The Co-ordination of Chitosan and Chitin Synthesis in *Mucor rouxii*

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Chitin synthetase preparations from cell walls and chitosomes of the fungus *Mucor rouxii* were tested for their ability to synthesize chitosan when incubated with uridine diphosphate *N*-acetyl-\(
\text{D}-\text{glucosamine}\) in the presence of chitin deacetylase. The most effective chitin synthetase preparation was one dissociated from cell walls with digitonin. The rate of chitosan synthesis by the wall-dissociated chitin synthetase was about three times that of an equivalent amount of cell walls. The chitosan-synthesizing ability of chitosomes was relatively low, but was more than tripled by treatment with digitonin. Presumably, digitonin improves chitosan yields by dissociating chitin synthetase. The dissociated enzyme would produce dispersed chitin chains that could be attacked by chitin deacetylase before they have had time to crystallize into microfibrils. The regulation of chitin and chitosan syntheses *in vivo* may be determined by the organization of chitin synthetase molecules at the cell surface. Those molecules that remain organized as a complex, similar if not identical to that found in chitosomes, would produce mainly chitin. Chitosan would be preferentially produced by chitin synthetase molecules which are dispersed upon reaching the cell surface.

INTRODUCTION

Chitin synthesis in fungi proceeds by the transfer of GlcNAc from UDP-GlcNAc into chitin, catalysed by chitin synthetase (EC 2.4.1.16; chitin synthase) (see review by Gooday & Trinci, 1980). In a preceding study with the dimorphic fungus *Mucor rouxii*, we showed that chitosan is also synthesized from UDP-GlcNAc, but by two consecutive enzymic reactions – glycosyl transfer by chitin synthetase to form chitin chains and deacetylation of the nascent chains by chitin deacetylase (EC 3.5.1.41) to produce chitosan (Davis & Bartnicki-Garcia, 1984).

The vegetative cell walls of *M. rouxii* contain both chitin and chitosan in an approximate ratio of 1:3 (Bartnicki-Garcia & Nickerson, 1962; L. L. Davis & S. Bartnicki-Garcia, unpublished results). The question of how chitin and chitosan syntheses are regulated *in vivo* must take into account the following: (1) the same glycosyl transferase is involved in both chitin and chitosan synthesis (Davis & Bartnicki-Garcia, 1984); (2) the chitin already present in the cell wall is not a precursor of chitosan since chitin deacetylase does not attack pre-formed chitin to any significant extent (Araki & Ito, 1975; Davis & Bartnicki-Garcia, 1984); and (3) chitosan synthesis requires the combined action of chitin synthetase and chitin deacetylase, operating in tandem (Davis & Bartnicki-Garcia, 1984).

Chitin synthetase activity is associated with several fractions from *M. rouxii* cells, including cell walls (McMurrough et al., 1971) and microvesicular structures called chitosomes (Ruiz-Herrera et al., 1975; Bracker et al., 1976). We previously synthesized chitosan *in vitro* utilizing a digitonin extract from mycelial walls of *M. rouxii* as the source of chitin synthetase (Davis & Bartnicki-Garcia, 1984). In the present study, we have explored the co-ordination of chitin and chitosan syntheses *in vivo* by comparing the ability of chitin synthetase preparations from cell...
walls and chitosomes of *M. rouxii* to synthesize chitosan when incubated in conjunction with a soluble chitin deacetylase. Studies on the effect of digitonin on chitosan-synthesizing systems provided the basis for the proposed co-ordination mechanism.

**METHODS**

**Culture conditions and cell-free extract preparation.*** *Mucor rouxii*, strain IM-80 (ATCC 24905), was maintained on solid YPG medium, consisting of 0.3% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glucose, 2.5% (w/v) agar, pH 4.5 (Bartnicki-Garcia & Nickerson, 1962). Spores were harvested in sterile distilled water and were used to inoculate three 2-l Erlenmeyer flasks, each containing 600 ml liquid YPG medium, to a final concentration of about 5 × 10⁷ spores ml⁻¹. A gas mixture of 70% N₂: 30% CO₂ was bubbled through the cultures to achieve the yeast form of *M. rouxii*. Cultures were incubated in a reciprocating shaker bath at 28 °C, and were harvested after 13 h by filtration through a Millipore SM 5 µm membrane. The cells were resuspended in about 20 ml 50 mM-KH₂PO₄/NaOH buffer, pH 6.5, containing 10 mM-MgCl₂ (phosphate/magnesium buffer), mixed with an equal volume of glass beads (0.45 mm diameter), and broken in a Braun MSK cell homogenizer (Bronwill Scientific, Rochester, NY, USA) for 30 s while the vessel was cooled with liquid CO₂. Cell walls were removed by centrifugation at 10000 g for 10 min.

