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CHITIN SYNTHASE ACTIVITY FROM THE *SLIME* VARIANT OF *NEUROSPORA CRASSA*

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Summary

Chitin synthase (UDP-2-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamido-deoxy-D-glucosyltransferase, EC 2.4.1.16) activity from the wall-less variant of *Neurospora crassa* (*slime*) was partially characterized. The *slime* enzyme activity was found to be similar to that reported for *slime*-like and wild-type chitin synthase activities with respect to the following: specific activity, particulate cell-fraction localization, activation by *N*-acetylglucosamine, apparent K_m with respect to substrate, pH optimum and ion requirement. It appears that the phenotype of *slime* cannot be solely accounted for by the absence of chitin synthase enzyme activity.

Introduction

Interest in this laboratory centers around cell-wall assembly in the filamentous Ascomycete *Neurospora crassa*. Wild-type *N. crassa* under routine laboratory conditions grows as a mycelium whose hyphal cell walls contain 25—35% β(1 → 3) glucan, 2—8% galactosamine polymer, 12—15% proteins and peptides, 15—20% undefined glucans and 10—15% chitin [1—8]. Chitin synthase (UDP-2-acetamide-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxy-D-glucosyltransferase, EC 2.4.1.16) catalyses the reaction: uridine-5′-diphospho-*N*-acetylglucosamine (UDPGlcNAc) + acceptor → acceptor-GlcNAc + UDP and has been implicated as an essential activity for normal cell-wall biosynthesis not only in *Neurospora* [9—11] but also in a number of taxonomically diverse

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fungi [12—17]. The general properties of the enzyme appear to be very similar amongst all fungi examined; e.g., Mg^{2+} requirement, activation by *N*-acetylglucosamine (GlcNAc), particulate fraction localization and inhibition by UDP and polyoxin [11—17].

Isolates of the *slime* variant *Neurospora* grow without the presence of detectable surface-bound cell-wall material, i.e., as populations of protoplasts [18—22]. Inquiries into the molecular basis of the *slime* phenotype have led to an examination of cell-wall-polymer biosynthetic enzymes. This study reports the partial characterization of chitin synthase activity from the *slime* variant and suggests that the wall-less nature of *slime* cannot be simply accounted for by the absence of chitin synthase activity.

Materials and Methods

Chemicals

Radioactive UDP[1-^{14}C-GlcN]GlcNAc was purchased from New England Nuclear and ICN. Diacetylchitobiose and tetraacetylchitotetraose were generous gifts of Dr. D.R. Sonneborn (University of Wisconsin, Madison). Biochemicals were purchased from Sigma Chemical Company; other chemicals were of reagent grade and distilled water was used throughout.

Growth of N. crassa wild-type and slime strains

The *slime* variant [*fz* (no number); *sg* (no number), *arg-1* (B369), *cr-1* (B123), *aur* (34508), *os-1* (B135), *A* — Fungal Genetics Stock Center No. 326] was obtained from Dr. R.L. Metzenberg (University of Wisconsin, Madison). This strain of *slime* grew as a homogeneous suspension of protoplasts. The *wild-type* used was 74-OR8-la (FGSC 988).

Cultures of the *slime* variant were grown using Nelson's Medium B in liquid shaken culture essentially as described by Nelson et al. [18]. Exponentially growing cultures were harvested by centrifugation (500 × *g*; 5 min) and washed twice with ice-cold fresh medium. Cell-pellets were quick-frozen in liquid N_2 and stored at −70°C until needed. No detectable change in enzyme specific activity was noted for storage periods up to one month.

Cultures of *wild-type* were initiated by conidial suspensions (final concentration $1 \cdot 10^5$ conidia/ml) into 2-liter flasks containing 300 ml of Nelson's Medium B [18]. Flasks were shaken (160 rev./min) on a New Brunswick Shaker Table for 24 h at 28°C. Cells were harvested by centrifugation, washed twice with ice-cold distilled water, frozen in liquid N_2 and stored at −70°C.

Preparation of particulate cell-fractions

Cells of *slime* were quantitatively disrupted by thawing cell pellets in ice-cold 50 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$. Crude lysates were centrifuged at 1000 × *g* for 5 min at 2°C. Supernatants were centrifuged for 60 min at 100 000 × *g* (average *g*) at 2°C; resulting pellets were resuspended in buffer by a brief (approx. 2—3 s) sonication treatment. Wild-type cells were disrupted by sonication (Ultrasonics Sonifier® equipped with a microtip; 10 × 15 s bursts at 50 W with 90-s cooling periods between bursts) at 4°C with 25 μm glass beads. Disruption was judged to be ⩾95% (phase contrast microscopy). High-speed pellets were prepared as described above.

