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 x 10^6 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 withstood 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 shadow-cast preparations of the enzyme contained particles ("granules") of about 350 to 1000 A 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

Culture Techniques and Cell-free Extract Preparation—*Mucor rouxii* IM-80 (ATCC 24965) 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 KH₂PO₄/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 further centrifuged at 1000 g for 5 min. The supernatant was centrifuged in a Beckman No. 30 rotor at 54,500 x g (R₂₀) for 45 min. The supernatant fraction was removed with a pipet; a floating lipid layer was excluded as much as possible. Chitosomes were purified from the supernatant fraction as described below. The pelleted or mixed membrane fraction was washed by centrifugation with buffer for 1 h at 78,800 g (R₁₀₀) and used for the experiments on crude chitin synthetase.

Chitin Synthetase 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 Reeve Angel 934 AH glass-fiber filters (2.4 cm in diameter) and washed with about 50 ml of 1 N 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 x 2.1 cm) of Sepharose 6B (Pharmacia, Uppsala, Sweden) or Bio-Gel A-5m (Bio-Rad, Richmond, Calif.). The column was eluted with

1 The abbreviations used are: GlcNAc, N-acetylglucosamine; Pipes, 1,4-piperazinediethanesulfonic acid; GlcNAc-P, N-acetyl-glucosamine phosphate.
microscopy (negative staining) revealed that ribosomes were far more abundant than chitosomes. For elimination of ribosomes, the crude supernatant fraction more than doubled after passage through Sepharose 6B and Sepharose 2B columns as described under "Materials and Methods.

Our colleagues at the University of California, Riverside, Calif., kindly provided the following: cowpea mosaic virus (J. S. Semancik), rabbit-reticulocyte 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.). Pipes and alkaline phosphatase (calf intestine) were obtained from Calbiochem, La Jolla, Calif. Trypsin was from NBCo, Cleveland, Ohio. Mexicain, a protease from Pileus mexicanus, was a gift from Professor M. Castaneda-Agullo, Instituto Politecnico Nacional, Mexico, D. F., Mexico. Renilase, a crude acid protease from Mucor miehei, was a gift from Novo Enzyme Corp, Mamaroneck, N. Y. Polyoxin D was kindly supplied by M. Sakamaki of the Kaken Chemical Co., Ltd., Tokyo, Japan. Uridine 5'-diphosphate N-acetyl-D-[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 the removal of ribosomes, the eluate (void volume fraction) from a Sepharose 6B column was digested with ribonuclease (see below). The precipitated basic proteins were removed by centrifugation at 12,000 × 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 × g (R20w) in a Beckman SW-27 rotor for 3 h. Gradients were fractionated with a density gradient fractionator, model 183 (ISCO, Lincoln, Neb.), into 1-ml fractions. Absorbance was monitored at 254 nm (A254). Chitin synthetase activity was measured in 50- or 100-μl samples (Fig. 2).

Chitosome isolation from [14C]choline labeled cells—Cultures of yeast cells of M. rouxii (total volume, 2 liters), grown as described above, received a dose of [1,2-14C]choline chloride (31.6 nmol; 1.85 μCi) 2 h before they were harvested. The cells were broken and suspended by sonication. Chitosomal chitin synthetase 3339

The crude supernatant fraction from yeast cells of M. rouxii was passed through Sepharose 6B and Sepharose 2B columns as described under "Materials and Methods."

<table>
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<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Chitin synthetase activity</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Purification</th>
<th>-fold</th>
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<tr>
<td>Crude supernatant fraction</td>
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<td>0.14</td>
<td>31.85</td>
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<td>Sepharose 6B peak (Fractions 5–10)</td>
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<td>Sepharose 2B peak (Fractions 21–28)</td>
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<td>16.39</td>
<td>7.70</td>
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</table>

Table I

Purification of chitosomal chitin synthetase from Mucor rouxii

Chitin synthetase activity (0) 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 synthetase zymogen.

