Properties of Chitin Synthetase in Isolated Chitosomes from Yeast Cells of *Mucor rouxii*  
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Chitin synthetase was isolated and purified 120-fold from the supernatant fraction (54,500 × g) of broken yeast cells of *Mucor rouxii*. The purified preparations consisted mainly of chitin synthetase particles (chitosomes) with an average size larger than 7 × 10⁶ daltons (by gel filtration) and an average sedimentation coefficient of 105 S. The samples also contained other enzyme complexes (fatty acid synthetase, pyruvate dehydrogenase, and, depending on method, ribosomes). Nearly all of the chitosomal chitin synthetase occurred in a zymogenic form that required proteolytic activation. In most properties, the chitosomal enzyme was similar to crude enzyme (54,000 × g sediment): kinetics, activation by proteases, response to metals, stimulation by N-acetylglucosamine, and inhibition by polyoxin or UDP. One major difference was the much greater stability of the chitosomal chitin synthetase zymogen against spontaneous activation and destruction. Product (chitin microfibril) and enzyme (chitin synthetase) remained associated in a complex that was readily separated by centrifugation.

In previous publications, the properties of chitin synthetase in crude fractions from mycelium (1-3) and yeast cells (4) of *Mucor rouxii* were examined. The salient difference between the enzymes of mycelium and yeast forms was stability. Mycelial enzyme was rapidly destroyed by endogenous proteases, whereas yeast enzyme increased its activity upon standing (4). The enzyme from yeast cells withstand treatments to separate it from other particles in the cell-free extract. With such purified preparations, we were able to synthesize chitin microfibrils in vitro (5) and determine that chitin synthetase contained particles ("granules") of about 350 to 1000 Å in diameter (6). A more recent ultrastructural study revealed that these granules have a distinct microvesicle-like morphology and that they undergo an extensive transformation during microfibril synthesis (7). These characteristic chitin synthetase-containing particles have been named "chitosomes" (7).

This communication describes the main properties of chitosomal chitin synthetase and compares them with the properties of crude enzyme from *M. rouxii* and, wherever pertinent, of crude chitin synthetases from other fungi.

MATERIALS AND METHODS

**Cultivation Techniques and Cell-free Extract Preparation**—*Mucor rouxii* IM-80 (ATCC 24905) was grown in the yeast form under an atmosphere of 70% prepurified N₂, 30% CO₂ for 13 h as previously described (4). The cultures were filtered through a coarse sintered glass filter until the volume was reduced to about 20 ml. Sufficient amounts of 1 M K₂HPO₄/NaOH buffer, pH 6.5, and 1 M MgCl₂ were added to obtain final concentrations of 50 and 10 mM, respectively. The cells (about 1 to 1.5 g, dry weight) were broken in a Braun MSK homogenizer for 30 s while the vessel was cooled with liquid CO₂ (4). The cell walls were removed by centrifugation at 1000 × g for 5 min. The supernatant was centrifuged in a Beckman No. 30 rotor at 54,500 × g (R,av) for 45 min. The supernatant fraction was removed with a pipet; a floating lipid layer was included as much as possible. Chitosomes were purified from the supernatant fraction as described below. The pellet or mixed membrane fraction was washed with centrifugation with buffer for 1 h at 78,800 × g (R,av) and used for the experiments on crude chitin synthetase.

**Chitosome Assay**—Chitin synthetase was activated by protease either before or during the assay. Unless otherwise stated, the proteolytic activation and assay were done simultaneously. The standard incubation mixture contained, unless otherwise stated: 0.42 mM UDP-[¹⁴C]GlcNAc (0.2 Ci/mol); 17 mM GlcNAc; 0.17 mM ATP; 10 mM MgCl₂; 5 or 10 µg of acid protease from *Rhizopus chinensis* (Miles Laboratories, Elkhart, Ind.; no longer available); and 50 mM KH₂PO₄/NaOH buffer, pH 6.5, in a final volume of 0.15 ml. After 30 or 60 min, 20 µl of glacial acetic acid were added to stop the reaction. The entire sample was filtered through Whatman GF/C or Whatman GF/C glass-fiber filters (2.4 cm in diameter) and washed with about 50 ml of 1 M acetic acid, 95% ethanol (8:2 by volume). The radioactivity was counted as previously described (4).