Enzyme assay

Chitin synthase activity was monitored in 25-μl reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 12.5 mM GlcNAc, 3.3 mM UDP[1-^{14}C]GlcNAc (19 000 dpm/mixture) and aliquots of resuspended high-speed pellets. Mixtures were incubated at 30°C and reactions terminated by boiling for 2—3 min. Reaction mixtures were separated by descending chromatography for 16—18 h using Whatman 1 paper and 1 M ammonium acetate (pH 7.5)/ethanol (3 : 7, v/v) as solvent. Chromatographically immobile radioactivity (chitin) was determined by liquid scintillation counting using Aquasol II (New England Nuclear). Chitin formation was linear with respect to time for 15 min and linear with respect to protein concentration (50—150 μg/reaction mixture). Less than 10% enzyme activity was present after 24 h incubation at either 4°C or 30°C; enzyme activity remained stable for up to 2 weeks after high-speed pellets were quick-frozen in liquid N_2 and stored at —70°C.

Chitinase treatments

For some experiments chromatographically immobile radioactivity was treated with chitinase (EC 3.2.1.14): dried areas from origins (1 cm × 2 cm) were immersed in water (5 ml) containing 3 units/ml chitinase, 100 μg/ml chloramphenicol, 100 μg/ml streptomycin, 1000 units/ml Penicillin G. After treatment for 72 h at 28°C with shaking (160 rev./min) in sealed tubes, the samples were washed twice with distilled water and dried in scintillation vials. Solutions were pooled, lyophylized, resuspended in water and spotted on Whatman 1 paper and separated by chromatography as described above. Distribution of radioactivity was determined by liquid scintillation counting of chromatograms cut into 1 cm pieces.

Miscellaneous procedures

Protein was determined by the method of Lowry et al. [23], using bovine serum albumin (Fraction V) as standard. Enzyme activity is reported in units (nmol GlcNAc incorporated into chitin/min) at 30°C.

Results

Chitin synthase activity in centrifugal fractions from wild-type and slime variant cells

Exponentially growing cultures of *wild-type* and the wall-less variant, *slime*, were harvested, disrupted, and centrifugal fractions assayed for chitin synthetase activity. The results in Table I demonstrate that although enzyme activity was detected in all fractions tested for both *slime* and *wild-type*, the majority of the activity was recovered in high-speed particulate fraction (100 000 × *g* pellets). It is noteworthy that a higher percentage of enzyme activity was recovered in 100 000 × *g* supernatants derived from *slime* cells (30%) than that recovered in supernatant fractions derived from *wild-type* cells (9%). Further, it was observed that the specific activities of *wild-type* and *slime* crude extracts were not significantly different.

TABLE I

DISTRIBUTION OF CHITIN SYNTHASE ACTIVITY IN CENTRIFUGAL FRACTIONS OF SLIME AND *WILD-TYPE* EXTRACTS

Crude extracts of *slime* and *wild-type* cells were prepared as described. Extracts were centrifuged at 1000 × *g* for 5 min at 2°C. Resulting supernatants were centrifuged at 100 000 × *g* for 60 min at 2°C. Fractions were assayed for chitin synthase activity and enzyme units normalized to account for the difference in protein content between *wild-type* and *slime* extracts.

Cell-type	Fraction	Chitin synthase activity	
		Total units	% Distribution
Wild-type	Crude lysate	159	—
	1 000 × *g* supernatant	133	86
	1 000 × *g* pellet	21	14
	100 000 × *g* supernatant	10	9
	100 000 × *g* pellet	107	91
Slime	Crude lysate	160	—
	1 000 × *g* supernatant	126	80
	1 000 × *g* pellet	30	20
	100 000 × *g* supernatant	33	30
	100 000 × *g* pellet	77	70

Formation of chitin by chitin synthase activity from high-speed particulate fractions of slime cells

Reaction mixtures containing aliquots of resuspended 100 000 × *g* pellets derived from *slime* cells and UDP[^{14}C]GlcNAc as substrate were incubated for various times and, after inactivation, mixtures were separated by descending chromatography. Three separate peaks of radioactivity were found when the distribution of radioactivity in chromatograms was determined. One peak corresponded to UDP[^{14}C]GlcNAc (unincorporated substrate), another was chromatographically immobile (chitin; see below) while the third was at an R_F GlcNAc of approximately 0.9. This peak had less than 10% of the radioactivity found in chitin and was not investigated further *. Only the UDP[^{14}C]GlcNAc peak was present in zero-time controls and in mixtures containing boiled extract. No radioactive GlcNAc was detected in mixtures containing active extracts. That the chromatographically immobile radioactivity was [^{14}C]-GlcNAc incorporated into chitin was determined by treating isolated areas from origins with chitinase. Greater than 98% of the radioactivity was rendered soluble by treatment with chitinase while <10% was solubilized in treatments lacking chitinase; greater than 97% of the solubilized radioactivity comigrated with authentic diacetylchitobiose.

