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Cell Wall Studies of *Histoplasma capsulatum* and *Blastomyces dermatitidis* Using Autologous and Heterologous Enzymes

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Enzymes capable of hydrolyzing cell walls of *Blastomyces dermatitidis* and chemotypes I and II of *Histoplasma capsulatum* were prepared in the laboratory or obtained from commercial sources. They included chitinases, β-1,3-glucanases, β-1,6-glucanase, and Pronase. Monosaccharides and disaccharides of glucose released from the cell walls by the enzymes were determined qualitatively by paper and gas-liquid chromatography, and monosaccharides were quantitated by the latter technique as well. An enzyme system isolated from *Streptomyces* sp. containing both chitinase and glucanase released maximum amounts of glucose and *N*-acetylglucosamine from the cell walls of *H. capsulatum* chemotype I. A chitinase preparation, free of glucanase, from *Serratia marcescens* released only chitobiose and *N*-acetylglucosamine from chemotype I cell walls, but the total quantity of *N*-acetylglucosamine released was about 60% less than that released by the *Streptomyces* system. A β-1,3-glucanase from *Bacillus circulans* hydrolyzed the cell walls of *H. capsulatum* chemotype I, but a β-1,6-glucanase failed to release glucose from the same walls. Autolytic enzymes, viz., β-1,3-glucanases and several glycosidases, were detected as constitutive enzymes in both yeast and mycelial phases of *B. dermatitidis* and *H. capsulatum* chemotypes I and II. No difference in the amount of activity was found between cell sap and culture filtrate preparations. The β-glucanases prepared from the *Histoplasma* and *Blastomyces* strains were active on the cell walls of the yeast phases of *H. capsulatum* chemotypes I and II, releasing laminaribiose and glucose, but were essentially inactive on the cell walls of *B. dermatitidis*. Chitinase, β-1,6-glucanase, α-glucanase, and α-glucosidase activities were absent from these fungal enzyme preparations.

The cell wall of *Histoplasma capsulatum*, a dimorphic fungus that infects both man and other animals, consists predominantly of chitin and glucan (12-14, 23, 24). Based on the ratio of chitin to glucan in the walls of the yeast phase, those strains of *H. capsulatum* studied to date can be divided into two chemotypes, I and II (14). Chemotype I cell walls contain more chitin and less glucan than chemotype II. Moreover, the evidence suggests that the glucan in chemotype II cell walls is predominantly linked in the α configuration with few β-glucans present, whereas the glucan of chemotype I is entirely β-linked.

Lytic enzymes have been used in the past to investigate the cell wall of *H. capsulatum*, albeit to a limited extent. For example, Domer et al. (12, 14) used a crude chitinase to attack the cell wall of *H. capsulatum*, but since the preparation contained not only chitinase but also glucanase activity, no conclusions concerning the role of chitin or glucan in wall structure could be drawn. Additionally, Kanetsuna et al. (23) employed a chitinase as well as a preparation containing several glucanases to lyse cell walls of *H. capsulatum*. They suggested that the wall of *H. capsulatum* consisted of an inner layer of chitin and an outer layer of glucan.

In the present investigation we decided to expand the studies of *H. capsulatum* by Domer (12, 14) in two directions: (i) using partially purified enzymes produced by other microorganisms in an attempt to further define the cell wall and (ii) searching for glucan hydrolases produced by the fungus itself. For comparative purposes, cell walls and cultures of *Blastomyces dermatitidis* were included in the studies as well.

MATERIALS AND METHODS

Microorganisms and cultivation. *H. capsulatum* SwA, *H. capsulatum* G184A, and *B. dermatitidis*

3489 were clinical isolates obtained from H. C. Sweany (Missouri State Sanatorium), M. D. Berliner (Harvard School of Public Health), and J. D. Schneidau, Jr. (Tulane University), respectively, and were maintained in the yeast phase by weekly transfer on brain heart infusion agar slants (Difco) at 37°C. *H. capsulatum* SwA was representative of chemotype I, whereas *H. capsulatum* G184A was representative of chemotype II. Cell walls as well as enzymes were derived from yeast- or mycelial-phase cultures incubated in modified Sabouraud liquid broth (Baltimore Biological Co.) that had been dispensed in 100-ml amounts in 500-ml Erlenmyer flasks. Additionally, enzymes were prepared from the yeast phase of *H. capsulatum* SwA grown in a modified synthetic medium (33) consisting of: 0.7 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.58 g of $MgSO_4 \cdot 7H_2O$, 0.018 g of $ZnSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 1.0 g of $(NH_4)_2SO_4$ dissolved in 1 liter of distilled water. The pH of the latter medium was adjusted to 7.0, and chitin or laminarin at a final concentration of 1% was added as the sole carbon source.

Inocula for the mass production of yeasts or mycelia were prepared in the following manner. Two slants of brain heart infusion agar were seeded from the stock culture and incubated for 5 days at 37°C. To prepare yeasts, the growth from both slants was washed from the surface and used to inoculate a single flask of modified Sabouraud liquid broth, which was incubated at 37°C for 3 days on a gyratory shaker at 160 rpm. A 5-ml portion of this culture was then inoculated into each of four flasks of modified Sabouraud liquid broth and incubated as above. Cultures were harvested by centrifugation at $1,000 \times g$. To prepare mycelia, the brain heart infusion agar slants were inoculated with yeast and used to seed the modified Sabouraud liquid broth, but the culture was subsequently incubated at 25°C for 14 days on a gyratory shaker. Complete conversion to the mycelial phase had occurred by the end of 14 days, and then four additional flasks of medium were inoculated from the 14-day-old culture. The fresh cultures were incubated for only 7 days and harvested by centrifugation.

Protoplasts of the *H. capsulatum* chemotype I isolate were produced and harvested by the method of Carbonell et al. (8), and the degree of protoplast formation was determined by phase-contrast microscopy and by the use of Calcofluor White M-2R fluorescent brightener (19).