In some experiments, the assay mixtures were analyzed by paper chromatography (4). One unit of chitin synthetase was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of GlcNAc into chitin in 1 min. Specific activity was calculated per mg of protein.

**Purification of Chitosomes by Gel Filtration**—The supernatant fraction (10 to 13 ml) was subjected to gel filtration at 2°C in a column (35 × 2.1 cm) of Sepharose 6B (Pharmacia, Uppsala, Sweden) or Bio-Gel A-5m (Bio-Rad, Richmond, Calif.). The column was eluted with 3338
0.05 M KH₂PO₄/NaOH buffer, pH 6.5, containing 10 mM MgCl₂ (phosphate/magnesium buffer), and fractions of 3 ml were collected. The absorbance of the fractions was measured at 260 nm. Samples of 50 or 100 μl were withdrawn for chitin synthetase assay. The enzyme eluted in the void volume. The five fractions with highest chitin synthetase activity were pooled and subjected to a second gel filtration in a column of Sepharose 2B (37.5 x 2.2 cm) as described above for Sepharose 6B. In Sepharose 2B, the enzyme was included into the gel bed and eluted as a broad peak (Fig. 1), which emerged before another broad peak of strong ultraviolet light-absorbing material (ribosomes). A small shoulder of chitin synthetase was sometimes observed at the void volume. Active fractions (21 to 28) that appeared at the void volume. Active fractions (21 to 28) that appeared

Although the fractions of chitin synthetase purified by Sepharose 2B gel filtration were chosen as far away as possible from the ribosome peak (Fig. 1), examinations of purified chitin synthetase by electron microscopy (negative staining) revealed that ribosomes were far more abundant than chitosomes. For elimination of ribosomes, the eluate (void volume fraction) from a Sepharose 6B 15 min. was digested with ribonuclease (see below). The precipitated basic proteins were removed by centrifugation at 12,000 x g for 20 min and the clear supernatant was concentrated to about 0.5 ml with an Amicon, model 202 ultrafiltration cell, equipped with an XM-300 membrane (Amicon, Lexington, Mass.). The membrane was washed several times with a total volume of 3 ml of phosphate/magnesium buffer. The concentrated sample and washings were mixed, and 3 ml were layered on a linear sucrose gradient (5 to 20%, 36 ml). This was centrifuged at 81,500 x g (Rₑₚ) in a Beckman SW-27 rotor for 3 h. Some samples (7 ml) were applied without concentration by ultracentrifugation onto a 5 to 20% sucrose gradient (32 ml). Gradients were fractionated with a density gradient fractionator, model 183 (ISCO, Lincoln, Neb.), into 1-ml fractions. Absorbance was monitored at 254 nm. Chitin synthetase activity was measured in 50- or 100-μl samples (Fig. 2).

**Chitosomal Chitin Synthetase Isolation**

Cultures of yeast cells of *M. rouxii* (total volume, 2 liters), grown as described under "Materials and Methods." The crude supernatant fraction from yeast cells of *M. rouxii* was digested with ribonuclease (see below). The precipitated basic proteins were removed by centrifugation at 12,000 x g for 20 min and the clear supernatant was concentrated to about 0.5 ml with an Amicon, model 202 ultrafiltration cell, equipped with an XM-300 membrane (Amicon, Lexington, Mass.). The membrane was washed several times with a total volume of 3 ml of phosphate/magnesium buffer. The concentrated sample and washings were mixed, and 3 ml were layered on a linear sucrose gradient (5 to 20%, 36 ml). This was centrifuged at 81,500 x g (Rₑₚ) in a Beckman SW-27 rotor for 3 h. Some samples (7 ml) were applied without concentration by ultracentrifugation onto a 5 to 20% sucrose gradient (32 ml). Gradients were fractionated with a density gradient fractionator, model 183 (ISCO, Lincoln, Neb.), into 1-ml fractions. Absorbance was monitored at 254 nm. Chitin synthetase activity was measured in 50- or 100-μl samples (Fig. 2).