To grow the mycelial form of *M. rouxii*, filtered air was bubbled through cultures prepared as above. The cultures were incubated in a reciprocating shaker bath at 22 °C, and were harvested after 13.5 h by filtration through a fine nylon net. Large batches (10 l) of *M. rouxii* mycelium were grown in a 14-l Microferm fermenter (New Brunswick Scientific) under similar conditions. The cells were resuspended in phosphate/magnesium buffer, mixed with an equal volume of glass beads (0.45 mm diameter), and broken in a Braun MSK cell homogenizer in three cycles of 15 s each. Cell walls were sedimented by centrifugation as above.

**Preparation of wall-associated (WA) chitin synthetase.*** Mycelial walls (1000 g pellet) were washed three times with phosphate/magnesium buffer, resuspended in an equal volume of the same buffer, and used directly as a source of WA chitin synthetase. Only fresh preparations of WA chitin synthetase were used, as it was difficult to get uniform suspensions with freeze–thawed preparations.

**Preparation of wall-dissociated (WD) chitin synthetase.*** Mycelial walls (1000 g pellet) were washed three times with phosphate/magnesium buffer, suspended in an equal volume of 1% (w/v) digitonin in phosphate/magnesium buffer, and incubated for 30 min at 22 °C, followed by centrifugation at 48000 g for 15 min. The supernatant was lyophilized and stored at -12 °C. Samples of the lyophilized WD chitin synthetase were redissolved in water before use.

**Preparation of chitosomes.** The procedure for isolating chitosomes was similar to that described previously (Bartnicki-Garcia et al., 1978). A cell-free extract from yeast cells of *M. rouxii* was centrifuged in a Beckman 30 rotor at 54000 g (Rₑₛₜ) for 45 min. The clear supernatant was removed and passed through a column (32.5 × 2.1 cm) of Bio-Gel A-5m (Bio-Rad). The column was eluted with phosphate/magnesium buffer, and fractions of 3 ml were collected. Chitin synthetase eluted in the void volume, and fractions with the highest synthetase activity were pooled. To eliminate ribosomes, the sample was digested with 30 μg pancreatic ribonuclease per mg ribosomes for 30 min at 30 °C. (Ribosome concentration was determined by absorbance at 260 nm.) The resulting precipitate was removed by centrifugation at 12000 g for 20 min, and the supernatant was concentrated to about 3 ml with Aquacide IIA (Calbiochem). The concentrated sample was layered on a ‘linear-log’ (about 6–33%) sucrose gradient (Brakke & Van Pelt, 1970). This was centrifuged at 81000 g (Rₑₛₜ) in a Beckman SW27 rotor for 3 h at 4 °C. Gradients were fractionated into 1 ml fractions with an ISCO model 183 density gradient fractionator. The six fractions with the highest chitin synthetase activity were pooled.

**Preparation of chitin deacetylase.*** Solid ammonium sulphate was gradually added to yeast cell-free extracts (1000 g supernatant) to 60% saturation. After 24 h at 4 °C, the slurry was centrifuged at 20000 g for 30 min. The supernatant was then adjusted to 85% ammonium sulphate saturation. After 24 h at 4 °C, the slurry was centrifuged as above. The pellet was dissolved in and dialysed against phosphate/magnesium buffer, followed by centrifugation in a Beckman 30 rotor at 54000 g (Rₑₛₜ) for 3.5 h. The supernatant was used as crude chitin deacetylase.