Source of substrates and biochemicals. Cell walls were obtained from yeast-phase cells of all three strains, which had been disrupted mechanically in a model MSK Braun cell homogenizer (Bronwill Scientific Co.) operating at 4,000 cycles/min, the inner chamber of which was cooled by intermittent blasts of carbon dioxide. Glasperlen beads (0.25 to 0.30 mm in diameter, Bronwill Scientific Co.) were added to cells in glass bottles in a ratio of approximately 3:1 (wt/vol), i.e., 30 g of beads per 10 g (wet weight) of fungus suspended in approximately 10 ml of 0.1 N sodium phosphate buffer at pH 7.0. Virtually 100% breakage of the yeast cells occurred after 4 min of agitation. The degree of breakage was determined by Gram staining, with unbroken cells being gram positive and broken cells being gram negative, and by culture of the debris on blood agar plates. Cell walls were separated from the mixture by centrifugation at $1,000 \times g$ and were washed at least 10 times with 0.2 M NaCl, once with 1 M NaCl, and 20 times with distilled water before lyophilization and storage in a vacuum desiccator at 25°C. A glycoprotein fraction, soluble in water, was extracted with ethylenediamine from these cell walls after lipid extraction (14).

Laminaribiose was prepared according to Wolfrom and Franks (40), but the remainder of the substrates were commercial preparations or gifts. Chitin, laminarin, *p*-nitrophenyl phosphate, 2-deoxyglucose, and *p*-nitrophenyl glycosides were purchased from Sigma Chemical Co., and pustulan, Azocoll, and Lipostrate-CB were purchased from Calbiochem. Laminarin is a β-1,3-glucan, and pustulan is a β-1,6-glucan. Chitobiose was prepared according to the method of Bailey and Pridham (3). Calcofluor White M-2R was a gift of the American Cyanimid Co.

Preparation and assay of heterologous enzymes. A chitinase was prepared from the culture filtrate of *Streptomyces* sp. ATCC 11238 (obtained from the American Type Culture Collection [ATCC]) by a method similar to that of Skujins et al. (36, 37) in which the crude enzyme preparations were chromatographed on a Sephadex G-100 column. A second chitinase was isolated from the culture medium of *Serratia marcescens* by precipitation with solid ammonium sulfate (32), followed by column chromatography on Sephadex G-100. Both chitinases were prepared from cultures incubated with chitin as the sole carbon source. To obtain β-1,3- and β-1,6-glucanases, *Bacillus circulans* was incubated in a medium containing laminarin as the sole carbon source and the glucanases were purified on a Sephadex G-100 column, using the method of Fleet and Phaff (17). Finally, a crude pullulanase containing α-1,4 and α-1,6 activities was prepared from *Aerobacter aerogenes* as previously described (5).

Several enzymes were purchased from commercial sources: chitinase (Calbiochem 22071, lot 400891); laminarinase (Calbiochem 427999, lot 400266); protease type VI from *Streptomyces griseus* (Sigma P-5130, lot 114C-0168); and grade B Pronase (Calbiochem 53702, lot 502133).

Enzymatic hydrolyses of cell walls, chitin, laminarin, chitobiose, or pustulan were performed in a phosphate-acetate buffer, pH 5.0 to 5.5, at 37°C. A 0.5- to 2.0-mg portion of substrate was added to 2 ml of buffer in screw-capped tubes fitted with Teflon-lined lids. A given quantity of enzyme, determined by preliminary assays to be the amount required for maximum hydrolysis, was added to the substrate and incubated for 24 to 48 h. The products of the hydrolyses were determined by paper and gas-liquid chromatography (see below). Since some of the substrates were insoluble, the tubes were incubated on a reciprocating shaker operating at approximately 100 excursions/min to allow maximum contact of enzyme and substrate. The total distance traveled per excursion was approximately 1.5 in (ca. 3.81 cm). Phosphatase, lipase, and protease activities were determined using *p*-nitrophenyl phosphate, Lipostrate-CB, and Azocoll, respectively, as sub-

strates according to the instructions of the manufacturers for the conditions of hydrolysis.

Preparation and assay of autologous enzymes. Glucanases were extracted from the culture filtrate (extracellular), the cell wall (cell wall-associated), and the cell sap (intracellular) fractions of all three strains of fungi in the following manner. Cell sap (i.e., all cellular constituents other than cell walls remaining in the supernatant after low-speed centrifugation after the homogenization of yeast cells) and culture filtrate were fractionated at 4°C by the addition of solid ammonium sulfate. The precipitate at 40% saturation was tested for glucanase activity, found to be negative, and discarded, and the concentration of ammonium sulfate was then raised to 75% saturation. The resultant precipitate was resuspended in 5 to 10 ml of 0.1 N sodium phosphate buffer, pH 7.0, dialyzed for 4 h against distilled water, lyophilized, and stored at −20°C. Cell wall-associated glucanase was removed from suspensions of cell walls by incubation under sterile conditions in 0.1 N sodium phosphate buffer, pH 7.0, for 7 days at 23°C. After centrifugation to remove the cell walls, the supernatant was fractionated and stored as above. Where appropriate, protein estimates were made by the method of Lowry et al. (29).

The enzymatic hydrolyses were performed as previously described (12), and the reaction was stopped by heating in a boiling-water bath. The residue was removed by centrifugation, and 50 μl of the supernatant was used for paper chromatography. A 1-ml portion of the remaining hydrolysate was divided equally between two vacuoles (Wheaton Glass Co.) and lyophilized. The lyophilized material in each vacuole was dissolved in 1 ml of warm 1.0 N methanolic HCl and incubated at 80°C for 3 h (10). The methanol was subsequently removed under nitrogen, and *O*-trimethylsilyl ether derivatives were prepared for gas-liquid chromatography (26, 38), using mannitol as an internal standard for quantitation. Using this two-step procedure, i.e., enzyme hydrolysis followed by acid hydrolysis, all of the glucose in the substrate could be quantitated, even that which was released by the enzyme in the form of oligomers.

Substrate specificity was determined by measuring the activity of enzyme preparations against a battery of *p*-nitrophenyl glycosides (28) as well as against laminarin, chitin, pustulan, and cell wall preparations. The effect of pH on glucanase activity was observed between pH 3.5 and 7.5, using 0.1 N sodium phosphate and 0.05 M phosphate citrate and phosphate acetate buffers. The effect of temperature on glucanase activity was observed between 23 and 80°C, and the effect of metal ions on enzyme activity was observed using 5×10^{-3} M Ca^{2+}, 5×10^{-3} M Cu^{2+}, 5×10^{-3} M Hg^{2+}, 5×10^{-3} M Mg^{2+}, or 5×10^{-3} M Mn^{2+}. The effect of 5×10^{-6} M ethylenediaminetetraacetic acid was also determined.

Chromatography. Carbohydrates released by enzymatic attack were determined qualitatively by descending paper chromatography. A 50-ml portion of the hydrolysate was spotted on Whatman no.1 chromatography paper and developed for 12 to 16 h in a system consisting of 1-butanol–pyridine–0.1 N HCl (5:3:2, vol/vol). The chromatograms were then air dried, dipped in benzidine reagent, and heated at 100°C for 1 min to visualize the carbohydrate products of the hydrolysis (6).