**Miscellaneous**

Protein was measured with Folin's phenol reagent (8). Ribosome concentration was estimated spectrophotometrically (9). Ribosomes were eliminated by digestion with 20 μg of pancreatic ribonuclease (5 x crystalline; Calbiochem, La Jolla, Calif.) per ml of ribosomes, at pH 6.5, in phosphate/magnesium buffer, for 30 min at 30°C.

Our colleagues at the University of California, Riverside, Calif., kindly provided the following: cowpea mosaic virus (J. S. Semancik), rabbit-erythrocyte ribosomes (J. A. Traugh), and tritium-labeled ribosomes from *Phytophthora palmivora* (J. V. Leary). Radioactivity of nonaqueous samples was counted with a toluene-based scintillation fluid (1). Aqueous samples were counted with Aquasol (New England Nuclear, Boston, Mass.). Peps and alkaline phosphatase (calf intestine) were obtained from Calbiochem, La Jolla, Calif. Tritpsin was from NBCo, Cleveland, Ohio. Mexican, a protease from *Pileus mexicanus*, was a gift from Professor M. Castaneda-Aguillo, Instituto Politecnico Nacional, Mexico, D. F., Mexico. Remilase, a crude acid protease from *Mucor miehei*, was a gift from Novo Enzyme Corp. Mamaroneck, N. Y. Polysomes D was kindly supplied by M. Sakamaki of the Kaken Chemical Co., Ltd., Tokyo, Japan. Uridine 5′-diphosphate N-acetyl-β-[1-14C]glucosamine was obtained from ICN, Irvine, Calif. N-Acetyl-d-[1-14C]glucosamine was purchased from New England Nuclear, Boston, Mass.

**RESULTS**

**Purification of Chitosomal Chitin Synthetase**

Upon purification by gel filtration, the specific activity of the chitin synthetase isolated from the supernatant fraction increased 120-fold (Table I). Note that the total chitin synthetase activity of the crude supernatant fraction more than doubled after passage through Sepharose 6B. This increase was most likely due to dilution effect.

![Fig. 1. Separation of chitosomal chitin synthetase by gel filtration in Sepharose 2B. The most active chitin synthetase fractions separated on a Sepharose 6B column (10 ml; 15 mg of protein) were applied to a Sepharose 2B column (37.5 x 2.2 cm) and eluted with phosphate/magnesium buffer. Fractions of 3 ml were collected. Absorbance was measured at 260 nm (O). Chitin synthetase activity (O) was measured in 100-μl samples and expressed as total units per fraction.](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein Concentration</th>
<th>Chitin synthetase activity (units)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude supernatant fraction</td>
<td>0.14</td>
<td>31.85</td>
<td>1</td>
</tr>
<tr>
<td>Sepharose 6B peak (Fractions 5-10)</td>
<td>2.46</td>
<td>67.40</td>
<td>18</td>
</tr>
<tr>
<td>Sepharose 2B peak (Fractions 21-28)</td>
<td>16.39</td>
<td>7.70</td>
<td>120</td>
</tr>
</tbody>
</table>

**Fig. 2. Sedimentation of chitosomal chitin synthetase in a sucrose density gradient.** Fractions eluted in the void volume of a Sepharose 6B column (15 ml; 20 mg of protein) were treated with ribonuclease as described in the text and concentrated to about 3.5 ml. Three ml (3.6 mg of protein) were placed on top of a linear 5 to 20% sucrose gradient in phosphate/magnesium buffer (36 ml) and centrifuged for 3 h at 81,500 x g (Rₑₚ). Gradients were fractionated into 1-ml fractions. Absorbance at 254 nm (O) was continuously recorded. Chitin synthetase activity (O) was measured in 100-μl samples and expressed as total units per fraction.
to removal of a low molecular weight protein, which is a strong inhibitor (16) of the activation of chitin synthetasezymogen.