**Preparation of glycol [acetyl-³H]chitin.** Partially O-hydroxyethylated chitin (glycol chitin), radiolabelled in N-acetyl groups, was prepared as described by Araki & Ito (1975). Partially O-hydroxyethylated chitosan (glycol chitosan; 80 mg) (Sigma) was combined with 800 mg NaHCO₃ and with 12.5 mM CI (462.5 MBq) [³H]acetic anhydride (100 Ci mol⁻¹) (New England Nuclear) and left for 24 h at 4 °C. Cold acetic anhydride (0.4 ml) was added and the mixture was left for a further 24 h at 4 °C. After thorough dialysis, the product, glycol [acetetyl-³H]chitin, was diluted with unlabelled glycol chitin, prepared as described above using unlabelled acetic anhydride.

**Chitin deacetylase assay.*** Assay mixtures contained glycol [acetetyl-³H]chitin (129 nmol; 45000 d.p.m.), 12.5 mM-TES/NaOH buffer, pH 5.5, and enzyme additions, as indicated, in a final volume of 100 μl. The mixtures were incubated in Eppendorf centrifuge tubes at 30 °C for 10 min. Reactions were terminated by adding 20 μl
0.2 M-HCl, 5 μl 1 M-acetic acid, and 50 μl water. To measure [3H]acetate released, the contents of each tube were extracted with ethyl acetate, as described below for chitosan assays.

**Chitosan and chitin synthesis assays.** The same standard assay mixtures were used to measure the synthesis of chitosan or chitin. The mixtures contained 0.4 mM-UDP-[acetyl-14C]GlcNAc, 20 mM-GlcNAc, 0.2 mM-ATP, 10 mM-MgCl₂, 50 mM-KH₂PO₄/NaOH buffer, pH 6.5, 1 mg reninase ml⁻¹ (Novo Enzyme Corp., Mamaroneck, NY, USA) and enzyme additions (chitin synthetase with or without chitin deacetylase as noted) in a final volume of 125 μl. The mixtures were incubated in Eppendorf centrifuge tubes at 22 °C for 30 min, unless otherwise indicated. Reactions were terminated by adding 60 μl glacial acetic acid. To measure chitosan synthesis, ethyl acetate (0.5 ml) was added to each tube and the contents were mixed vigorously. The mixtures were centrifuged at 8000 g for 1 min in a Brinkmann 3200 centrifuge. The ethyl acetate layer was removed to a scintillation vial, and the extraction procedure was repeated once more. Radioactivity in the combined ethyl acetate extracts was determined by liquid scintillation. To measure chitin synthesis, a filtration method was used (Ruiz-Herrera & Bartnicki-Garcia, 1976). The terminated reaction mixtures were filtered through Reeve Angel 934 AH or Whatman GF/C glass-fibre filters (2.4 cm diameter) and washed with about 50 ml 1 M-acetic acid/95% ethanol (4:1, v/v). Radioactivity retained on the filters was determined by liquid scintillation. In calculating the amount of chitosan or chitin synthesized it was assumed that these polysaccharides are pure homopolymers of GlcN or GlcNAc, respectively, though this may not be exactly the case (Datema et al., 1977; Davis & Bartnicki-Garcia, 1984, and unpublished results).

**Miscellaneous.** Radioactive by-products from the chitosan synthesis assays were separated and quantified by paper chromatography (Davis & Bartnicki-Garcia, 1984). Protein was measured with Folin's phenol reagent using bovine serum albumin as a standard. Digitonin was purchased from Sigma.

**RESULTS**

The following chitin synthetase preparations were tested for their ability to synthesize chitosan in conjunction with chitin deacetylase.

**Wall-dissociated chitin synthetase**

WD chitin synthetase, extracted from *M. rouxii* mycelial walls with digitonin, is an effective co-participant in chitosan synthesis (Davis & Bartnicki-Garcia, 1984). Rennilase increased the rate of chitosan synthesis threefold by activation of zymogenic chitin synthetase (Davis & Bartnicki-Garcia, 1984). The effect of rennilase on chitin deacetylase was not tested but there is no reason to suspect that the protease adversely affects the deacetylase since the rates of chitosan synthesis during the assay were essentially linear either in the presence or absence of rennilase.