The monosaccharides and disaccharides released were also detected as *O*-trimethylsilyl ether derivatives in a Barber-Coleman gas-liquid chromatograph equipped with a flame ionization detector and a glass tubular column (6 ft [ca. 182.88 cm]) containing 3% OV-1 on 80/100 Chromosorb W-HP (Supelco, Inc.). Nitrogen was the carrier gas. *N*-acetylglucosamine and glucose were the only carbohydrates that had been quantitated. They were quantitated by this method, using mannitol as the internal standard. The detector response to glucose was equivalent to that of mannitol on a weight-to-weight basis so that calculations could be done directly without the use of a factor. The detector response to an equal amount of *N*-acetylglucosamine, however, was 2.1 times less than the response to mannitol. That factor, therefore, was used in all calculations involving *N*-acetylglucosamine.

When the hydrolytic mixture contained both mono- and disaccharides, they were separated in a single operation with the following program. A constant temperature of 170°C was used to elute the monosaccharides from the column during the first 15 min of operation. At the end of that time, the temperature was increased to 220°C at a rate of 10°C/min and held there for 40 min, during which time the disaccharides were eluted. Table 1 contains the retention indices, based on α-glucose, which was arbitrarily assigned the value of 1.0, of 10 mono- and disaccharides that were separated by this procedure. One or two major peaks were observed with each compound, as indicated in the table. Several minor peaks occurred when mannose was tested, but they were proportionately insignificant compared to the major peak. When cell walls were hydrolyzed with methanolic HCl, however, only one anomeric form of mannose was observed. No minor peaks were observed with glucose, *N*-acetylglucosamine, or the disaccharides.

RESULTS

Enzymatic hydrolyses with heterologous enzymes. The products of enzymatic hydrolyses of the yeast phase cell walls of *H. capsulatum* and of selected additional substrates of known composition, which were determined using the heterologous enzymes prepared as described above or purchased from commercial sources, are presented in Table 2. The products were identified by paper and gas chromatography after 4 h of incubation. If the mixtures were incubated for 48 h, only monosaccharides could be detected. The chitinase produced by *Streptomyces* sp. ATCC 11238 was contaminated with glucanase activity, and attempts to purify it by column chromatography were unsuccessful. Therefore, *N*-acetylglucosamine, chitobiose, and glucose were released from the fungal cell walls. The

same enzyme system released glucose and laminaribiose from laminarin and *N*-acetylglucosamine and chitobiose from chitin. Larger oligomers of both glucose and *N*-acetylglucosamine were released from laminarin and chitin, respectively, during the early hours of incubation but had been hydrolyzed to monomers or dimers by 24 h. A commercial chitinase also contained glucanase activity and produced a similar hydrolysis pattern from the cell walls.

Three of the enzyme preparations isolated in this study, a chitinase-glucanase mixture from *Streptomyces* sp. ATCC 11238, a chitinase from *S. marcescens*, and a β-1,3-glucanase from *B. circulans*, were used to quantitate by gas-liquid chromatography the release of glucose and *N*-acetylglucosamine from the cell walls of the yeast phase of *H. capsulatum* chemotype I (Table 3). The *Streptomyces* mixture released 9 to 12% of the cell wall as glucose and 25 to 38% as *N*-acetylglucosamine. Chitobiose could be detected in hydrolysates, as verified by co-gas-liquid chromatography, when the incubation period was less than 24 h, but it was not quantitated. Chitobiase activity was demonstrated in the enzyme mixture, since *N*-acetylglucosamine was released from chitobiose. Moreover, the enzyme mixture from *Streptomyces* was free of lipase, phosphatase, and protease activity.

The chitinase system prepared from *S. marcescens* was free of glucanase activity, and only *N*-acetylglucosamine was released from the cell walls of *H. capsulatum* after 24 h (Table 3). As with the *Streptomyces* mixture, the chitinase from *S. marcescens* contained chitobiase and was free of lipase, phosphatase, and protease activity. Using the *S. marcescens* system, however, only 12 to 15% of the cell wall was hydrolyzed to *N*-acetylglucosamine. Pretreatment of cell walls with β-1,3-glucanase from *B. circulans* prior to hydrolysis with this chitinase resulted in only a moderate increase in the amount of *N*-acetylglucosamine released.

The glucanase prepared from *B. circulans* contained no chitinase but did contain both β-1,3- and β-1,6-glucanase activities. The two ac-

TABLE 1. *Retention indices of O-trimethylsilyl ether derivatives of mono- and disaccharides separated in a single run by gas-liquid chromatography*[a]

Sugar	Retention index
Mannose	0.70
α-Glucose	1.00
Mannitol	1.25
β-Glucose	1.50
N-acetylglucosamine	2.32
Sucrose	3.40
Cellobiose	4.00, 6.00
Laminaribiose	7.00, 7.40
Gentibiose	8.80, 9.20
Chitobiose	11.10

[a] The following chromatographic conditions and equipment were used: flame ionization detector; 3% OV-1 column on 80/100 Chromosorb W-HP; isothermal operation, 15 min at 170°C followed by programmed increase, 10°C/min, to 220°C; injector temperature, 265°C; detector temperature, 235°C.

TABLE 2. *Products resulting after 4 h of incubation of selected enzyme preparations with various polysaccharide substrates*[a]

Source of enzymes	Substrates[b]			
	Cell walls	Chitin	Laminarin	Pustulan
Chitinases				
Streptomyces sp.	Glc(tr), GlcNAc $(GlcNAc)_2$	GlcNAc $(GlcNAc)_n$	Glc(tr)	None
S. marcescens,	GlcNAc	GlcNAc	None	None
Commercial (Calbiochem)	Glc(tr), GlcNAc $(GlcNAc)_2$	GlcNAc $(GlcNAc)_2$	Glc(tr)	None
Glucanases				
B. circulans (crude)	Glc, GlcNAc(tr)	None	Glc, $(Glc)_n$	Glc
B. circulans (β-1,3-glucanase)	Glc, GlcNAc(tr)	None	Glc, $(Glc)_n$	None
B. circulans (β-1,6-glucanase)	None	None	None	Glc
Commercial (Calbiochem)	Glc, GlcNAc(tr)	None	Glc, $(Glc)_n$	Glc(tr)
Pronases				
Commercial (Sigma)	Glc, $(Glc)_n$	None	Glc, $(Glc)_n$	None
Commercial (Calbiochem)	None	None	None	None

[a] Products were identified by paper chromatography. Monosaccharides and disaccharides were confirmed by gas chromatography.