The density gradient centrifugation procedure afforded higher yields of purified chitin synthetasezymogen with higher specific activities than those obtained in the gel filtration method. The pooled fractions (16 to 22) at the peak of chitin synthetase activity (Fig. 2) contained about 0.3% of the total protein and 30% of the estimated total chitin synthetase activity in the cell-free extract. Because of the RNase treatment, these preparations of chitin synthetasezymogen were free of ribosomes. Electron microscopic observations described in detail elsewhere (7) revealed that the dominant particles in the preparations were microvesicle-like structures termed chitosomes (7). The preparations also contained other enzyme particles of comparable size, namely pyruvate dehydrogenase and the fatty acid synthetase complex (7). The chitosomal samples prepared by the gel filtration method (no RNase treatment) contained many ribosomes.

In cells labeled with [14C]choline and fractionated by the density gradient centrifugation method, the peak fractions of chitin synthetase, which contained 6.15% of the total chitin synthetase in the cell-free extract, had only 0.068% of the [14C]choline-labeled phospholipids in the cell-free extract. Accordingly, we have estimated that the entire chitin synthetase activity of the cells is associated at most with 1% of the total protein and 1% of the choline-labeled phospholipids in the cell-free extract.

General Properties of Chitosomal Chitin Synthetase—With the standard assay mixture, the rate of chitin synthesis was linear with time up to 60 min. Activity was also a linear function of protein concentration up to 60 μg/ml. The chitosomal preparation used in these tests had a specific activity of 8.2 units (mg of protein)-1.

Plots of enzyme velocity versus substrate concentration revealed sigmoidal kinetics (Fig. 3). A Kᵣ of 0.5 mM was estimated from values at high substrate concentration (Fig. 3). This value was the same as that obtained for the crude mycelial enzyme of Mucor rouxii (2).

The effect of pH was tested on chitosomes preactivated with acid protease at pH 6.5. The pH optimum for chitin synthetase activity was 6.5. This value coincides with that obtained for the crude mycelial enzyme of Mucor rouxii (2).

The effect of temperature on chitin synthetase activity was tested on chitosomes that had been preactivated with acid protease. Maximum chitin synthetase activity occurred at 24°C. The effect of temperature on stability of chitin synthetase was tested on chitosomes not previously activated with acid protease. The chitosome suspensions were heated at various temperatures for 5 min and then assayed in the presence of acid protease. Chitosomal chitin synthetase resisted heating at 40°C for 5 min. The detrimental effect of Mn²⁺ at high concentration occurred at low UDP-GlcNAc concentration. Maximum stimulation occurred at low UDP-GlcNAc concentration and vice versa. When a high concentration of GlcNAc was used, the sigmoidal nature of velocity-substrate concentration plots disappeared.

Incorporation of free GlcNAc into chitin by chitosomal enzyme was tested in parallel assay mixtures containing either proteolytic activity of acid protease or trypsin against hemoglobin or casein, respectively (data not shown); hence, Mn²⁺ probably sensitizes chitin synthetase to proteolytic destruction.

GlcNAc was a stimulator of crude chitin synthetase from the mycelium (2) and yeast cells of M. rouxii. Likewise, GlcNAc stimulated the activity of chitosomal chitin synthetase (Fig. 6). The stimulation of chitosomal chitin synthetase by GlcNAc depended on UDP-GlcNAc concentration. Maximum stimulation occurred at low UDP-GlcNAc concentration and vice versa. When a high concentration of GlcNAc was used, the sigmoidal nature of velocity-substrate concentration plots disappeared.

Incorporation of free GlcNAc into chitin by chitosomal enzyme was tested in parallel assay mixtures containing either

4.8 mM UDP-[\textsuperscript{14}C]GlcNAc and 0.108 \mu M GlcNAc or 4.8 mM UDP-GlcNAc and 0.108 \mu M [\textsuperscript{14}C]GlcNAc (0.5 \mu Ci). Only 1 mol of free GlcNAc was incorporated per 2.8 \times 10^6 residues of GlcNAc polymerized.