Properties of slime chitin synthase activity

A pH optimum of pH 8.0—8.2 for *slime* chitin synthase activity was determined by lysing cells in buffer adjusted to various pH (ranging from pH 7.2—8.5) and assaying resuspended pellets for chitin synthase activity. Essentially

* This peak most likely represents a mixture of chitodextrins [16].

identical results were obtained when cells were lysed in pH 8.0 buffer and 100 000 X *g* pellets resuspended in buffers adjusted to various pH.

Slime chitin synthase activity was found to be active in vitro without added metal divalent cations but maximum activity occurred in the presence of 1 mM $MgCl_2$. Neither Mn^{2+} nor Ca^{2+} could effectively substitute for Mg^{2+}. That chitin synthase activity may exist in extracts as an ion-enzyme complex is suggested by complete inhibition of activity by 10 mM EDTA.

The effect on enzyme activity of varying substrate (UDPGlcNAc) concentrations was determined at four GlcNAc concentrations and the data plotted by the standard Lineweaver-Burk method (Fig. 1). Note that GlcNAc stimulates enzyme activity and there exists a non-linear relationship between reciprocal velocity and reciprocal substrate concentration at each of GlcNAc concentrations tested; only at a high GlcNAc concentration (12.5 mM) was a linear relationship approximated. By extrapolating the linear portions of the curves (high substrate concentrations) it was found that the apparent K_m for UDPGlcNAc was related to GlcNAc concentration (Fig. 2). When the data in Fig. 1 were replotted as reciprocal velocity versus reciprocal GlcNAc concentration, it was found that the x-axis intercepts ($-K_a$ GlcNAc) were related to substrate concentrations.

Specificity of activation of slime chitin synthase activity by GlcNAc

Various compounds structurally related to GlcNAc were added to reaction mixtures containing resuspended high-speed particulate fractions and mixtures

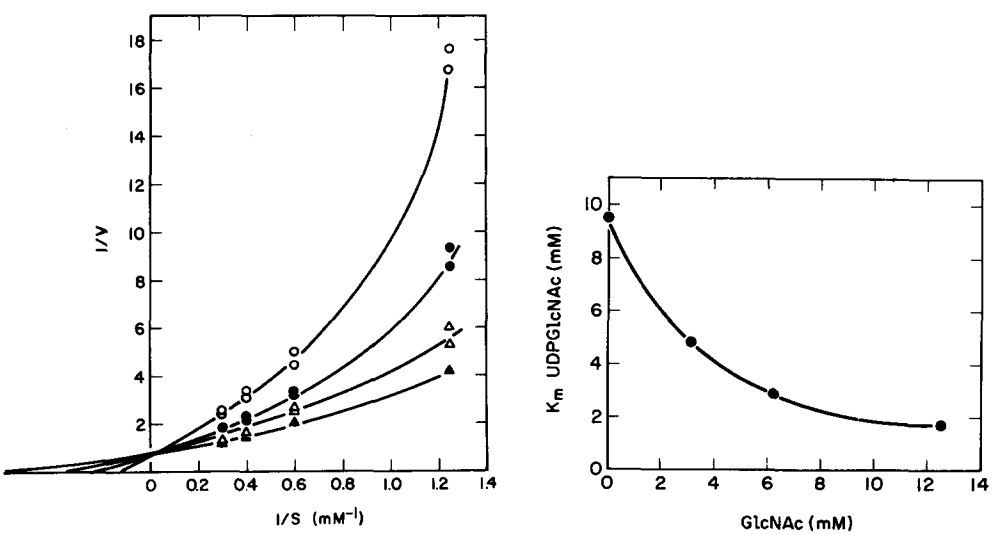

Fig. 1. Effect of varying substrate (UDPGlcNAc) concentrations at fixed GlcNAc concentrations on *slime* chitin synthase activity. Resuspended high-speed particulate fractions were incubated in mixtures containing the indicated UDPGlcNAc concentrations and the following GlcNAc concentrations: ○———○, none; ●———●, 3.1 mM; ▲———▲, 6.25 mM; △———△, 12.5 mM. After incubation for 7.5 and 15 min at 30°C, the amount of chitin formed was determined, velocity (nmol product/min) calculated and the data replotted in the double reciprocal form. Each point represents an individual determination. The x-axis intercepts ($-K_m$) were determined by linear regression analysis of the linear portions of the curves.