[b] Abbreviations: Glc, glucose; $(Glc)_n$, laminaribiose and higher oligomers of glucose; GlcNAc, *N*-acetylglucosamine; $(GlcNAc)_2$, chitobiose; $(GlcNAc)_n$, higher oligomers of *N*-acetylglucosamine; and tr, trace.

TABLE 3. *Quantities of glucose and N-acetylglucosamine released from the yeast-phase cell walls of H. capsulatum by selected enzymes*

Enzymes	Quantities released (mg/2 mg of cell walls)	
	Glucose	*N*-acetyl-D-glucosamine
Chitinase-glucanase (*Streptomyces* sp.)	0.25[a]	0.75
Chitinase (*S. marcescens*)	0.00	0.25
β-1,3-Glucanase (*B. circulans*)	0.50	0.13

[a] Sugars were detected and quantitated by gas chromatography. See legend to Table 1 for conditions.

tivities were subsequently separated by column chromatography on Sephadex G-100. Both partially purified glucanases were free of protease, lipase, and phosphatase activities. When cell walls were hydrolyzed with partially purified β-1,3-glucanase, 22 to 23% of the wall was released as glucose and approximately 2% was released as *N*-acetylglucosamine (Table 3). Pretreatment of cell walls with the *Serratia* chitinase prior to attack by the β-1,3-glucanase did not result in increased amounts of glucose being released. The β-1,6-glucanase did not release glucose from cell walls, and neither it nor the crude *B. circulans* enzyme from which it was derived released gentiobiose. Both preparations, however, released glucose and gentiobiose from pustulan after short periods of incubation. The β-1,3-glucanase released glucose and β-1,3-oligomers of glucose from laminarin but released only glucose from cell walls.

A commercial laminarinase, which should theoretically be active only on laminarin, was active both on laminarin and pustulan. It released glucose and a trace of *N*-acetylglucosamine from the cell walls of *H. capsulatum*. A crude pullulanase prepared from *A. aerogenes* was ineffective against the cell walls.

A commercial Pronase (Sigma P-5130) was also found to contain β-1,3-glucanase activity. It released laminaribiose from both laminarin and cell walls, but only trace amounts of glucose appeared in the hydrolysates, even after 48 h of incubation. A second Pronase (Calbiochem 53702), free of glucanase, released neither glucose nor *N*-acetylglucosamine from cell walls. Pretreatment of cell walls with the latter Pronase did not significantly enhance cell wall hydrolysis by either glucanase or chitinase.

Enzymatic hydrolyses with autologous enzymes. Initially, glucanase activity was demonstrated in the culture filtrate of the chemotype I isolate of *H. capsulatum* when it was in a synthetic medium with laminarin as the sole carbon source. Subsequently, however, glucanase was found to be a constitutive enzyme, not requiring laminarin for expression, and glucanase activity was demonstrated in the culture filtrates of both the yeast and mycelial phases of the same isolate as well as in the other two strains examined, viz., an isolate of *H. capsulatum* chemotype II and one of *B. dermatitidis*. The culture filtrate of the mycelial phase of the chemotype I isolate of *H. capsulatum* contained approximately 20% more activity than did the corresponding yeast-phase culture filtrate. Moreover, protoplasts of the yeast phase of that same isolate liberated glucanase into the culture medium as well. None of the fungi was ever successfully cultivated using chitin as the sole carbon source, and chitinase activity was never detected in cultures of fungi grown on laminarin or glucose.

Glucanase was also detected in the cell sap derived from both yeast- and mycelial-phase cells of all three strains. The cells had been thoroughly washed to remove any extracellular enzyme prior to breakage. The recovery of β-1,3-glucanase activity, as measured by the hydrolysis of laminarin, from the yeast-phase cells of the chemotype I strain of *H. capsulatum* is presented in Table 4. It should be noted that approximately 60% of the activity was not liberated into the culture medium. Glucanase closely associated with the cell wall was detected in suspensions of washed walls that had been incubated for 7 days, so that at least some of the glucanase remained firmly attached to the wall after homogenization of the whole cells. Glucose was present in the suspensions at the end of the incubation period as well, perhaps the result of the autolytic property of the glucanase. Such activity, however, could only be detected with cell walls that had not been lyophilized but had been incubated directly for a week after their preparation. The incubation of lyophilized cell wall preparations with enzymes not capable of attacking the walls; i.e., Pronase from Calbiochem and β-1,6-glucanase from *B. circulans* did not result in the release

TABLE 4. *β-1,3-Glucanase activity from yeast-phase cultures of H. capsulatum chemotype I*

Source of activity	Units (μmol of glucose from laminarin/30 min)	Protein (mg)[a]	Sp act
Culture filtrate	586.0	12.5	46.8
Cell sap fraction	729.0	14.9	48.7
Cell wall-associated	61.0	4.3	14.2

[a] Lowry method (reference 29).

of glucose from the walls after 48 h of incubation.

All of the fungal glucanases hydrolyzed laminarin, a β-1,3-glucan, but not pustulan, a β-1,6-glucan, when tested over a wide range of temperature and pH values. A comparison of the activity of one of the fungal glucanases, that from the yeast phase of the chemotype I isolate of *H. capsulatum* with a β-1,3-glucanase prepared from *B. circulans*, is presented in Table 5. It can be seen that both enzymes hydrolyzed laminarin and a glycoprotein extracted from the cell wall of *H. capsulatum* chemotype I and that neither hydrolyzed pustulan. Moreover, the enzyme isolated from *B. circulans* was moderately active on the cell walls of the yeast phase of *H. capsulatum* chemotype I, whereas that isolated from the fungus released only trace amounts of glucose from its own cell wall. To determine the glycosidase activities of the crude glucanase preparations, the enzyme preparations from culture filtrates of all three strains were tested against a battery of *p*-nitrophenyl derivatives (Table 6). α-Glucosidase activity was not exhibited by any of the fungi. Both isolates of *H. capsulatum* maximally hydrolyzed *p*-nitrophenyl-β-D-glucoside, and *H. capsulatum* chemotype I and *B. dermatitidis* 3489 significantly hydrolyzed *p*-nitrophenyl-α-D-mannoside as well. The wide disparity of activities was suggestive of more than one glycosidase being produced by each fungus. Protease activity was present in all preparations, particularly in the cell sap fractions.