The effect of variable proportions of WD chitin synthetase and chitin deacetylase on chitosan synthesis was investigated. Addition of small amounts of WD chitin synthetase to a constant amount of chitin deacetylase produced marked increases in chitosan synthesis (Fig. 1a); beyond this initial effect, increasing quantities of the synthetase brought about less pronounced and approximately linear increases in the rate of chitosan synthesis. The WD chitin synthetase had an absolute requirement for chitin deacetylase before significant chitosan synthesis occurred (Fig. 1b). With a constant amount of WD chitin synthetase, the rate of chitosan synthesis increased linearly with increasing amounts of chitin deacetylase until saturation was apparently achieved (Fig. 1b); further addition of chitin deacetylase had little effect on the rate of chitosan synthesis.

**Wall-associated chitin synthetase**

A cell wall suspension was used directly as a source of WA chitin synthetase. In general, the rates of chitosan synthesis obtained with WA chitin synthetase were much lower than those obtained with an equivalent amount of WD chitin synthetase (Fig. 1a,b). With a constant amount of chitin deacetylase, the rate of chitosan production was directly proportional to the amount of WA chitin synthetase (Fig. 1a). The cell wall suspension was able to synthesize a small amount of chitosan even in the absence of exogenous deacetylase (Fig. 1b). Furthermore, the rate of chitosan synthesis with the WA chitin synthetase was not affected by the addition of exogenous chitin deacetylase (Fig. 1b).
Fig. 1. Effect of variable amounts of cell wall chitin synthetase and chitin deacetylase on chitosan synthesis. A washed cell wall suspension was divided into two equal portions. One portion was treated with 0.5% digitonin and the resulting extract was used as wall-dissociated (WD) chitin synthetase. The untreated portion was used as wall-associated (WA) chitin synthetase. Chitosan synthesis was determined in standard assay mixtures containing UDP-[acetate-l-14C]GlcNAc, chitin deacetylase (15-90 mg protein ml⁻¹), and either WD chitin synthetase (1.15 mg protein ml⁻¹) (○), or WA chitin synthetase (3.25 mg protein ml⁻¹) (●), in a total volume of 250 µl. (a) The amount of chitin deacetylase was held constant (25 µl) and the amount of each of the two chitin synthetases was varied. (b) The amount of each chitin synthetase was held constant (50 µl) and the amount of chitin deacetylase was varied. Chitosan synthesis is expressed as nmol acetate released min⁻¹.

Table 1. Chitosan and chitin synthesis by chitosomal chitin synthetase and chitin deacetylase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Chitosan synthesis (nmol min⁻¹)</th>
<th>Chitin synthesis (nmol min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitin deacetylase</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Chitosomal chitin synthetase</td>
<td>0.002</td>
<td>0.220</td>
</tr>
<tr>
<td>Chitosomal chitin synthetase + chitin deacetylase</td>
<td>0.018</td>
<td>0.082*</td>
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* This rate refers to chitin accumulation rather than to chitin synthesis since, in the presence of chitin deacetylase, some of the chitin that is synthesized is converted to chitosan.

Chitosomal chitin synthetase

Incubation of chitosomal chitin synthetase alone with UDP-GlcNAc did not produce chitosan (Table 1), whereas a considerable amount of chitin was synthesized. But when chitosomal chitin synthetase and chitin deacetylase were incubated simultaneously with UDP-GlcNAc, a significant amount of chitosan was produced (Table 1). Concomitantly, there was a reduction in the amount of chitin recovered. The total polymer (chitin + chitosan) recovered when both chitosomal chitin synthetase and chitin deacetylase were incubated with UDP-GlcNAc was 55% less than that obtained from chitosomes incubated alone with the substrate (Table 1). The cause of this decrease in total polymer production is not entirely clear though a small loss – estimated by paper chromatography to be <5% – can be attributed to chitin destruction by traces of chitinase introduced with the crude deacetylase. Chitinase is present in the cytosol of M. rouxii and can cause significant chitin losses during conventional chitin synthetase assays (Lopez-Romo et al., 1982).