Fig. 2. Dependence of apparent K_m of *slime* chitin synthase activity on GlcNAc concentration. The x-axis intercepts ($-K_m$) in Fig. 1 were plotted as function of GlcNAc concentration.

TABLE II

EFFECT OF VARIOUS SUGARS ON *SLIME* CHITIN SYNTHASE ACTIVITY

Reaction mixtures containing aliquots of resuspended *slime* high-speed pellets, 3.3 mM UDP[^{14}C]GlcNAc and the indicated additions were incubated for 15 min and the radioactivity incorporated into chitin determined.

Additions to reaction mixture	Radioactivity incorporated (cpm)
None	185
12.5 mM *N*-acetylmannosamine	245
12.5 mM *N*-acetylgalactosamine	130
12.5 mM glucosamine	270
12.5 mM galactosamine	215
12.5 mM glucose	215
12.5 mM galactose	170
12.5 mM GlcNAc	1140
10 mM diacetylchitobiose	325
10 mM tetraacetylchitotetraose	530
10 mM diacetylchitobiose, 10 mM tetraacetylchitotetraose	500
12.5 mM GlcNAc, 10 mM diacetylchitobiose	1270
12.5 mM GlcNAc, 10 mM tetraacetylchitotetraose	1290
12.5 mM GlcNAc, 10 mM diacetylchitobiose, 10 mM tetraacetylchitotetraose	1320

assayed for chitin synthase activity. The results presented in Table II demonstrate that none of the compounds tested was as effective as GlcNAc in stimulating *slime* chitin synthase activity. However, diacetylchitobiose and tetraacetylchitotetraose stimulated enzyme activity by 1.7 and 3-fold, respectively. The presence of 10 mM tetracetylchitotetraose in reaction mixtures did not alter the effects of varying substrate concentrations at different GlcNAc concentrations on enzyme activity, i.e., non-Michealis-Menten enzyme kinetics observed in Fig. 1; however, the presence of tetraacetylchitotetraose in reaction mixtures altered the effect on enzyme activity of varying GlcNAc concentrations at fixed substrate (UDPGlcNAc) concentration. The K_a GlcNAc was found to be independent of substrate concentration and was estimated to be 2.6 mM.

TABLE III

EFFECT OF NUCLEOSIDE PHOSPHATES ON *SLIME* CHITIN SYNTHASE ACTIVITY

Reaction mixtures containing 12.5 mM GlcNAc, 3.3 mM UDP[^{14}C]GlcNAc, resuspended *slime* high-speed pellets, and the indicated concentrations of each of the listed compounds were incubated for 15 min and the radioactivity incorporated into chitin determined.

Addition	Radioactivity incorporated (cpm)
None	1115
2 mM UTP	690
5 mM UTP	115
5 mM UDP	460
2 mM UMP	850
5 mM UMP	440
2 mM ATP	910
5 mM ATP	225

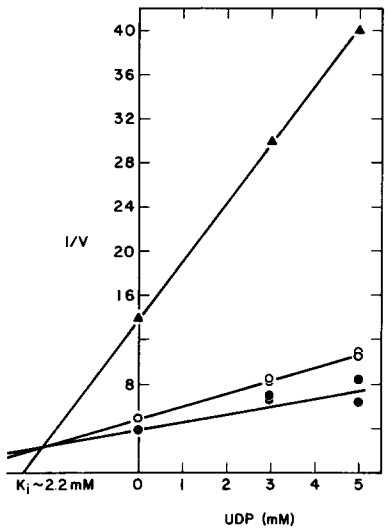

Fig. 3. Competitive inhibition of chitin synthase activity by UDP. Reaction mixtures containing the indicated UDP concentrations and the following UDPGlcNAc concentrations: ●———●, 3.3 mM; ○———○, 2.5 mM; ▲———▲, 0.8 mM; were incubated for 7.5 and 15 min. The amount of chitin formed was determined and the results were plotted by the method of Dixon [25]. Each point represents an individual determination.

Effect of various phospholipids and nucleoside phosphates on slime chitin synthase activity

Phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol (25 μg/reaction mixture) were each tested for their ability to stimulate chitin synthetase activity (see ref. 15). Phosphatidylinositol stimulated enzyme activity by 4.5 fold while phosphatidylserine and phosphatidylethanolamine increased activity by 2.8 and 1.7 fold, respectively; phosphatidylcholine was without significant effect. Whether stimulation of activity by phospholipids (and especially phosphatidylinositol) is due to stabilization of enzyme activity, to solubilization of enzyme activity rendering it more available to substrate or to the provision of a limiting lipid acceptor (intermediate?) is not known.

