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IN VITRO INHIBITION OF STABLE 1,3-β-D-GLUCAN SYNTHASE ACTIVITY FROM *NEUROSPORA CRASSA*

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Glucan synthase activity of *Neurospora crassa* was isolated by treatment of protoplast lysates with 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate and 0.5% octylglucoside in 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, containing 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 200 mM inorganic phosphate, 10 μM GTP, 1 mM DTT, 10 mM sodium fluoride, and 600 mM glycerol. Resulting activity was partially purified by sucrose gradient density sedimentation. Approximately 70% of enzyme activity in the sucrose gradient peak fraction was soluble and enzyme activity was purified 7.3-fold. Partially purified enzyme activity had a half-life of several weeks at 4°C, and a $K_{m\,app}$ of 1.66 ± 0.28 mM.

Inhibitors (Cilofungin, papulacandin B, aculeacin A, echinocandin B, sorbose and UDP) of 1,3-β-D-glucan synthase activity were tested against crude particulate and detergent treated enzyme fractions and the $K_{i\,app}$ of each inhibitor determined. It seems likely that this stable preparation of glucan synthase activity may be useful for *in vitro* enzyme screens for new glucan synthase inhibitors.

KEY WORDS: 1,3-β-D-Glucan synthase, anti-fungal drugs, glucan synthase inhibitors, *Neurospora crassa*.

INTRODUCTION

An important approach towards understanding growth and morphogenesis in fungi is to study the enzymes involved in the synthesis and assembly of fungal cell walls. *Neurospora crassa* is a filamentous Ascomycete for which two enzyme activities have been identified as essential for normal cell wall assembly, namely, chitin synthase and 1,3-β-D glucan synthase.[1,2,3,4,5,6,7,8] 1,3-β-D-Glucan synthase (EC 2.4.1.34; UDP-Glucose: 1,3-β-D-glucan 3 β-D-glucosyltransferase) catalyzes the synthesis of a linear 1,3-β-D glucan polymer from UDP-glucose which, along with other carbohydrate polymers (e.g. chitin), is assembled into the cell wall. Glucan synthase activity is associated with the plasma membrane (transmembrane) and the active site for substrate hydrolysis is cytoplasmic facing.[8,9,10,11,12] Enzyme activity *in vitro* forms glucan chains which hydrogen bond with adjacent chains, resulting in the assembly of glucan microfibrils.[8,13] Glucan synthase activity is essential for cell-wall assembly and fungal

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Abbreviations used: CHAPS, 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; OG, octylglucoside; PMSF, phenylmethylsulfonylfluoride; UDP-glucose, uridine 5-diphosphoglucose; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid.

growth since drug treatments that inhibit glucan synthesis result in inhibition of growth,[13,14] and mutations that alter the level of glucan synthase activity result in mutants that form abnormal cell walls.[15]

Anti-fungal compounds which target glucan synthase activity are potentially important since glucan synthase is an integral, plasma membrane enzyme found in most fungi, but is completely absent in humans and other animals.[8,16,17] However, to date none of these drugs has proved to be useful for clinical applications.

It seemed to us that a stable glucan synthase preparation would be very useful for an *in vitro* screen for new enzyme inhibitors. We isolated glucan synthase from *N. crassa* protoplast lysates by treatment with detergents, followed by a low-speed centrifugation to remove cellular debris. The resulting supernatant was separated by sucrose density sedimentation and enzyme activity characterized. This preparation of glucan synthase could be stored at 4°C for at least two weeks and enzyme activity was linear with time *in vitro* for 4 h at 25°C. We tested the effects of several glucan synthase inhibitors[9,18] against crude particulate and sucrose-gradient purified enzyme activity, and report here the resulting K_{iapp} for Cilofungin, papulacandin B, aculeacin A, echinocandin B, sorbose, and UDP.

