9.7×10^{-4} g/l
$ZnSO_4.7H_2O$	4.43 g	4.4×10^{-3} g/l
$CuSO_4.5H_2O$	0.20 g	2.0×10^{-4} g/l
$MnSO_4.H_2O$	31.0 mg	3.1×10^{-5} g/l
$Na_2MoO_4.2H_2O$	50.0 mg	5.0×10^{-5} g/l
$Na_2B_4O_7.10H_2O$	89.0 mg	8.9×10^{-5} g/l
$CoCl_2.6H_2O$	20.2 mg	2.0×10^{-5} g/l
distilled H_2O	95 ml	

Procedure - Use 20.0 ml of Stock Solution I and 0.1 ml of Stock Solution II for one liter of medium. Adjust to pH 6.5 and autoclave. Glucose was sterilized separately in water and then added to the salts and trace elements medium to give a final glucose concentration of 10 g/l.

(d) Malt Extract Agar - Difco Manual of Dehydrated Culture Media and Reagents Difco Laboratories, Detroit, Mich. 1953

Malt Extract (Difco)	30 g
Agar	15 g
distilled H_2O	to 1000 ml

Table I. continued

(e) Potato Agar - Soc. of Am. Bact. Manual of Microbiological Methods McGraw Hill 1957

Potatoes, infusion from	250 g
Agar	15 g
distilled H_2O	to 1000 ml

The potato infusion is made by slicing 250 g of peeled potatoes in 500 ml of distilled water. Bring to boiling. Allow to steep for 30 minutes. Filter through cheesecloth and make up to 1000 ml.

(f) Potato Dextrose Agar -

Potato Dextrose Medium (Difco)	39 g
Agar	15 g
distilled H_2O	to 1000 ml

TABLE IIa. SCREENING FOR LYTIC FUNGI

Organism	Plate clearing[1]	% Utilization[2] *Rhizopus rhizopodiformis* as C. source	Degradation[3] of *Mucor* walls	Degradation[4] of *Rhizopus* walls
Aspergillus sclerotiorum (QM 6732)	Definite	46%	0	0.07
Fungal sp. C 7405	Definite	48%	0	0.22
Fungal sp. Soil 5A	NT	59%	0.44	0.22
Metarrhizium anisoplias (QM 192)	Definite	46%	0	0
Monospora brevis (QM 1243)	Light	48%	0	0
Penicillium avellaneum (QM 1849)	Overgrowth	43%	0.27	0.39
Penicillium citrinum (QM 6748)	Overgrowth	51%	0.12	0.33
Penicillium islandicum (QM 7571)	NT	29%	0.16	0.35
Penicillium lilacinum (QM 1034)	Definite	40%	0.05	0.14
Rhizopus nigricans (QM 387)	Overgrowth	47%	0	0
Rhizopus rhizopodiformis (QM 9395)	Overgrowth	42%	0	0.03
Streptomycete sp. (QM TC11)	Definite	45%	0	0.29
Streptomycete sp. (QM TC77)	Light	42%	0	0.28

[1]Clearing of 0.2% *Rhizopus* walls + salts agar plates

[2]Organisms grown for 14 days at 29° C on 0.9% *Rhizopus rhizopodiformis* walls 0.01% peptone, 0.01% yeast extract in TT (Table I). Dry weight of mixture determined after incubation.

[3]mg. Reducing groups as Glucose released from cell walls measured by dinitrosalicylic acid (DNS) reagent.
Assay 0.5 ml cell walls (1% *Mucor ramosissimus* ball milled hyphae) in 0.1 M Na citrate buffer pH 6.0
0.5 ml enzyme (culture filtrate from organisms grown on *Rhizopus* walls.)
Incubated at room temperature for 24 hours.
Merthiolate (0.01%) final concentration used as an antimicrobial agent.

[4]Same assay as [3] 1% *Rhizopus rhizopodiformis* (QM 9395) ball milled hyphae
Incubated at 37° C for 24 hours.