The indicated concentration of the nucleoside phosphates listed in Table III was added to reaction mixtures and mixtures assayed for chitin formation. All compounds tested inhibited enzyme activity. Since UDP is a product of chitin synthetase activity, inhibition by UDP was investigated further. The data in Fig. 3 show that UDP behaved as a competitive inhibitor (nomenclature of ref. 24) with respect to the substrate, UDPGlcNAc. An inhibition constant (K_i) of 2.2 mM was graphically determined (average K_i from 3 experiments was 2.4 ± 0.2 mM).

Discussion

Cell wall construction in *Neurospora crassa* and other filamentous fungi occurs by deposition of carbohydrate polymers and other materials at the

growing hyphal apex [26—33]. Although many mutants exist which confer aberrant morphology upon the organism as well as a chemically altered cell wall [see Refs. 1, 34, 35], analysis of such mutants has shed little light upon the molecular details of cell-wall assembly. Since the variants *slime* and its derivative, *slime-like* [20,36], possess extreme phenotypes, i.e., grow a protoplasts devoid of surface-bound cell-wall material, it was suspected that the molecular block(s) in these strains might be at a key point in cell-wall biosynthesis, perhaps, even at an initial step. From a number of studies using a variety of filamentous fungi it appears that one of the first polymers to be laid down is chitin and that chitin synthase is an essential enzyme activity for continued cell-wall biosynthesis (see Ref. 33). Thus the molecular defect responsible for the *slime* phenotype could have been an absence of chitin synthase activity.

However, chitin synthase activity of *slime* and *slime-like* resembles that described from a number of fungi and that of *wild-type Neurospora crassa* (this report, and Refs. 11 and 36). All three enzyme activities were primarily recovered in particulate fractions of cell lysates and the specific activities of crude extracts were not significantly different from those of *wild-type* [11,36]. That a larger percentage of *slime* and *slime-like* enzyme were recovered in 100 000 $\times g$ supernatants than that recovered for *wild-type* (Table I; Ref. 36) may be a direct consequence of the wall-less nature of these mutants. *N*-Acetylglucosamine acts as a specific allosteric effector of *slime* variant (Fig. 1 and Table II), *slime-like* and *wild-type* enzyme activities [11,36]. All three enzyme activities are stimulated by Mg^{2+} [11,36] and those from *wild-type* and *slime* were inhibitable by polyoxin, an analogue of UDPGlcNAc (Refs. 9, 10 and Selitrennikoff, C.P., unpublished results). The pH optimum of the *slime* enzyme was pH 8.0—8.2, that for the *slime-like* enzyme was pH 7.8 [36] and that for *wild-type* has been variously reported to be pH 7.5 [11] and pH 7.8 [36]; in light of the differences in experimental conditions between these determinations, it is likely that these differences are not significant. The apparent K_m with respect to UDPGlcNAc for *wild-type* enzyme activity has been reported to be 1.4 mM [9], 2.1 mM [11], and 4 mM [36]. The apparent K_m for the *slime-like* enzyme was 3.7 mM [36] while that for the *slime* enzyme was found to be dependent upon GlcNAc concentration (Fig. 2); the K_m was 2.2 mM when extrapolated to be the conditions used for *wild-type* [11]. Further, the *slime* enzyme shares with other fungal chitin synthase the following properties: competitive inhibition by UDP [15,17,28], and stimulation of activity by phospholipids [15]. Taken together these data point to a general similarity of chitin synthase enzyme activity from *slime*, *slime-like* and *wild-type*.

The presence of active chitin synthase in extracts from *slime* and *slime-like* does not exclude the possibility that in vivo the enzyme is inactive. The lack of enzyme activity could be the result of a block in precursor biosynthesis (hexosamine biosynthetic pathway) or to a defect in the activation of chitin synthase (see Refs. 14, 37—39), for activation of zymogenic chitin synthase activity by intra-cellular proteases). These notions seem excluded by the following observations: In *slime-like* the enzymes of the hexosamine biosynthetic pathway were found to be present at essentially the same specific activities as found in *wild-type* [36] and the pool sizes of hexosamine intermediates and UDPGlcNAc were similar to those found in *wild-type* [36].

Finally, when cultures of *slime* were grown in the presence of [^{14}C]GlcN, radioactively labeled chitin was formed (Selitrennikoff, C.P., unpublished, and Braymer, D., personal communication).

It seems unlikely that the wall-less nature of *slime* and *slime-like* can be accounted for by an absence of chitin synthase acitivity.

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