MATERIALS AND METHODS

Chemicals

UDP-[$U^{14}C$]-glucose (250 mCi/mmol) was purchased from ICN (Irvine, CA), Bio-Rad protein reagent from Bio-Rad Laboratories (Richmond, CA), scintillation fluid (Complete Counting Cocktail, Budget-Solve) from RPI Corporation (Mt. Prospect, IL) and Novozym 234 from Novo Laboratories (Danbury, CT). Cilofungin was obtained from Dr. C.J. Fouts-Johnson of Eli Lilley and Company, Indianapolis, Indiana. Papulacandin B, aculeacin A and echinocandin B were generous gifts of Ciba-Geigy Limited, Basel, Switzerland, Toyo Jozo Co., Ltd., Tokyo, Japan, and Sandoz, Ltd., Basel, Switzerland, respectively. All other chemicals and enzymes were from Sigma Chemical Company (St. Louis, MO). Distilled deionized water was used throughout.

Isolation of glucan synthase activity

Wild-type *Neurospora crassa* (74-OR8-1a) was obtained from the Fungal Genetics Stock Center, Kansas City, Kansas. Protoplasts of wild-type *N. crassa* were prepared as previously described.[19,20] Briefly, protoplasts were obtained by treating 16 h germinated macroconidia with Novozym 234 for 2 h at 25°C. Resulting protoplasts were harvested as described[20,21] and stored frozen at −70°C.

Glucan synthase activity was isolated using modifications of the procedure of Hrmova *et al.*[21] Frozen cell-pellets (obtained as described above) were lysed in ice cold buffer A (25 mM HEPES, pH 7.4, 5 mM EDTA, 10 mM NaF, 600 mM glycerol, 1 mM PMSF, 200 mM NaH_2PO_4, 1 mM DTT, 10 μM GTP) to obtain a final protein concentration of 10 mg/ml. Resulting cell lysates were mixed 1:1 with buffer A (as a control) or with buffer B (buffer A containing 1% [w/v] OG and 0.2% [w/v] CHAPS). Cell lysates were centrifuged (500 × g) for 20 min, at 4°C. Alternatively, for comparison studies, cell lysates (in buffer B) were centrifuged at 100,000 × g for 1 h at 4°C as previously described.[20,21] Supernatant fractions and pellets were separated and

pellets were re-suspended in 150 μl buffer A. Supernatants were mixed with 170 μl buffer A containing 10% (w/v) sucrose to reach a final concentration of 0.5% (w/v) sucrose, and layered on top of freshly prepared 2–20% linear sucrose gradients (34 mls) containing buffer A or buffer A plus 1/4 detergents (0.125% OG, 0.025% CHAPS). Gradients were centrifuged at 22,400 × g, 4°C, for 15 h and fractions were collected at 4°C.

The refractive index of each fraction was determined using an American Optical ABBE refractometer. The protein content of each fraction was measured using the Bradford protein assay[22] with bovine serum albumin (fraction V) as standard. Fractions were assayed for 1,3-β-D-glucan synthase activity (see below) and fractions containing the peak enzyme activity were pooled. Pooled sucrose gradient fractions (obtained from 500 g supernatants) were stored at 4°C and glucan synthase activity monitored at various times of storage. A 200 μl aliquot of the pooled fractions was centrifuged at 107,000 xg (R_{av}), 4°C for 20 min*. Supernatant and pellets were separated, and each assayed for glucan synthase activity.

For inhibitor studies, frozen protoplasts (− 70°C) were lysed in buffer A and mixed 1:1 with buffer A plus 12% sucrose (to equal the sucrose concentration of the pooled gradient fractions) and assayed for glucan synthase activity (see below).

1,3-β-D-Glucan Synthase Assay

1,3-β-D-glucan synthase (EC 2.4.1.34; UDP-Glucose: 1,3-β-D-glucan 3-β-D-glucosyltransferase) activity of various cell fractions was determined as previously described.[20,21,23] Briefly, 25 μl reaction mixtures contained 50 μg α-amylase (Sigma type IIA), 10 μM GTP, various concentrations of UDP-[^{14}C]-glucose and inhibitors dissolved in water or 50% DMSO; water and 50% DMSO were used as controls. Cilofungin, papulacandin B, echinocandin B and aculeacin A were dissolved in 50% DMSO; sorbose and UDP were dissolved in water — all solutions were stored at − 20°C. Cell protein was added to ice-cold reaction mixtures and incubated at 25°C. Reactions were stopped by the addition of 50 μl 5% (w/v) TCA. The incorporation of [^{14}C]-glucose from UDP-[^{14}C]-glucose into 1,3-β-D-glucan was monitored using the millipore filter method described by Gooday and de Rousset-Hall.[24] Enzyme kinetic data were processed using the EZ-FIT computer program developed by Perella.[25]