TABLE IIb. SCREENING FOR LYTIC BACTERIA

Organism	Plate clearing[1]	% Utilization[2] *Rhizopus rhizopodiformis* as C. source	Degradation[3] of *Mucor* walls	Degradati of *Rhizop* walls
Bacterial sp. (QM B1589)	Definite	61%	0	0
Bacterial sp. Soil 5	NT	76%	0.03	0.11
Bacterial sp. Soil 6E2	Definite	64%	0.02	0.13
Streptomycete sp. + *Bacillus cereus*	Definite	52%	0	0
Streptomycete sp. (0143)	NT	72%	0	0
Streptomyces globisporus (NRRL-B2872)	NT	73%	0	0
Streptomyces griseus	NT	61%	0	0
Streptomyces parvus (NIHJ 1115)	NT	68%	0	0
Streptomyces satsumaensis (1399)	NT	72%	0	0

[1]Clearing of agar plate 0.2% *Rhizopus* walls

[2]1% *Rhizopus*, 0.01% yeast extract, 0.025% peptone in TT (Table I). Dry weight measured after incubation for 14 days at 29° C.

[3]mg reducing groups as glucose released from cell walls measured by dinitrosalicylic acid (DNS) reagent.
Assay 0.5 ml 1% *Mucor ramosissimus* in 0.1 M Na citrate buffer pH 6.0
0.5 ml enzyme (culture filtrates from organisms grown on *Rhizopus* walls.)
Incubated at room temperature for 12 hours.
Merthiolate (0.01%) final concentration used as an antimicrobial agent.

[4]Assay [3] using 1% *Rhizopus rhizopodiformis* walls (ball milled).

TABLE III. CHITOSANASE FROM *Penicillium islandicum* QM 7571

Removal of inhibitor by dialysis

	Units/ml[2]
Crude enzyme[1]	0.056
Dialysed enzyme	0.105

Concentration stages

	Units/ml	Volume (ml)	Total units	% Recovery
Crude dialyzed broth	0.105	8000	840	100%
Amicon concentration (PM 30 membrane)	1.80	400	720	85%
Amm. sulfate (60-100%) + dialysis	11.6	55.2	640	89%

[1]Enzyme prepared by growing *P. islandicum* in an 8 l fermentor at 30°C for 9 days with *R. rhizopodiformis* cell walls as a carbon source.

[2]Assay 0.5 ml colloidal chitosan (Moretex) (1.0%) in 0.05 M acetate buffer pH 4.5.

0.5 ml enzyme. Incubate 3 hours at 30°C Assay reducing groups by Somogyi-Nelson method.

1 enzyme unit = 1 µMole of reducing sugar equivalent as glucosamine/min at 30°C.

TABLE IV. ORGANISMS LYSING LIVE *Rhizopus rhizopodiformis* CULTURES

Organism	5 DAY	6 DAY	7 DAY	10 DAY	15 DAY	≥ 30 DAY
Aspergillus sclerotiorum (QM 6732)	-	-	-	-	-	+
Bacterial sp. Soil 6E2	-	-	-	-	-	+
Beauveria bassiana(QM 7435)	±	+	+	+	+	+
Fungal sp. Soil 5A	±	+	+	+	+	+
Penicillium islandicum (QM 7571)	±	+	+	+	+	+
Penicillium lilacinum (QM 1034)	±	+	+	+	+	+
Penicillium wortmanni (QM 7323)	-	-	-	±	+	+
Streptomyces satsumaensis (1399)	-	-	-	±	±	+

Negatives

Actinomycete sp. (QM TC52), *Bacillus cereus*, Bacterial sp. (QM B1589), Bacterial sp. 2Δ, Fungal sp. (C 7405), *Metarrhizium anisopliae* (QM 192), *Monospora brevis* (QM 1243), *Penicillium avellaneum* (QM 1849), *Streptomycete* sp. + *Bacillus cereus*, *Streptomycete* sp. (0143), *Streptomycete* sp. (QM TC11), *Streptomyces globisporus* (NRRL-B2872), *Streptomyces parvus* (NIHJ 1115)

TABLE V. SUGARS RELEASED BY ACID HYDROLYSIS OF ZYGOMYCETE CELL WALLS[1]

	Mucor genevensis RC 46	*Rhizopus arrhizus* QM 1032
N-acetyl glucosamine[2,3,4,5]	++	++
Glucosamine[2,3,4]	++	++
Galactose[2,7]	++	+++
Mannose[2,7]	+++	+++
Fucose[2,3,5]	++	++
Glucuronic Acid[2,3,6,8,9]	++	+

[1] Composite results from acid hydrolysis of cell wall material at 100°C e.g. 1 N H_2SO_4 6, 12, 24 hours; 0.5 N HCl 3, 6, 24 hours.