Addition of increasing concentrations of chitosomal chitin synthetase or chitin deacetylase to a constant amount of the other resulted in linear increases in chitosan synthesis until saturation was apparently achieved (Fig. 2a,b).
Co-ordination of chitosan and chitin synthesis

Fig. 2. Effect of variable amounts of chitosomal chitin synthetase and chitin deacetylase on chitosan synthesis. Chitosan synthesis was determined in standard assay mixtures containing UDP-[acetate-14C]-GlcNAc, chitosomal chitin synthetase (0.24 mg protein ml⁻¹), and chitin deacetylase (15-15 mg protein ml⁻¹), in a total volume of 250 µl. (a) The amount of chitin deacetylase was held constant (25 µl) and the amount of chitosomal chitin synthetase was varied. (b) The amount of chitosomal chitin synthetase was held constant (50 µl) and the amount of chitin deacetylase was varied. Chitosan synthesis is expressed as nmol acetate released min⁻¹.

Fig. 3. Effect of digitonin on chitosan and chitin synthesis. Chitosomal chitin synthetase was pre-incubated with digitonin, at the concentrations shown, for 30 min at 22 °C. Standard chitosan assay mixtures contained UDP-[acetate-14C]-GlcNAc, the digitonin-treated chitosomal chitin synthetase, and chitin deacetylase. Chitosan synthesis (●) is expressed as nmol acetate released min⁻¹. Standard chitin assay mixtures contained UDP-[acetate-14C]-GlcNAc and the digitonin-treated chitosomal chitin synthetase. Chitin synthesis (○) is expressed as nmol GlcNAc incorporated into chitin min⁻¹.

Digitonin-treated chitosomal chitin synthetase

Since digitonin had a beneficial effect on the ability of cell wall chitin synthetase to participate in chitosan synthesis, we investigated whether digitonin would have a similar effect on chitosomal chitin synthetase. Chitosomes were tested both in the presence of chitin deacetylase (to measure chitosan synthesis) or in its absence (to measure chitin synthesis) (Fig. 3). At concentrations below 0.05%, digitonin produced a slight stimulation of chitin synthesis followed by sharp inhibition at higher concentrations. In contrast, chitosan synthesis was stimulated at all concentrations of digitonin tested; this stimulation was maximal (three- to five-fold) at 0.1% digitonin (Fig. 3). Higher digitonin concentrations caused only a slight decrease in
chitosan formation. Pre-incubation of chitosomes with digitonin (at 4 °C for 0.5–24 h) did not significantly improve chitosan synthesis compared to assay mixtures where digitonin was added directly.

We wanted to test the possibility that *M. rouxii* chitosomes contain inactive chitin deacetylase that is somehow activated by digitonin. Chitosomes were pre-incubated with 0.01–0.50% digitonin at 22 °C for 30 min. The digitonin-treated chitosomes were then assayed for chitin deacetylase activity. Digitonin-solubilized chitosomes had no activity toward glycol-[acetyl-3H]-chitin, regardless of the digitonin concentration employed. Likewise, non-digitonin treated chitosome controls had no chitin deacetylase activity.

**DISCUSSION**

Chitin deacetylase catalyses the synthesis of chitosan by deacetylation of nascent chitin (Davis & Bartnicki-Garcia, 1984); this enzyme has little activity on pre-formed chitin. We previously proposed that chitin deacetylase cannot penetrate the crystalline structure of pre-formed chitin, but must act before the nascent chitin chains crystallize into microfibrillar bundles (Davis & Bartnicki-Garcia, 1984). Effective chitosan synthesis requires both chitin synthetase and chitin deacetylase operating in tandem (Davis & Bartnicki-Garcia, 1984).

**Stimulation of chitosan synthesis by digitonin**

The substantial stimulation of chitosan synthesis by digitonin may be explained in the light of previous observations on the effect of digitonin on chitin synthetase from *M. rouxii*. Low digitonin concentrations (below those tested in the present study) result in up to 50% stimulation of chitosomal chitin synthetase activity (Ruiz-Herrera et al., 1980). Digitonin dissociates chitin synthetase from *M. rouxii* into 16S subunits (about 500000 Dal); both the enzyme located in microvesicles (chitosomes) and in the cell wall can be dissociated in this manner (Bartnicki-Garcia et al., 1977; Ruiz-Herrera et al., 1980). Ruiz-Herrera et al. (1980) speculated that digitonin may bind to chitosome sterols causing the chitosome to dissociate into subunits.