Product characterization of glucan formed in glucan synthase reactions was carried out as previously described.[21] Briefly, glucan synthase reactions were incubated for

*Solubilization was defined as enzyme activity found in the supernatant fraction after centrifugation at 100,000 xg for 1 h. In this case, a Beckman TLA100.1 rotor was used. For larger volumes the Beckman SW55 Ti rotor was used and the relative centrifugal force was kept at 100,000 xg by adjusting the revolutions per minute following the equation:

$$RCF = 1.12\,r\,(\text{rpm}/1000)^2$$

where RCF = relative centrifugal force, r = radius (millimeters) of the specific rotor, and rpm = revolutions per min. Furthermore, the centrifugation time was adjusted to compensate for the individual rotor's relative pelleting efficiency, known as the K factor. The following equation was used to determine the minimum centrifugation time:

$$t_a/t_b = K_a/K_b$$

where t = time in min, and K = K factor of specific rotor. The K factors obtained from the Beckman rotor instruction manuals (Spinco Division of Beckman Instruments, Inc. Palo Alto, CA) for the TLA1000.1 and SW55 Ti rotors are 12 and 48, respectively.

four hours in siliconized (Sigmacote) 1.5 ml microfuge tubes, terminated by boiling for 10 min, and centrifuged at 8800 × g for 30 min at 4°C. Pellets were washed by centrifugation with distilled, deionized water and treated with 100 μl 50 mM acetate buffer, pH 5.4, containing either (1) 50 μg bovine serum albumin, (2) 50 μg α-amylase, or (3) 50 μg exo-1,3-β-D-glucanase (Mollusk). Hydrolysis reactions were terminated by the addition of 25 μl glacial acetic acid diluted 1:1 with water. Mixtures were centrifuged 8800 × g for 30 min at 4°C and the radioactivity remaining in pellets was determined by liquid scintillation counting.

RESULTS

Preparation of stable glucan synthase activity

Several methods have been developed in our laboratory concerning the isolation of 1,3-β-D-glucan synthase activity. These included isolating protoplasts from *N. crassa* wild-type hyphae using either a mixture of cell wall hydrolyases,[26] or Novozym 234[19] and harvesting cells using SS medium (Vogel's medium N plus 7.5% (w/w) sorbitol, 1.5% (w/v) sucrose).[27] Glucan synthase activity has been isolated in low speed (500 x g) pellets by lysing frozen protoplasts in FAGE buffer (25 mM HEPES, pH 7.4, 5 mM EDTA, 10 μM GTP, 100 mM glycerol, 10 mM NaF, 1 mM DTT, 1 mM PMSF, and 1% (w/v) BSA) followed by re-suspension and assay in GEF buffer (FAGE buffer without BSA).[20,23] Unfortunately, the specific activity of this enzyme preparation varied dramatically from experiment to experiment. We substituted SSH buffer (7.5% (w/v) sorbitol, 1.5% (w/v) sucrose, 25 mM HEPES, pH 7.4) for the SS medium for harvesting protoplasts which resulted in a 3-fold increase in yield of protoplasts.[21] We found that protoplasts could be lysed in buffer A (FAGE buffer without BSA, but with increased levels of glycerol, 1 mM DTT, and 200 mM NaH_2PO_4), and the crude lysate used as an enzyme source instead of the 500 × g pellet. The resulting enzyme preparation had activity with a longer half-life at 4°C [from 190 min to 250 min (500 × g pellets versus crude lysates)]. Using these crude lysates as enzyme sources, we were able to solubilize *ca* 50% of enzyme activity and began to purify glucan synthase activity.[21]