	Solvent system	Developing Agent
[2]	n-Butanol/Acetic Acid/water (12/3/5)	p-anisidine - spray
[3]	n-Butanol/Acetic Acid/water (12/3/5) (Z. Dische Methods in Carbohydrate Chem. Vol. I p 597.)	naphthoresorcinol - dip
[4]	n-Butanol/Acetic Acid/water (12/3/5)	Elson Morgan - dip
[5]	Isoamylacetate/Pyridine/water (3/3/0.9)	p-anisidine - spray
[6]	n-Butanol/Acetic Acid/water (12/3/5)	orthoamino biphenyl - dip
[7]	Pyridine/Ethanol/water (10/4/3)	p-anisidine - spray

[8] Onozooka - crude cellulase - release of uronic acid from cell wall

[9] *Pseudomonas syringiae* Uronate dehydrogenase - specific for glucuronic and galacuronic acid monomers
(Bateman *et al.* 1970. Arch. Biochem. Biophys. 136, 97-105.)

TABLE VI. CHITOSAN: CHITIN RATIOS IN FUNGI

Organism	Age of culture (days)	Soluble aminosugar* (chitosan µg/1 mg wall)	Insoluble aminosuga (chitin µg/1 mg wal
Phycomycetes			
Comycetes			
Karlingia rosea QM 517	10	26	16
Zygomycetes			
Mucorales			
Absidia ramosa QM 8b	3	71	80
Absidia sp. QM 45b	6	5	14
Circinella muscae QM 629	3	11	41
Cunninghamella echinulata QM 35c	3	49	62
Helicostylum piroforme QM 546	3	25	102
Mucor genevensis RC 46	2(f)	50	64
Mucor heterosporus QM 615	3	28	57
Mucor oblongisporus QM 776	3	42	58
Rhizopus arrhizus QM 1032	2(f)	68	65
Syncephalastrum racemosum QM 709	3	26	60
Ascomycetes			
Chaetomium globosum QM 104a	7	3	67
Nectria gliocladioides QM 7434	4	5	0
Neocosmospora vasinfecta QM 9154	4	5	88
Neurospora crassa FGSC 262	3	5	30
Peziza ostracoderma QM 7795	4	0	15
Trichoderma viride QM 6a	4	0	67
Trichophyton mentagrophytes QM 248	4	1	37
Basidiomycetes			
Flammulina (Collybia) velutipes QM 1012	5	3	66
Fomes pinicola QM 511	22	4	60
Polyporus betulinus QM 8387	10	0	8
Polyporus cinnabarinus QM 8846	7	19	22
Poria placenta QM 1010	19	0	2
Schizophyllum commune QM 812	4	3	87
Ustilago maydis (U. zeae) QM 990	4	2	7
Deuteromycetes			
Alternaria sp. QM 7771	3	19	64
Aspergillus parasiticus QM 883	6	5	80
Aspergillus parasiticus QM 884	4	6	95
Aspergillus parasiticus var. globosus QM 9363	4	8	57
Botryodiplodia sp. QM 7092	4	3	34
Cephaliophora tropica QM 596	8	15	126
Dactylium dendroides QM 508	4	4	120
Metarrhizium anisopliae QM 192	4	6	34
Penicillium melinii QM 1931	4	9	77

(f) - grown in 10 l fermentor system

* a factor covering losses due to the hydrolytic procedure has not been applied.

TABLE VII. DEGREE OF ACETYLATION OF CHITIN AND CHITOSAN

	% Acetylation[1]
Chitin (Lobster - Kodak)	101.8
Chitin (Lobster purified - Kodak)	95.9
Chitin (Crustacean - Calbiochem)	84.9
Chitin (Shrimp - Lab. preparation)	65.1
Chitosan (Pfanstiehl)	31.6
Chitosan (Calbiochem)	17.0

[1]Using the Tracey method (3)

n. b. α-chitin is generally considered to possess up to 12.5% deacetylated residues (4)