**Formation of Byproducts**—Besides chitin, crude mycelial fractions synthesized diacetylchitobiose when incubated with UDP-GlcNAc (2). Preparations of yeast mixed membrane fractions, incubated in standard assay mixtures, formed three products: chitin, diacetylchitobiose, and N-acetyl-\(\alpha\)-glucosaminylphosphate (GlcNAc-P) in approximate ratios of 3:1:2. These products were separated by paper chromatography in 95% ethanol, 1 M acetic acid (20:80 by volume) or isooamyl alcohol:pyridine:water (1:1:0.8 by volume). Alkaline phosphatase treatment converted the suspected GlcNAc-P into a substance with the paper chromatographic mobility of GlcNAc. In contrast to the above, preparations of chitosomal chitin synthetase produced neither GlcNAc-P nor diacetylchitobiose, only chitin.

**Competitive Inhibitors**—The antibiotic polyoxin (11) was a strong competitive inhibitor of chitosomal chitin synthetase. The kinetics of inhibition and \(K_I\) (0.65 \mu M) were similar to those found for crude fractions from mycelium (3) or yeast cells of \(M. rouxii\) (not shown). UDP, a product of chitin synthesis, also inhibited the reaction. The kinetics of inhibition (Dixon plots) were nonlinear (Fig. 7) except at the lower concentration of inhibitor. At such concentrations the inhibition was competitive with a \(K_I\) of -0.4 mM. UDP caused the same inhibition of chitin synthetase on crude mixed membrane fractions of yeast cells. Other nucleotides, ATP, ADP, AMP, UTP, and UMP (all at 6.25 mM), inhibited chitin synthetase by 70 to 95%. At 0.2 mM, ATP was slightly stimulatory.

**Activation**—Chitosomal chitin synthetase was mostly present in a "zymogenic" form and showed little activity unless treated with proteases. The zymogen was highly stable; purified chitosome preparations kept at 28° for 4 h did not change.

**Fig. 6 (right). Effect of three proteases on the activation of chitosomal chitin synthetase.** Samples of chitosomes purified by gel filtration through Sepharose 2B (0.1 ml; 8.7 \mu g of protein) were incubated with two concentrations of UDP-[\textsuperscript{14}C]GlcNAc and variable concentrations of UDP; all other reagents in the standard assay were unchanged. After 60 min, radioactivity incorporated into chitin was measured. \(A\), inhibition of chitin synthetase by UDP at the higher UDP-GlcNAc concentration; \(B\), Dixon plots of activity.

**Fig. 7. Effect of UDP on activity of chitosomal chitin synthetase.** Samples of chitosomes purified by gel filtration through Sepharose 2B (0.1 ml; 8.7 \mu g of protein) were incubated with two concentrations of UDP-[\textsuperscript{14}C]GlcNAc and variable concentrations of UDP; all other reagents in the standard assay were unchanged. After 60 min, radioactivity incorporated into chitin was measured. \(A\), inhibition of chitin synthetase by UDP at the higher UDP-GlcNAc concentration; \(B\), Dixon plots of activity.
commercially unavailable, a crude acid protease from *Mucor miehei* (Rennilase) was employed in more recent experiments. Ten times more Rennilase was needed to give the activation produced by pure acid protease.

**Chitin Synthetase Particle Size**—The chitosomal enzyme was excluded from Sepharose 6B. In Sepharose 4B and 2B, the enzyme was included in the gel bed and the peak of activity appeared before that of a marker sample of cowpea mosaic virus (MW = 7 × 10^6).

The sedimentation of chitosomes in 5 to 20% sucrose density gradients (see "Materials and Methods") was compared with that of ribosomes and their subunits from rabbit reticulocytes, *M. rouxii*, and *Phytophthora palmivora*. Accordingly, chitosomes have an average sedimentation coefficient of 105 S.