The density gradient centrifugation procedure afforded higher yields of purified chitin synthetase zymogen 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 synthetase zymogen 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 chitosome 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 $K_m$ 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 reported for crude mycelial enzyme (2). Little activity was noticed at pH values below 5 and above 8.

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 at 22°C for 30 min and divided into 150-µl aliquots. Each one was added to the concentrations shown. Final volume was 0.3 ml. After 60 min, radioactivity incorporated into chitin was measured. Chitin synthetase activity is expressed in units (mg of protein)^{-1}.

![Figure 3. Effect of substrate concentration on chitosomal chitin synthetase.](image)

![Figure 4. Effect of Mg^{2+} and Mn^{2+} on chitosomal chitin synthetase.](image)

$[\text{UDP-GlcNAc}] (\text{mM})$ vs $[\text{UDP-GlcNAc}] (\text{mM})$ curve

$[\text{Mg}^{2+}] (\text{mM})$ vs $[\text{Mn}^{2+}] (\text{mM})$ curve

Chitosomal Chitin Synthetase was stimulated by GlcNAc (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 proteolytic activity of acid protease or trypsin against hemoglobin or casein, respectively (data not shown); hence, Mn^{2+} 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-[14C]GlcNAc and 0.108 µM GlcNAc or 4.8 mM UDP-GlcNAc and 0.108 µM [14C]GlcNAc (0.5 µCi). Only 1 mol of free GlcNAc was incorporated per 2.8 x 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-

The products were separated by paper chromatography in 95% ethanol, 1 m acetic acid (20:80 by volume) or isoaamyl 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 µM) 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°C for 4 h did not change their basal or potential chitin synthetase activities (measured without or with added proteases, respectively). (On prolonged incubation (>1 day), a slow self-activation of chitosomal chitin synthetase zymogen has been observed.) This is in sharp contrast to the behavior of crude mixed membrane fractions from yeast cells whose chitin synthetase activity increased several-fold upon incubation for 4 to 6 h at 28°C (4).

Three proteases, active against crude chitin synthetase (4), were tested as activators of purified chitosomes. The acid protease of Rhizopus chinensis was the most effective. Maximum activation was obtained with 30 to 130 µg of acid protease/ml (Fig. 8) and in less than 5 min (not shown). Upon acid protease treatment, the chitin synthetase activity of chitosomes increased about 20-fold. Trypsin was most effective at 4 to 5 µg/ml, but the level of activation attained was only about one-third of that obtained with acid protease, probably because trypsin also destroyed chitin synthetase quite rapidly (Fig. 8). Because the pure acid protease of R. chinensis became
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 × 10⁶).

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 (45 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 ¹⁴C-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 (Rₛₑₛ) for 20 min and were fractionated. The radioactivity of fractions from the sample preincubated with radiolabeled substrate was measured to determine the position of ¹⁴C-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 activity along the gradients. From the sample preincubated with UDP-[¹⁴C]GlcNAc, a band of radioactivity collected near the bottom of the tube (Fig. 9A). This band 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 of the gradient a part of the chitin synthetase present in the incubation mixture. This suggests an intimate and lasting association between enzyme and product. A nonspecific 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 × 10⁶ daltons; 300 Å in diameter) but somewhat smaller than the stipulated exclusion limit for the gel filtration bed (20 × 10⁶ 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. 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 protein 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 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.


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.
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 (4). Nevertheless, the chitosomal enzyme is deficient in the protease(s) responsible for the conversion of zymogen to active enzyme and 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 itself but by contaminating enzymes: GlcNAc, by chitinase and GlcNAc-P probably by a UDP-GlcNAc pyrophosphatase (24). These findings support an earlier conclusion (2) that the formulation of diacetylchitobiose in chitin synthetase reaction mixtures should not be construed as evidence that diacetylchitobiose 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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