The glucanase preparations from the culture filtrates of all three fungi liberated glucose and laminaribiose from the yeast phase cell walls of both isolates of *H. capsulatum*, but, at most, only a trace of glucose could be removed from *B. dermatitidis* cell walls by any of the enzymes. The glucanase prepared from *H. capsulatum* chemotype I hydrolyzed the glycoprotein fraction from its own cell walls and that from the cell walls of *H. capsulatum* chemotype II but failed to hydrolyze the glycoprotein prepared from the walls of *B. dermatitidis* (Fig. 1). The glucanase from *H. capsulatum* chemotype II (not pictured) exhibited the same pattern of hydrolysis toward the glycoprotein fractions as did the other two strains. The enzyme prepared from *B. dermatitidis* also hydrolyzed the glycoprotein fractions from both *H. capsulatum* strains but not that prepared from its own walls. All three enzyme preparations hydrolyzed laminarin under the same conditions.

N-acetylglucosamine and chitobiose were not liberated from any cell walls incubated with any of the fungal glucanase preparations. The hydrolysis of the glycoprotein fraction from the cell walls of *H. capsulatum* chemotype I by its own glucanase preparations was monitored at selected intervals during incubation, and it was found that laminaribiose and higher oligomers of glucose appeared after 1 or 2 h of incubation but that laminaribiose and glucose were the predominant sugars thereafter (Fig. 2). The same enzyme hydrolyzed laminarin even more vigorously when examined at the same time intervals. Therefore, a β-1,3-glucanase and a β-glucosidase were present, but there may also have been an exo-β-1,3-glucanase in the preparation. Glucose and laminaribiose were demonstrated by paper and gas-liquid chromatography in the hydrolysates of the fungal walls, but mannose, galactose, gentiobiose, and other mono- or disaccharides were not present.

The glucanase obtained from the culture filtrate of the yeast-phase culture of *H. capsulatum* chemotype I was active over a broad pH range, but optimum activity occurred at pH 5.0 (Fig. 3A). Enzymes stored for 1 h at selected temperatures before assay showed rapid inactivation above 60°C (Fig. 3B). Optimum hydrolysis of laminarin occurred at 55°C (Fig. 3C). The glucanase was inhibited by Hg^{2+} but not by ethylenediaminetetraacetic acid. Two attempts to purify the crude glucanase by chromatography on a Sephadex G-100 column were unsuccessful, since β-D-glucosidase activity was recovered and no β-1,3-glucanase was found in the eluent.

DISCUSSION

The carbohydrate components of the cell wall of the yeast phase of *H. capsulatum* chemotype I were confirmed as β-1,3-linked glucan and β-

TABLE 5. *Hydrolyses of various substrates by β-1,3-glucanases isolated from B. circulans or the yeast phase of H. capsulatum chemotype I*

Enzyme source	Substrate			
	Unextracted wall[b]	Wall glycoprotein[b]	Laminarin	Pustulan
H. capsulatum	0.05	0.75	1.05	0.00
B. circulans	0.42	0.78	1.48	0.00

[a] Milligrams of glucose released per 2 mg of substrate after 24 h of incubation at 37°C and pH 5.5 using 100 μg of protein per assay.

[b] Both substrates were isolated from *H. capsulatum* chemotype I.

1,4-linked chitin, as suggested by Domer (12) and Kanetsuna et al. (23), inasmuch as dimers of both β-1,3-linked glucose and β-1,4-linked *N*-acetylglucosamine were isolated from cell wall hydrolysates and identified by paper and gas-liquid chromatography. No α-1,4- or α-1,6-linked glucose was detected, and α-glucan has not been found by others (12, 23). *H. capsulatum* chemotype II walls, on the other hand, appear to contain a mixture of α- and β-glucans (23), and although there was no enzymatic data presented here to confirm those conclusions, the acid solubility characteristics of the cell walls would support that contention (12).

TABLE 6. *Activity of enzymes isolated from filtrates of yeast-phase cultures on p-nitrophenyl substrates*

p-Nitrophenyl substrates	Source of enzymes (μmol of nitrophenol/30 min)[a]		
	H. capsulatum		*B. dermatitidis*
	Chemotype I	Chemotype II	
Phosphate	0.04	0.00	0.00
N-Acetyl-β-D-glucosaminide	0.40	0.40	0.40
α-D-Mannoside	1.60	0.00	1.60
α-D-Glucoside	0.00	2.00	0.00
β-D-Glucoside	2.80	2.00	0.04
α-D-Galactoside	0.80	0.00	0.01
β-D-Galactoside	0.00	0.00	0.00

[a] One unit was equivalent to 0.1 μmol of nitrophenol/30 min at 37°C at pH 5.5 with 25 μg of protein per assay.

The architectural arrangement of the chitin and glucan within the cell walls remains unsolved, although Kanetsuna et al. (23) suggested that there was an inner layer of chitin in the wall of the yeast phase of *H. capsulatum*. Our own studies indicated that at least part of the *N*-acetylglucosamine in the yeast-phase wall was linked to glucan in some fashion, since a small amount of *N*-acetylglucosamine was released from the cell walls when they were hydrolyzed by a glucanase free of chitinase activity and since maximal amounts of *N*-acetylglucosamine were released only in the presence of both glucanase and chitinase. On