**Association of Chitin Synthetase with Microfibrils**—One-ml samples (43 μg of protein) of chitosomes purified by the sucrose gradient method were activated with Rennilase (5 mg/ml) for 30 min and preincubated at 22° with either ^14^C-labeled substrate or unlabeled substrate. The samples were then layered over linear sucrose gradients. A control sample of chitosomes was activated with Rennilase and incubated at 22° without addition of substrate. The gradients were centrifuged at a relatively low speed of 12,200 × g (R_{uv}) for 20 min and were fractionated. The radioactivity of fractions from the sample preincubated with radiolabeled substrate was measured to determine the position of ^14^C-chitin along the gradient. The fractions from the samples preincubated with unlabeled substrate or without substrate were assayed to determine the distribution of chitin synthetase along the gradients. From the sample preincubated with UDP[^14]^C-GlcNAc, a band of radioactivity coincided with a zone of fibrous material apparent to the naked eye. (The radioactivity in the upper portion of the tube (Fig. 9A) represents unused substrate.) A determination of chitin synthetase in a parallel tube (Fig. 9B) showed that most of the chitin synthetase activity in the incubation mixture remained above the gradient. But, significantly, a portion of the enzyme moved down into the gradient and sedimented at the same place as the radioactive fibrils. In the control sample, which was not preincubated with substrate, no chitin synthetase activity moved to the bottom of the tube (Fig. 9C). Clearly, the dense chitin microfibrils had carried down to the bottom part of the gradient a portion of the chitin synthetase present in the incubation mixture. This suggests an intimate and lasting association between enzyme and product. A non-specific adsorption of chitin synthetase to the fibrils is not a likely explanation of the cosedimentation, since unincubated enzyme does not adsorb to chitin.

**DISCUSSION**

Chitin synthetase can be isolated from the cytoplasm of yeast cells of *Mucor rouxii* as discrete, characteristic particles, or chitosomes (7), with an average sedimentation coefficient of 105 S and an average molecular size larger than that of the cowpea virus marker (7 × 10^6 daltons; 300 Å in diameter) but somewhat smaller than the stippled exclusion limit for the gel filtration bed (20 × 10^6 daltons). These values are in agreement with electron microscopic measurements; chitosomes measured from 350 to 1000 Å in diameter with a mean value of about 600 Å (7).

By the procedures described herein, up to 30% of the total chitin synthetase activity of the cell-free extract was recovered in the final pool of purified chitosomes. A major loss occurred during preparation due to the deliberate elimination of the less active fractions at each step of the purification sequence. By a different procedure involving direct centrifugation of the cell-free extract (1000 × g supernatant) on a sucrose density gradient, most (approximately 70%) of the total chitin synthetase activity of the extract was recovered in a broad band with the sedimentation velocity of chitosomes.² We have calculated from the relative proportions of chitin synthetase activity, protein, and choline-labeled phospholipids in the purified chitosome preparations versus the cell-free extract that the entire chitosome population from a cell would represent a minute portion of the cell mass (<1% of the total protein¹ and 1% of the choline-labeled phospholipids in the cell-free extract). Although these are admittedly gross approximations, they can be construed as evidence against the possibility that the isolated chitosomes are mere fragments of some major membrane component of the cell, produced during cell rupture. For instance, Durán et al. (12) have reported that the bulk of the chitin synthetase zymogen is in the plasma membrane fraction of *Saccharomyces cerevisiae*. Whether some of the chitin synthetase activity of *M. rouxii* is associated with the plasma membrane has not been established.

Chitosomal chitin synthetase is largely in a zymogenic state. The zymogenicity of chitin synthetase was first noted in *S. cerevisiae* (13), but the exact nature of the latent enzyme is

² In view of the presence of other enzyme complexes in the purified chitosome samples, the actual chitosome protein content must be somewhat lower than that measured.