However, our purification procedure (~6-fold) resulted in an enzyme activity that had relatively short *in vitro* activity, and a short half-life (60 h)[21] at 4°C. Therefore, we modified and simplified our procedure. Detergent-treated lysates were centrifuged at 500 × g (instead of 100,000 × g), 4°C, for 20 min to remove large debris and resulting low-speed supernatants were layered on freshly prepared 2–20% linear sucrose gradients. Gradients were centrifuged at 22,400 × g for 15 h and fractions collected. As shown in Figure 1, the peak of glucan synthase activity was found between 11 and 13% sucrose. Recovery and purification of enzyme activity using this scheme are shown in Table 1. Note that the pooled peak-enzyme activity from the sucrose gradient was purified 7.3-fold and 26% of the enzyme activity was recovered. When a sample of the pooled peak fraction was centrifuged at 107,000 × g (R_{av}) as described under Materials and Methods, 73% of the recoverable enzyme activity was found in the resulting supernatant, i.e., enzyme activity was soluble. Three subsequent experiments, using the same procedure, resulted in a solubility range of 63–78% (results not shown).

Characterization of product formed in reaction mixtures using the sucrose gradient peak fractions as enzyme sources, showed that greater than 99% of radioactivity

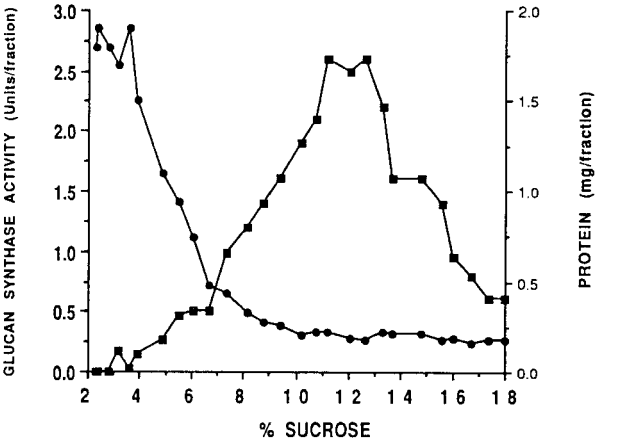

FIGURE 1. Sucrose density sedimentation of 500 × g supernatant fractions. Protoplasts were lysed in buffer A to obtain a 10 mg/ml protein concentration and diluted 1:1 with buffer B. The resulting crude lysate was centrifuged at 500 × g for 20 min at 4°C, and supernatant carefully removed. Sucrose, in buffer A, was added to a final concentration of 0.5% (w/v) and the supernatant (3.4 ml) was layered onto a freshly prepared 2–20% linear sucrose gradient prepared in buffer A containing 0.125% OG and 0.025% CHAPS. The gradient was centrifuged for 15 h at 22,400 × g, 4°C, and fractions collected as described in Materials and Methods. Fractions were assayed for protein content (●—●) and glucan synthase activity (■—■). Reaction mixtures contained 2 mM UDP-[^{14}C]-glucose (50,000 cpm/assay), 50 μg α-amylase, 10 μM GTP, 2–23 μg cell protein, and were incubated for 0 and 30 min at 25°C. Reactions were terminated by the addition of 50 μl 5% (w/v) TCA and amounts of glucan formed determined as described in Materials and Methods. The refractive index of each fraction was determined and compared to a standard curve of sucrose concentration prepared in buffer A containing 0.125% OG and 0.025% CHAPS. One unit of glucan synthase activity is defined as the amount that catalyzes the incorporation of 1 nmol glucose per min at 25°C into 1,3-β-D-glucan.[21]

TABLE I
Partial purification of (1,3)-β-D-glucan synthase activity

Fraction	Total protein (mg)	Total activity (Units)	Specific activity (U/mg)	Recovery (%)	Purification factor
Crude Lysate A	18	26	1.5	100	1.0
Crude Lysate B	18	75	4.2	290	2.8
500 × g supernatant B	20	59	3.0	230	2.0
500 × g pellet B	0.15	5.6	37	22	25
Sucrose gradient peak	0.60	6.8	11	26	7.3