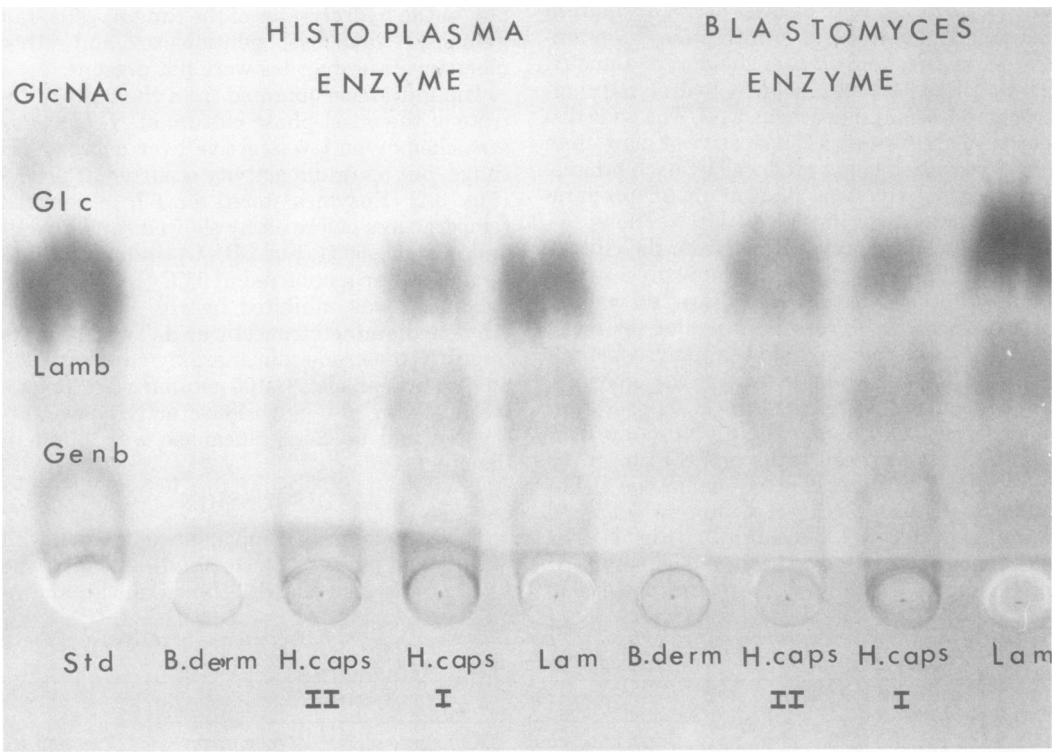

FIG. 1. *Hydrolysis of wall glycoprotein from the yeast phases of H. capsulatum chemotype I, H. capsulatum chemotype II, and B. dermatitidis 3489 by the extracellular glucanases prepared from H. capsulatum chemotype I and B. dermatitidis 3489. Substrates were incubated with the enzyme at 37°C, pH 5.5, for 24 h. Abbreviations: Glc, glucose; GlcNAc, N-acetylglucosamine; Lamb, laminaribiose; Genb, gentiobiose; I, II, B. derm, glycoproteins extracted from H. capsulatum chemotypes I and II and B. dermatitidis, respectively; Lam, laminarin; Std, standard.*

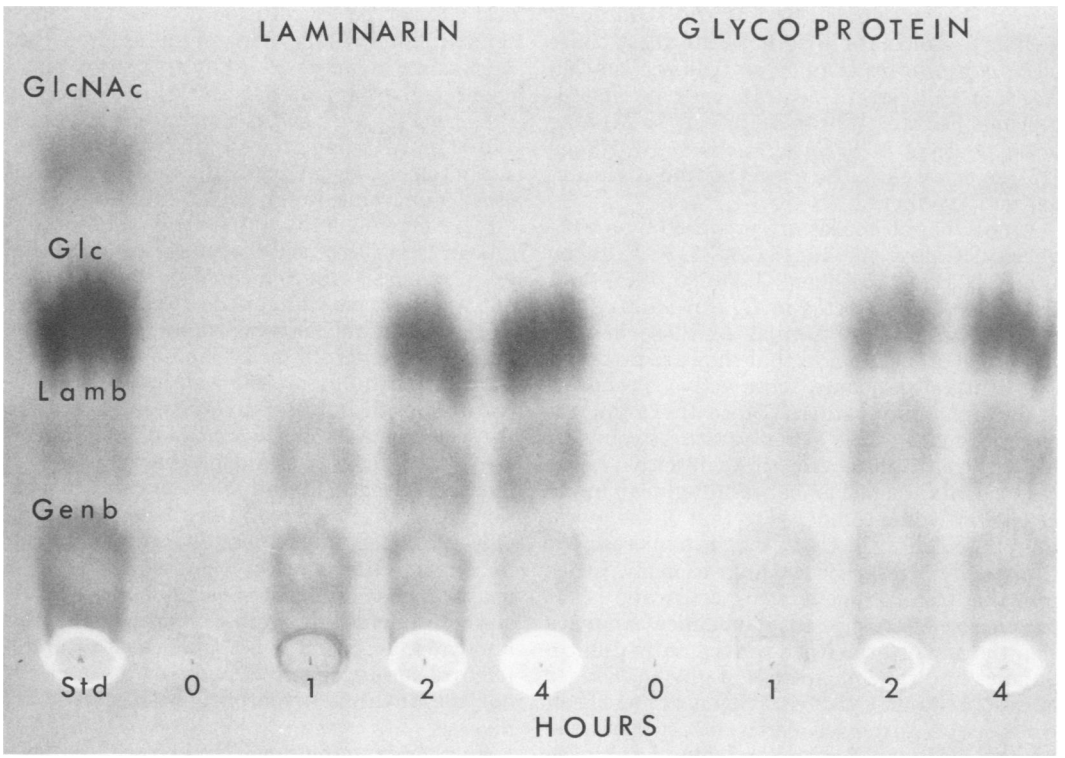

FIG. 2. *Hydrolysis of laminarin and wall glycoprotein from the yeast phase of H. capsulatum chemotype I by a glucanase prepared from the culture filtrate of the yeast phase of H. capsulatum chemotype I. Substrates were incubated with the enzyme for 0, 1, 2, and 4 h at 37°C, pH 5.5. Abbreviations: Glc, glucose; GlcNAc, N-acetylglucosamine; Lamb, laminaribiose; Genb, gentiobiose; Std, standard.*

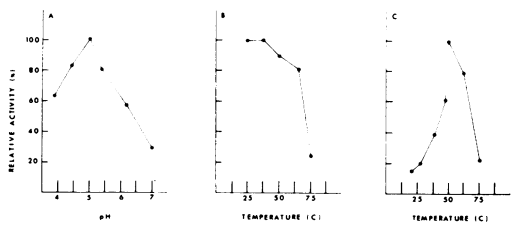

FIG. 3. *Effect of various pH values (A), various temperatures of storage (B), and various temperatures of incubation (C) on the extracellular glucanase prepared from H. capsulatum chemotype I.*

the contrary, maximal amounts of glucose were released in the presence or absence of chitinase. Protein, if present, did not interfere with the release of maximal quantities of glucose and *N*-acetylglucosamine, since prior treatment of the cells with protease did not alter the amounts of either sugar released. An outer layer of glucan has been reported for two other fungi, *Neurospora* (21) and *Aspergillus* (36), but many of the studies designed to determine the cell wall structure of *H. capsulatum* with the use of lytic enzymes were complicated by the use of enzyme preparations containing extraneous activities (12, 14, 23). In fact, most of the commercial enzymes tested in this study were contaminated by one or more additional activities.