TABLE VIIIa. CHITOSANASE/CHITINASE ACTIVITIES

Organism	Chitinase[1]	Chitosanase[2]	Chitosanase/Chitinase Ratio
Aspergillus sclerotiorum (QM 6732)	0.14	0.40	2.86
Bacterial sp. (QM B1559)	0.06	0.56	9.34
Bacterial sp. 2Δ	0.10	0.12	1.20
Bacterial sp. Soil 2	0.12	1.04	8.67
Bacterial sp. Soil 6E2	0.10	1.56	15.6
Beauvaria bassiana (QM 7435)	0.16	0.30	1.87
Fungal sp. (C 7405)	0.27	0.76	2.81
Fungal sp. Soil 5A	0.43	0.92	2.14
Penicillium avellaneum (QM 1849)	0.22	1.30	5.91
Penicillium citrinum (QM 6748)	0.16	1.07	6.69
Penicillium islandicum (QM 7571)	0.30	0.56	1.87
Penicillium lilacinum (QM 1034)	0.08	0.20	2.50
Rhizopus nigricans (QM 387)	0	0.52	∞
Streptomycete sp. + *Bacillus cereus*	0.16	0.96	6.00
Streptomycete sp. (QM TC11)	0.19	1.44	7.58
Streptomyces globisporus (NRRL-B2872)	0.32	0.74	2.31
Streptomyces parvus (NIHJ 1115)	0.16	0.58	3.62
Streptomyces satsumaensis (1399)	0.13	0.60	4.61
Actinomycete sp. (QM TC52)	0.43	0.14	0.326
Metarrhizium anisopliae (QM 192)	0.42	0.24	0.571
Monospora brevis (QM 1243)	0.12	0	0
Serratia marcescens (QM B1466)	0.23	0.13	0.464
Streptomycete sp. (0143)	0.60	0.19	0.317

[1] mg of reducing groups as glucose by DNS/ml/18 hr at 37° C.
Assay 0.5 ml Shrimp chitin (1% wiley milled) in 0.1 M Na citrate buffer pH 5.5
0.5 ml enzyme (culture broth from organisms grown in the presence of *R. rhizopodiformis*)
Merthiolate added as an antimicrobial agent to 0.01%.

[2] Assay as above utilizing 1% chitosan (Pfanstiehl colloidal)

TABLE VIIIb. OTHER CHITOSANASE PRODUCERS

Organism	% Utilization of [1] *Rhizopus rhizopodiformis* hyphae as C. substrate	Chitosanase[2]
Arthrobacter sp. Ballou (GMJ-1)	28%	0.70
Aspergillus candidus (QM 9371)	44%	0.60
Aspergillus flavipes (QM 1994)	13%	1.02
Aspergillus fumigatus (QM 45h)	31%	0.68
Aspergillus giganteus (QM 1970)	31%	0.84
Aspergillus ruber (QM 360)	38%	1.04
Aspergillus sclerotiorum (QM 6732)	46%	0.59
Cokeromyces recurvatus (QM 1248)	27%	1.08
Gliocladium nigrum (QM 1240)	37%	1.11
Penicillium islandicum (QM 7571)	29%	0.78
Penicillium purpurogenum (QM 1960)	NT	0.84
Penicillium wortmanni (QM 7323)	18%	0.98
Rhodotorula sannici (QM 8223)	27%	0.90
Stachybotrys lobulata (QM 1370)	38%	1.28
Streptomycete sp. (QM TC77)	42%	0.80
Trichoderma lignorum (QM 1275)	30%	0.98
Trichurus spiralis (QM 834)	38%	1.04
Zygorrhynchus moelleri (QM 856)	26%	0.78

[1] Growth on 0.9% *Rhizopus rhizopodiformis* walls as carbon source salts medium (TT) 0.01% peptone, 0.01% yeast extract, at 29° C for 14 days. 30 ml media in 150 ml erlenmeyer shaken at 200 rpm.

[2] as mg. Reducing groups as glucose by DNS reagent/ml/24 hr at 37°.
Assay 0.5 ml Chitosan (1%-Pfanstiehl colloidal) in 0.1 M Na citrate buffer pH 5.5
0.5 ml enzyme culture filtrate
Merthiolate (0.01%) final concentration.