Protoplasts were lysed in buffer A to obtain 10 mg/ml protein and diluted 1:1 with buffer B. Crude lysate with centrifuged at 500 × g for 20 min at 4°C, and supernatant and pellet fractions isolated. The resulting pellet was resuspended in 150 μl buffer A. The supernatant fraction was separated by rate-zonal density centrifugation (as described in Materials and Methods). Glucan synthase reaction mixtures contained 2 mM UDP-[^{14}C]-glucose (50,000 cpm/assay), 50 μg α-amylase, 10 μM GTP and cell protein. Crude lysates (84 μg/assay), supernatant (15 μg/assay), and pellet (92 μg/assay) fractions were incubated for 0, 4, 6 and 30 min at 25°C. Sucrose gradient fractions (2 μg/assay) were incubated for 0 and 30 min at 25°C. Reactions were terminated and the amount of glucan formed determined as described in Materials and Methods.

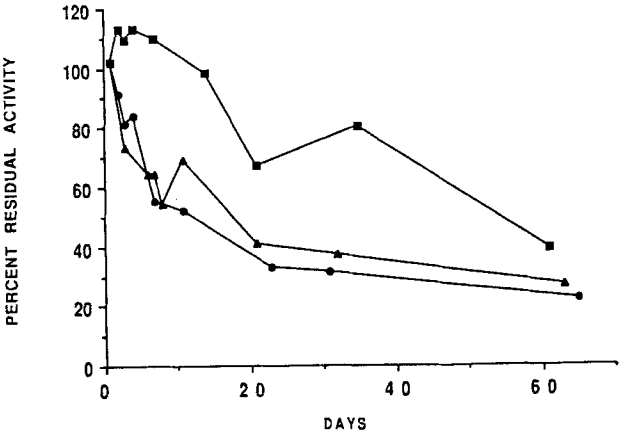

FIGURE 2. Half-life of sucrose gradient peak fractions at 4°C. Sucrose gradient peak fractions from three different experiments (isolated as described in the legend to Figure 1) were separately pooled, stored at 4°C, and assayed for glucan synthase activity at the indicated times. Reaction mixtures contained 2 mM UDP-[^{14}C]-glucose (50,000 cpm/assay), 50 μg α-amylase, 10 μM GTP and 2 μg cell protein. Reactions were incubated for 0 and 60 min at 25°C, and were terminated by the addition of 50 μl 5% (w/v) TCA. The amount of glucan formed was determined as described in Materials and Methods.

incorporated into glucan was rendered soluble by exo-β(1-3) glucanase treatment and less than 2% was solubilized by α-amylase treatment (data not shown); that is, the *in vitro* synthesized product was $\beta(1 \rightarrow 3)$-linked glucan.

The pooled sucrose gradient fractions from three separate experiments were individually stored at 4°C for various times and glucan synthase activity assayed. These

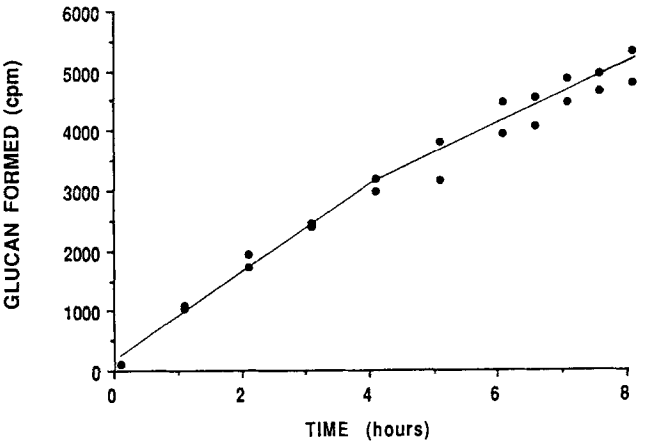

FIGURE 3. *In vitro* glucan synthase activity of sucrose gradient peak fractions. Sucrose gradient peak fractions were prepared as described in Materials and Methods, and used as an enzyme source to determine the amount of glucan formed at various times in reaction mixtures. Mixtures contained 2 mM UDP-[^{14}C]-glucose (50,000 cpm/assay), 50 μg α-amylase, 10 μM GTP, and 2 μg cell protein. Reactions were incubated at 25°C and terminated at the indicated times by the addition of 5% (w/v) TCA. The amount of glucan formed was determined as described in Materials and Methods.