B. dermatitidis and representatives of chemotypes I and II of *H. capsulatum* produced β-1,3-glucanase and several glycosidases constitutively when growing in either the yeast or mycelial phase. β-1,6-Glucanase, chitinase, β-glucanase, and α-glucosidase could not be detected. One might expect to find β-1,3-glucanase in both phases of the two chemotypes of *H. capsulatum*, since each has β-glucan in its yeast-phase cell (14) and some hydrolysis of glucan would be expected to occur during budding or hyphal extension, but it was somewhat surprising to find high levels of β-1,3-glucanase activity in the yeast phase of *B. dermatitidis*, since its cell walls contain little β-glucan. In fact, Kanetsuna et al. (22) found less than 5% of the glucan in the cell wall of the yeast phase of *B. dermatitidis* 3489 to be β-linked. Perhaps the ability to produce such high levels of β-glucanase activity in the virtual absence of β-glucan in the cell wall represents an evolutionary link between *Histoplasma* and *Blasto-*

myces. We anticipated finding α-glucanase as well as β-glucanase activity in the yeast phase of *H. capsulatum* chemotype II, since the cell walls of that strain contain both α- and β-glucans. Further, glucan in the cell walls of the yeast phase of *B. dermatitidis* is 95% α-linked (22), so one would also expect to find α-glucanase activity there.

Autolytic glucanases are produced by a wide variety of fungi (15, 16, 18, 20, 32; A. E. Stone and B. A. Stone, Biochem. J. 96:793, 1965), and the glucanases detected in *H. capsulatum* and *B. dermatitidis* are similar to those demonstrated in other fungi in that they are produced constitutively and are most active at moderately high temperatures. Since the β-glucanases are more active at temperatures compatible with yeast-phase growth, their activity may result in diminished amounts of β-glucan in the yeast wall when compared to that of the mycelial-phase cell wall. Using electron microscopy, Carbonell (9) observed the migration of vesicles into the area of regenerating cell walls of *H. capsulatum* and showed by chemical analysis that the newly formed cell wall contained chitin bundles and random strands of glucan. Kreger and Kopecka (25), studying regeneration of cell walls of *Saccharomyces cerevisiae*, carried such studies one step further and found that the newly formed chitin and glucan were resistant to attack by autolytic enzymes. Such enzymes hydrolyzed the intact wall, however, and it would appear that part of the cell wall glucan of the yeast might always be resistant to autolytic glucanases. It was demonstrated here, for example, that intact cell walls of *H. capsulatum* were relatively resistant to autologous β-glucanases but that extraction of the walls with ethylenediamine yielded a fraction that was highly susceptible to attack.

Morphogenesis of the fungal cell wall is thought to require the cooperation of both lytic and synthetic enzymes (7, 27), and glucanases but not chitinases have been found to be localized on or near the fungal cell wall (16, 18). Moreover, Sentandreu et al. (35) isolated a glucan synthetase from cultures of *S. cerevisiae*. Bacon (2) in a recent review suggested that the presence of enzymes involved in glucan hydrolysis may indicate that the state of the glucan and not chitin determines the mechanical properties and integrity of the wall.

There exists some disagreement in the literature on the percentage of hexose in the yeast-phase cell wall of *H. capsulatum* (1, 12, 23, 24), but there is general agreement that the yeast-phase cell walls of *H. capsulatum* chemotype I and *B. dermatitidis* contain less β-glucan than the mycelial-phase cell walls. Since *H. capsulatum* chemotype I strains do not possess α-glucans in their walls, one might assume that decreasing amounts of α-glucan in the yeast-phase wall are replaced by chitin. In chemotype II cell walls, α-glucan is replaced by β-glucan and/or chitin when growing in the yeast phase. A similar replacement might also be true for other dimorphic fungi. DeVries and Wessels (11) have demonstrated that the synthesis of β-glucan is required for the initiation of hyphal morphogenesis in *Schizophyllum commune*, but whether this is due to decreased synthesis, increased hydrolysis, or a combination of both has yet to be determined. One might speculate that extracellular or cell wall-associated glucanases result in lesser amounts of β-glucan in the yeast phases of *Histoplasma* and *Blastomyces* isolates and could play a role in wall morphogenesis. Alteration in glucan composition could affect cell wall thickness or stacking of wall components, or possibly even be a factor in determining whether the cell grows as a yeast or mycelium. However, since the diversity of fungal cell wall composition is well known (4, 30, 39), it is probable that the architectural arrangement of components and the enzymes involved in morphogenesis are equally diverse.

Further examination of the extracellular glucanases of these pathogenic fungi is warranted because: (i) characterization of individual glycohydrolases could offer insight into cell wall structure and (ii) purified glucanases would be useful in the preparation of cell wall glucans for linkage analysis. Moreover, the manipulation of enzyme activity in growing cells could provide information on the mechanisms involved in dimorphism. Recently, a purified enzyme of *Schistosoma mansoni* was used as a skin test reagent for the diagnosis of schistosomiasis (34), and since extracellular glucanases such as those produced by *H. capsulatum* and *B. dermatitidis* might stimulate the defense mechanism of the infected host, the purified enzymes could potentially be excellent antigens for detection of infection.

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LITERATURE CITED

1. **Anderson, K. L., R. W. Wheat, N. R. Conant, and W. Clingenpeel.** 1974. Composition of the cell wall and other fractions of the autolyzed yeast form of *Histoplasma capsulatum*. Mycopathol. Mycol. Appl. 54:439-451.