![Fig. 9. Association of chitin synthetase with microfibrils. Samples of chitosomes purified by sucrose gradient centrifugation (43 μg of protein) in phosphate/magnesium buffer were incubated with 0.25 ml of Rennilase (5 mg/ml) for 30 min at 22° and then incubated with 20 mM GlcNAc and either 1.2 mM UDP[^14]^C-GlcNAc (A) or 1.2 mM UDP-GlcNAc (B); a third sample received buffer but no substrate or GlcNAc (C). Final volume was 2 ml. After 30 min, the samples were layered on top of sucrose gradients (9.5 ml of a 10 to 30% linear sucrose gradient plus 1 ml of 60% sucrose cushion), centrifuged in an SW-41Ti Beckman rotor at 12,200 × g (R_{uv}) for 20 min, and fractionated into 1-ml aliquots. Radioactivity of fractions from the sample preincubated with UDP[^14]^C-GlcNAc was measured in 0.2-ml aliquots.](http://www.jbc.org/)
not yet known. Chitosomes synthesize microfibrils after activation with protease and incubation with UDP-GlcNAc and a divalent metal cofactor. The properties of the chitosomal chitin synthetase, such as pH, optimum temperature, response to divalent metals, and $K_m$, were similar to those from the crude mixed membrane fraction preparations. Likewise, the activation of chitin synthetase zymogen of both chitosomal and crude preparations was inhibited by a low molecular weight protein, irrespective of the protease used for activation (4, 10). The one major difference between crude enzyme and chitosomal enzyme was stability. Preparations of chitosomes could be left for several hours at 22 to 28°C without any appreciable change in their basal or potential activity, but crude preparations (mixed membrane fractions) exposed to these conditions underwent marked spontaneous activation of the zymogen followed by net destruction of enzyme activity (4). Seemingly, the chitosomal enzyme is deficient in the protease(s) responsible for the conversion of zymogen to active enzyme and for its eventual destruction.

The stimulation of chitosomal chitin synthetase by GlcNAc was strictly dependent on substrate concentration: high stimulations were obtained at low levels of UDP-GlcNAc and vice versa. GlcNAc is a marked stimulator of crude chitin synthetase from different fungi (2, 14-17), but, interestingly, in two studies where the enzyme was solubilized with butanol (14) or with digitonin (17), the effect of GlcNAc was absent or minimal, respectively. Our data support the suggestion that GlcNAc acts as an allosteric activator (2, 15, 17). Furthermore, since relatively high concentrations of GlcNAc are needed for substantial stimulation and since UDP-GlcNAc in much lower concentrations produces the same stimulatory effect, it is conceivable that the substrate itself is the natural allosteric effector and GlcNAc simply mimics its effect. The exceedingly small amount of free GlcNAc incorporated into chitin by the chitosomes disputes the possibility (18) that free GlcNAc may be a chitin chain initiator; this finding confirms a conclusion reached with crude preparations of chitin synthetase (2, 15).

UDP is a competitive inhibitor of chitosomal chitin synthetase of M. rouxii. UDP is also a competitive inhibitor of crude chitin synthetase from Piricularia oryzae (19), Coprinus cinereus (17), and Aspergillus flavus (20). It also inhibits other UDP-glycosyltransferases, e.g. mammalian glycogen synthetase (21, 22). Since other nucleotides also inhibit chitin synthetase, it is unlikely that UDP, a product of the reaction, is acting as a mass action inhibitor of a possible reversible reaction that might involve a lipid intermediate. The existence of a lipid intermediate in chitin biosynthesis is still undecided (2); so far, we have been unable to detect a lipid intermediate comparable to that found in other fungal polysaccharide synthetases (23).

Purified chitin synthetase did not form radioactive products other than chitin from UDP-$^{14}$C]GlcNAc, whereas crude preparations synthesized $^{14}$C]diacetylchitobiose and $^{14}$C]GlcNAc-P in addition to $^{14}$C] chitin. These results suggest that both (GlcNAc)$_2$ and GlcNAc-P are not formed by chitin synthetase itself but by contaminating enzymes: (GlcNAc)$_2$ by a chitinase and GlcNAc-P probably by a UDP-GlcNAc pyrophosphatase (24). These findings support an earlier conclusion (2) that the formulation of diacetylcchitobiose in chitin synthetase reaction mixtures should not be construed as evidence that diacetylcchitobiose is an intermediate in chitin biosynthesis (18). Our observation that the chitin microfibrils, produced in an incubation mixture, co-sediment with part of the chitin synthetase activity from the mixture suggests that the chitin chains of a microfibril may remain intimately associated with the enzyme complex that synthesized them. This view is consistent with electron microscopic observations that show individual microfibrils arising from individual chitosomes (6, 7).

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