results are presented in Figure 2. Surprisingly, the half-life of enzyme activity at 4°C from the three preparations ranged from 10–50 days. As shown in Figure 3, *in vitro* glucan synthase activity was linear for 4 h and nearly linear for 8 h at 25°C! Glucan synthase activity showed a $K_{m\,app}$ of 1.66 ± 0.28 mM (data not shown).

Effect of inhibitors

Previous work from our laboratory concerning the effects of putative inhibitors of glucan synthase activity used crude, particulate enzyme preparations[13,18,28]. However, the lipophilic nature of most of these inhibitors (e.g., papulacandin B, Cilofungin, aculeacin A, and echinocandin B) has suggested the possibility that these compounds inhibit glucan synthase by perturbing the membrane surrounding the enzyme, rather than interacting with the glucan synthase protein(s) directly. To test this idea, we examined the effects of each inhibitor on the crude particulate enzyme preparation, the 100,000 × g supernatant fractions of detergent-treated lysates, and the pooled sucrose gradient fractions prepared from detergent-treated lysates.

We first determined the type of inhibition and the $K_{i\,app}$ for each inhibitor using crude lysates and the sucrose gradient peak fractions as enzyme sources. These results are presented in Table II. Several points are noteworthy; (1) the type of inhibition shown by each compound remained unchanged, (2) UDP and sorbose inhibited both preparations similarly, although the $K_{i\,app}$ using the sucrose gradient purified enzyme preparation was somewhat higher, (3) Cilofungin and echinocandin B inhibited the crude lysate preparation to a significantly greater extent (six-fold) than the sucrose gradient preparation; in contrast, aculeacin A inhibited both preparations to nearly the same extent, and (4) surprisingly, papulacandin B inhibited the sucrose gradient peak fraction 5-fold greater than the crude enzyme preparation.

The effect of each inhibitor on soluble glucan synthase (100,000 × g supernatants) was also tested and compared to that of the crude lysate and the peak sucrose gradient fraction. Papulacandin B showed greater inhibition of glucan synthase present in the sucrose gradient and the soluble 100,000 × g supernatant preparations compared to

TABLE II
$K_{i\,app}$ of inhibitors of glucan synthase activity

Inhibitor	Inhibition[a] Type	K_i crude	K_i sucrose gradient	K_i sucrose[b]/ K_i crude
Papulacandin B	Noncompetitive	365 μM ± 78	72 μM ± 10	0.2
Cilofungin	Noncompetitive	13 μM ± 1	70 μM ± 9	6
Echinocandin B	Noncompetitive	4 μM ± 0.5	24 μM ± 5	6
Aculeacin A	Noncompetitive	24 μM ± 5	16 μM ± 4	0.67
Sorbose	Uncompetitive	40 mM ± 8	70 mM ± 14	1.8
UDP	Competitive	625 μM ± 63	1000 μM ± 120	1.6

[a]Inhibition type refers to the nomenclature of Cleland.[31] [b]The ratio of $K_{i\,app}$ of the sucrose gradient fractions to that of the crude enzyme preparation.

Crude lysates prepared in buffer A containing 12% sucrose and sucrose gradient peak fractions were assayed with 0.4 mM, 0.8 mM and 1.2 mM UDP-[^{14}C]-glucose (50,000 cpm/assay), 50 μg α-amylase, 10 μM GTP, and various concentrations of inhibitors (dissolved in 50% DMSO or water). Reaction mixtures were incubated at 25°C for 0, 3 and 6 min for crude lysates and for 0, 1 and 2 h for the sucrose gradient peak fractions. The amounts of glucan formed were determined as described and converted to velocity (nmol glucose incorporated/min). Resulting data was analyzed using the EZ-FIT program (see Materials and Methods).

inhibition of the crude lysate preparation; in contrast, Cilofungin inhibited the crude lysate preparation to a greater extent than the sucrose gradient and 100,000 × g supernatant preparations (data not shown). The results for these and the other inhibitors (data not shown) were qualitatively similar to those presented in Table II. That is, for each inhibitor, the soluble enzyme preparation (100,000 × g supernatant) was inhibited either similarly (for aculeacin A, sorbose, and UDP), greater (papulacandin B), or less (Cilofungin and echinocandin B) than the crude lysate preparation.