TABLE IX. EFFECT OF SUBSTRATE ON CHITOSANASE PRODUCTION

Substrate	Bacterial sp. S6E (pH 5.5) **	*Pen. islandicum* (pH 4.5) **
Chitosan (0.5%) (reppt)	0.88	0.05
Mushroom (1.0%)	0.43	0.25
Candida utilis (0.5%)	0.13	0.05
Lepiota procera (0.5%)	0.15	0.24
Mucor ramosissimus (0.5%)	0.41	0.68
Mucor ramosissimus (1.0%)	0.80	0.92

** . . . . mg RS/ ml/ hr at 50° C

TABLE X. EFFECT OF POLYOXIN D ON FUNGAL GROWTH[1]

	µg/ml Polyoxin D						
	C	1.0	5.0	10.0	50	100	500
Rhizopus rhizopodiformis QM 9395	++++	++++	+++	+++	+++	+++	++
Rhizopus arrhizus QM 1032	++++	++++	++++	++++	++++	++++	+++ N.S.
Mucor genevensis RC 46	+++	+++	+++	+++	+++	+++	+++ 3 days = (½)
Basidiomycete sp. QM 806	+++	+++	+	+	(+)	tr	tr

1. all at 6 days
2. N.S. - not sporing
3. tr - trace of growth

Assay

Fungi were added as 1 drop of a spore suspension to 1 ml of yeast nitrogen base (Difco) medium in test tubes. The fungi were then cultured for two weeks at room temperature with periodic observation.

Polyoxin D was filter sterilized and added as a concentrated solution to the aliquots of the YNB medium to achieve a range of concentrations from 0.5 to 500 µg/ml (0.95 mM).

PUBLICATIONS

1. Amer. Soc. Microbiol. 72nd Annual Meeting, Philadelphia, 1972.

G218 Involvement of Chitosanase in the Lysis of *Rhizopus rhizopodiformis*. R. L. MONAGHAN, D. E. EVELEIGH (Rutgers Univ., New Brunswick, N.J.) and E. T. REESE (U.S. Army, Natick, Mass.)

Two hundred strains of fungi and bacteria were screened, using a plate clearing assay and a reducing sugar assay, for ability to degrade the cell walls of *Rhizopus rhizopodiformis*, a human burn pathogen. Six bacterial and three fungal isolates were selected for further testing; certain of these cultures being able to lyse the live pathogen. Extracellular lytic enzymes were obtained by growing the cultures in the presence of either dead or living *R. rhizopodiformis*. The presence of a new class of enzymes, chitosanases, has been demonstrated in these lytic enzyme preparations. Our crude chitosanase preparations exhibit considerably greater activity towards chitosan than chitin. The best producers of chitosanase *per se* were *Penicillium islandicum* and an unknown bacterial isolate. *P. islandicum* produces the greatest yields of chitosanase when grown on cell walls of *R. rhizopodiformis* or *Mucor rouxianum* as a substrate. In addition to these substrates, the bacterium produces high enzyme levels when grown on reprecipitated chitosan.

2. 4th International Fermentation Symposium, Kyoto, 1972

LYSIS OF THE PATHOGEN RHIZOPUS RHIZOPODIFORMIS

G10-3

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A study has been initiated on the control of pathogenic fungi infecting burn tissue, by therapeutic use of cell wall degrading enzymes (CWDE). The screening of cultures for their ability to degrade the cell walls of a pathogenic strain of Rhizopus rhizopodiformis QM 9395, was carried out using a primary "agar plate clearing"assay and subsequent testing of isolates in liquid culture. Test organisms were obtained by the soil enrichment technique or from stock collections. 50 cultures (40 fungi and 10 bacteria) were obtained that showed lytic activity towards the cell walls by the"plate clearing" test. Further selection from these lytic organisms was made on the basis of their ability to utilize R.rhizopodiformis cell walls as a sole carbohydrate source in liquid culture. These organisms included 5 streptomycetes, 2 penicillia, 3 bacteria (unidentified) and 1 fungus (unidentified), which produced extracellular CWDE with varying degrees of activity against cell wall preparations. R.rhizopodiformis was also able to utilize its own cell walls as a carbohydrate source.

3. Monaghan, R.L., D.E. Eveleigh and E.T. Reese. 1972. Chitosanase- A novel enzyme. In preparation.

4. Mr. Monaghan was awarded the prize for best student presentation at the Society for American Microbiologists, New Jersey Branch General Presentations Meeting, 1972.