2. **Bacon, J. S. D.** 1973. The contribution of β-glucanases to the lysis of fungal cell walls, p. 61-74. *In* J. R. Villanueva, I. Garcia-Acha, S. Gascon, and F. Uruburu (ed.), Yeast, mould, and plant protoplasts. Academic Press Inc., New York.
3. **Bailey, R. W., and J. B. Pridham.** 1962. The separation and identification of oligosaccharides. Chromatogr. Rev. 4:114-136.
4. **Bartnicki-Garcia, S.** 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu. Rev. Microbiol. 22:87-108.
5. **Bender, H., and K. Wallenfels.** 1966. Pullulanase (an amylopectin and glycogen debranching enzyme) from *Aerobacter aerogenes*, p. 555-559. *In* E. F. Neufeld and V. Ginsburg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
6. **Block, R. J., R. LeStrange, and G. Zweig.** 1952. Paper chromatography, a laboratory manual. Academic Press Inc., New York.
7. **Cabib, E.** 1975. Molecular aspects of yeast morphogenesis. Annu. Rev. Microbiol. 29:191-214.
8. **Carbonell, L. M., M. D. Berliner, and F. Gil.** 1972. Kinetic and morphological observations on the yeast phase of *Histoplasma capsulatum* during protoplast formation. J. Bacteriol. 110:731-738.
9. **Carbonell, L. M., F. Gil, and F. Yegres.** 1973. Cell wall regeneration of protoplasts of *Histoplasma capsulatum*, p. 93-103. *In* R. J. Villanueva, I. Garcia-Acha, S. Gascon, and F. Uruburu (ed.), Yeast, mould, and plant protoplasts. Academic Press Inc., New York.
10. **Chambers, R. E., and J. R. Clamp.** 1971. An assessment of methanolysis and other factors used in the analysis of carbohydrate-containing materials. Biochem. J. 125:1009-1018.
11. **DeVries, O. M. H., and J. G. H. Wessels.** 1975. Chemical analysis of cell wall regeneration and reversion of protoplasts from *Schizophyllum commune*. Arch. Microbiol. 102:209-218.
12. **Domer, J. E.** 1971. Monosaccharide and chitin content of cell walls of *Histoplasma capsulatum* and *Blastomyces dermatitidis*. J. Bacteriol 107:870-877.
13. **Domer, J. E., and J. G. Hamilton.** 1971. The readily extracted lipids of *Histoplasma capsulatum* and *Blastomyces dermatitidis*. Biochim. Biophys. Acta 231:465-478.
14. **Domer, J. E., J. G. Hamilton, and J. C. Harkin.** 1967. Comparative study of the cell walls of the yeastlike and mycelial phase of *Histoplasma capsulatum*. J. Bacteriol. 94:466-474.
15. **Farkas, V., P. Biely, and S. Bauer.** 1973. Extracellular β-glucanases of the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta 321:246-255.
16. **Fleet, G. H., and H. J. Phaff.** 1974. Glucanases in *Schizosaccharomyces*. J. Biol. Chem. 249:1717-1728.
17. **Fleet, G. H., and H. J. Phaff.** 1974. Lysis of yeast cell walls: glucanases from *Bacillus circulans* WL-12. J. Bacteriol. 119:207-219.
18. **Fleet, G. H., and H. J. Phaff.** 1975. Glucanases in *Schizosaccharomyces*. Isolation and properties of an exo-β-glucanase from the cell extracts and culture fluid of *Schizosaccharomyces japonicus* var. versatilis. Biochim. Biophys. Acta 401:318-332.
19. **Gull, K., P. M. Moore, and A. P. J. Trinci.** 1972. Preparation of protoplasts from *Goetrichum lactis* and use of fluorescence to detect cell walls. Trans. Br. Mycol. Soc. 59:79-85.
20. **Holten, V. Z., and S. Bartnicki-Garcia.** 1972. Intracellular β-glucanase activity of *Phytophthora palmivora*. Biochim. Biophys. Acta 276:221-227.
21. **Hunsley, D., and J. H. Burnett.** 1970. The ultrastructural architecture of the walls of some hyphal fungi. J. Gen. Microbiol. 62:203-218.
22. **Kanetsuna, F., and L. M. Carbonell.** 1971. Cell wall composition of yeastlike and mycelial forms of *Blastomyces dermatitidis*. J. Bacteriol. 106:946-948.
23. **Kanetsuna, F., L. M. Carbonell, F. Gil, and I. Azuma.** 1974. Chemical and ultrastructural studies on the cell walls of *Histoplasma capsulatum*. Mycopathologia 54:1-13.
24. **Kobayashi, G. S., and P. L. Guiliacci.** 1967. Cell wall studies of *Histoplasma capsulatum*. Sabouraudia 5:180-188.
25. **Kreger, D. R., and M. Kopecka.** 1975. On the nature and formation of the fibrillar nets produced by protoplasts of *Saccharomyces cerevisiae* in liquid media: an electron-microscopic, X-ray diffraction and chemical study. J. Gen. Microbiol. 92:207-220.
26. **Laine, R. A., W. J. Esselman, and C. C. Sweeley.** 1972. Gas-liquid chromatography of carbohydrates, p. 159-167. *In* D. B. McCormick and L. D. Wright (ed.), Methods in enzymology, vol. 18. Academic Press Inc., New York.
27. **Lampen, J. O.** 1968. Yeast cell wall. Antonie van Leeuwenhoek J. Microbiol. Serol. 34:1-18.
28. **Li, Y.-T., and M. R. Shetlar.** 1964. Occurrence of α-galactosidase from *Calvatia cyathiformis*. Arch. Biochem. Biophys. 108:523-530.
29. **Lowry, O. H., N. Rosebrough, A. Farr, and J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
30. **Matile, P., H. Moor, and C. F. Robinow.** 1969. Yeast cytology, p. 220-302. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, vol. 1. Academic Press Inc., New York.
31. **Monreal, J., and E. T. Reese.** 1969. The chitinase of *Serratia marcescens*. Can. J. Microbiol. 15:689-696.
32. **Page, W. J., and J. J. Stock.** 1974. Sequential action of cell wall hydrolases in the germination and outgrowth of *Microsporum gypseum* macroconidia. Can. J. Microbiol. 20:483-489.
33. **Reynolds, D. M.** 1954. Exocellular chitinase from a *Streptomyces* sp. J. Gen. Microbiol. 11:150-159.
34. **Senft, A. W., and S. E. Maddison.** 1975. Hypersensitivity to parasite proteolytic enzyme in schistosomiasis. Am. J. Trop. Med. Hyg. 24:83-89.
35. **Sentandreu, R., M. V. Elorza, and J. R. Villaneuva.** 1975. Synthesis of yeast wall glucan. J. Gen. Microbiol. 90:13-20.
36. **Skujins, J. J., H. J. Potgieter, and M. Alexander.** 1965. Dissolution of fungal cell walls by a Streptomycete chitinase and β-1,3-glucanase. Arch. Biochem. Biophys. 111:358-364.
37. **Skujins, J., A. Pukite, and A. D. McLaren.** 1970. Chitinase of *Streptomyces* sp: purification and properties. Enzymologia 39:353-370.
38. **Stimson, W. H.** 1971. Gas-liquid chromatography of hexosamines. FEBS Lett. 13:17-20.
39. **Taylor, I. E. P., and D. S. Cameron.** 1973. Preparation and quantitative analysis of fungal cell walls: strategy and tactics. Annu. Rev. Microbiol. 27:243-259.
40. **Wolfrom, J. L., and N. E. Franks.** 1965. Partial acid hydrolysis. Preparation of polymer-homologous oligosaccharides; xyloglycoses from xylan. Methods Carbohydr. Chem. V:276-280.