DISCUSSION

The isolation of glucan synthase activity from *N. crassa* described in this paper used crude lysates as starting materials which, after the addition of detergents and rate zonal sucrose gradient centrifugation, yielded a 7.3-fold purified enzyme preparation that remained active at 4°C for at least several weeks. Glucan synthase activity has been reported from other fungal and plant sources, but to date, our preparation has resulted in the most stable enzyme activity. Kang and Cabib[29] have isolated particulate glucan synthase activity from *Saccharomyces cerevisiae* which was stable for several months, but only when stored at −80°C. A nearly comparable activity was reported by Eiberger *et al.*,[30] using glucan synthase activity from red beets: 76% of enzyme activity remained after 10 days at 4°C. Our *N. crassa* glucan synthase preparation was also linear in activity *in vitro* for up to four hours at 25°C, which, to our knowledge, is significantly longer than any other reported preparation.

The most significant difference between this preparation and previously published protocols from this laboratory (and others), is that 500 × g supernatants, rather than 100,000 × g supernatant fractions, were layered on sucrose gradients.[20,21] This difference may be responsible for the dramatic increase in the half-life and *in vitro* stability of the resulting enzyme preparation. The factor(s) responsible for the wide range of enzyme activity half-life observed from experiment to experiment is not known. However, one consistent observation warrants further study, namely, that the half-life of the enzyme was longer when stimulation by detergents of the crude lysate activity was higher. For example, in the preparation which showed a half-life of ~ 50 days at 4°C, the activity of the crude lysate was increased ~ 2.9-fold by the addition of detergents. In contrast, when the half-lives of glucan synthase activity in the sucrose gradient peaks were closer to ~ 10 days, the activities of the crude lysates were increased by only ~ 1.8-fold by addition of detergents. Therefore, detergent stimulation of the crude lysate is weakly correlated with the half-life of the sucrose gradient peak enzyme fractions. However, a half-life of ~ 10 days is a significant increase over the half life of the 100,000 × g supernatant (6.2 ± 0.2 h) and the resulting sucrose gradient fraction (60 h), published previously.[21] Thus, this procedure represents a significant and useful technical advance.

The effects of several glucan synthase inhibitors were tested against the crude lysate (without detergents), sucrose gradient (with detergents) and soluble (100,000 × g supernatant with detergents) enzyme preparations. Two points are interesting. Firstly, the type of inhibition (i.e., noncompetitive, competitive) exhibited by each inhibitor was not altered from preparation to preparation. Secondly, a clear, general pattern did not emerge. That is, the presence of detergents did not predictably change the potency of inhibitors. For example, echinocandin B, aculeacin A and Cilofungin share the same nucleus and differ only in their fatty acid side chains. Yet, two of these

showed significantly less inhibition of the detergent-treated soluble enzyme preparation compared to the crude lysate fraction, while for the other compound, inhibition remained unchanged. In sharp contrast, papulacandin B inhibited the detergent-treated preparation significantly better than the crude, particulate enzyme preparation. Whether this reflects an increase in the access of papulacandin B to the enzyme or other factors is not known, but is worthy of additional study. Overall, these results suggest that these inhibitors may not act directly on the enzyme itself.

In summary, this preparation of stable glucan synthase activity should permit *in vitro* screening of potential enzyme inhibitors. In addition, the glucan synthase activity of this preparation is sufficiently stable to use as starting materials for further purification of enzyme activity. Results of these experiments will be described elsewhere (Selitrennikoff, *et al.*, in preparation).

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