The spatial organization of chitin synthetase molecules seems crucial for chitosan synthesis. Ruiz-Herrera et al. (1980) proposed that the arrangement of chitin synthetase units in chitosomes provides the spatial organization necessary for the production of long chitin microfibrils. We speculate that the spatially ordered production of chitin chains from these organized assemblies of chitin synthetase molecules would allow rapid crystallization of the nascent chains either during or immediately after polymerization; the nascent chitin stage would be too short to permit the deacetylase to interact with free chitin chains. This is compatible with our finding that chitin accumulation greatly exceeds chitosan synthesis when intact chitosomes are used as a source of chitin synthetase in the chitosan-synthesizing mixtures. We further speculate that digitonin-dissociated chitin synthetase produces dispersed chitin chains that do not crystallize instantly; here the nascent stage of chitin would be sufficiently long to allow chitin deacetylase to attack the chitin chains. This speculation is compatible with our finding that digitonin treatment of cell walls or chitosomes greatly increased their ability to participate in chitosan synthesis. It is also compatible with our previous observation that chitosan formation is much greater than chitin accumulation in incubation mixtures containing digitonin-extracted WD chitin synthetase (Davis & Bartnicki-Garcia, 1984).

**Inhibitory effects of digitonin**

We confirmed the finding of Ruiz-Herrera et al. (1980) that digitonin stimulates chitin synthesis at low concentrations but is markedly inhibitory at higher concentrations. Yet, in the same concentration range, we found no inhibitory effect on chitosan synthesis. The most likely explanation for this seeming discrepancy is that the crude deacetylase preparation introduced a large amount of protein into the chitosan assay mixtures, diluting the effect of digitonin. Accordingly, in the presence of chitin deacetylase, increasing concentrations of digitonin were either stimulatory or caused only a minor inhibition of chitin synthetase while allowing a greater proportion of chitin to be deacetylated.
Co-ordination of chitosan and chitin synthesis

Chitin synthetase and deacetylase in cell walls

Isolated mycelial walls of *M. rouxii* have a high chitin synthetase activity (McMurrough et al., 1971). We found that the walls also have substantial chitosan synthetizing activity. The WA chitin synthetase did not respond to the addition of exogenous deacetylase, suggesting that there is sufficient chitin deacetylase associated with the cell wall so that this enzyme does not limit chitosan synthesis in vitro. Chitin synthetase preparations extracted from the walls with digitonin, on the other hand, had practically no chitin deacetylase activity. Thus, digitonin appears to selectively extract chitin synthetase from cell walls, leaving chitin deacetylase behind.

Although the cell wall fractions used in this study were washed several times before use, or before digitonin treatment, it is possible that the walls had some cytoplasmic contamination. It cannot be stated unequivocally, therefore, that the origin of the WA or WD chitin synthetase was, in fact, the cell wall. McMurrough et al. (1971) did find, however, that repeated breaking and washing failed to remove chitin synthetase from cell walls, and suggested that chitin synthetase is intimately associated with, if not an integral component of, the cell wall.

Regulation of chitin and chitosan synthesis in vivo

Our findings and conclusions on the role of digitonin in chitosan synthesis in vitro serve as the basis for the following hypothesis on the regulation of chitin and chitosan syntheses in vivo. This hypothesis takes into account that chitin synthetase is the glycosyl transferase in both chitin and chitosan biosynthesis (Davis & Bartnicki-Garcia, 1984). Accordingly, we propose that the ratio of chitosan to chitin in the cell wall is established by the degree of organization of chitin synthetase molecules at the cell surface. Most of the chitin synthetase in fungal cells is in the cytoplasm in the form of chitosomes; these microvesicles are thought to convey latent chitin synthetase to its final destination (Bartnicki-Garcia et al., 1979). Part of the chitosomal chitin synthetase that reaches the cell surface may remain tightly associated in a complex that retains all or much of the enzyme organization present in the chitosome; this enzyme would produce chitin microfibrils, as proposed above. Part of the chitosomal chitin synthetase, however, may become dispersed upon reaching the cell surface; the chitin chains produced by this enzyme would be susceptible to deacetylation into chitosan. Since chitosan is approximately three times more abundant than chitin in vegetative cell walls of *M. rouxii* (Bartnicki-Garcia & Nickerson, 1962; L. L. Davis & S. Bartnicki-Garcia, unpublished results), we suggest that a major portion of the chitin synthetase at the cell surface operates in a dissociated state.

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REFERENCES


